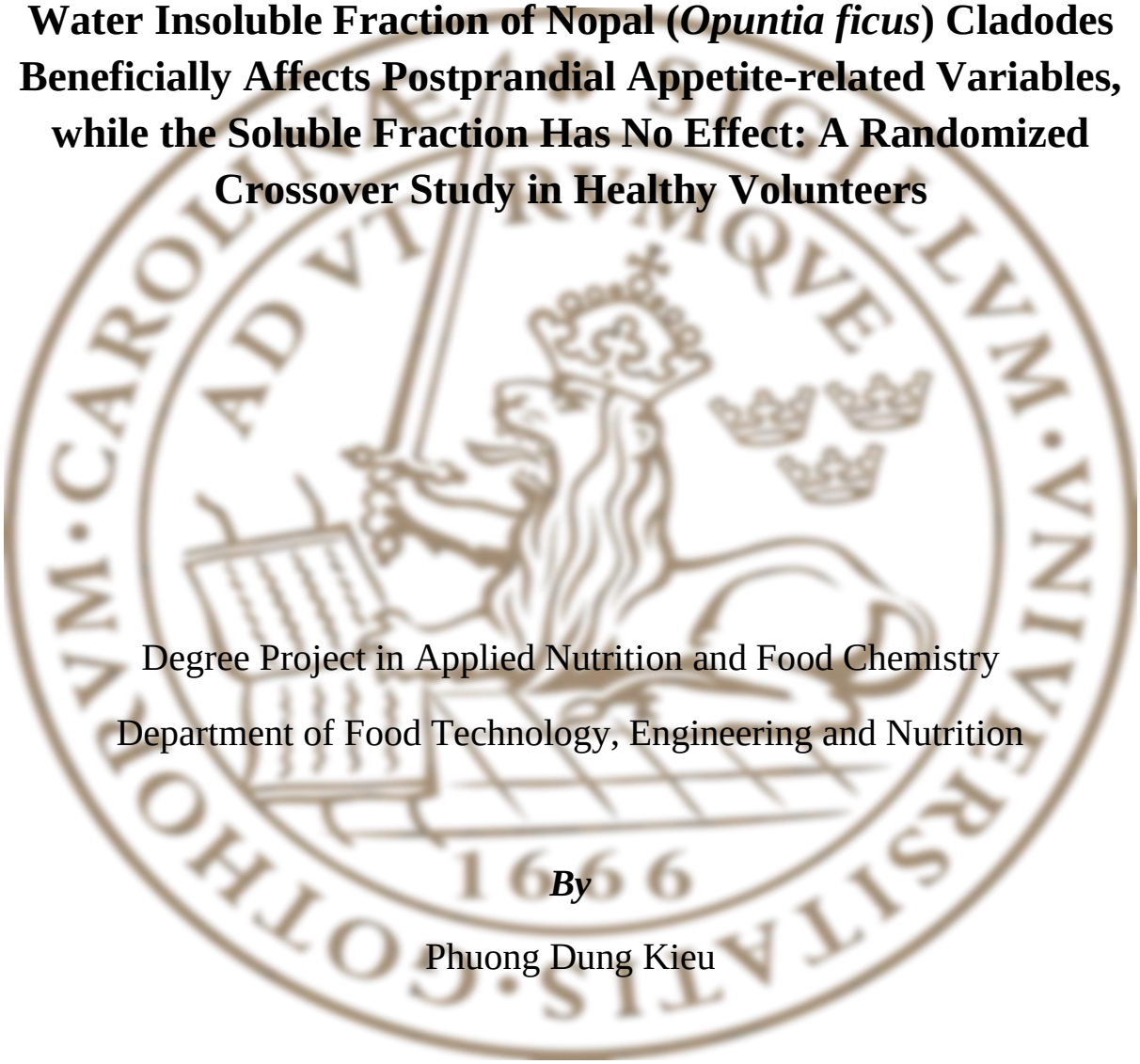
Water Insoluble Fraction of Nopal (*Opuntia ficus*) Cladodes Beneficially Affects Postprandial Appetite-related Variables, while the Soluble Fraction Has No Effect: A Randomized Crossover Study in Healthy Volunteers



Degree Project in Applied Nutrition and Food Chemistry

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA	-	Analysis of Variance
AUC	-	Area Under the Curve
BMI	-	Body Mass Index
^{o}C	-	Celsius
CCK	-	Cholecystokinin
CVD	-	Cardiovascular Disease
DF	-	Dietary Fiber
DM	-	Dry Matter
DNS	-	3,5-Dinitrosalicylic acid
DS1	-	Dry Substance 1
DS2	-	Dry Substance 2
GI	-	Glycaemic Index
GLP1	-	Glucagon-like Peptide 1
HI	-	Hydrolysis Index
INB	-	Insoluble fraction Nopal Bread
MetS	-	Metabolic Syndrome
OXM	-	Oxyntomodulin
pH	-	Potential of Hydrogen
PYY	-	Peptide tyrosine-tyrosine
SD	-	Standard Deviation
SEM	-	Standard Error of the Mean
SNB	-	Soluble fraction Nopal Bread
T2D	-	Type 2 Diabetic
VAS	-	Visual Analog Scale
WHO	-	World Health Organization
WB	-	White wheat Bread

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1. Abstract

Introduction: The world has witnessed a global trend of increasing obesity, which is directly linked to non-communicable diseases, such as type 2 diabetes (T2D). The epidemic of obesity is largely associated with consumption of high calorie diets and low physical activity. Food choice and exercise have a big impact to prevent obesity and T2D making these diseases highly related to personal lifestyle. The diet is one of the most important modifiable lifestyle factors that may be used to prevent obesity. Nopal (*Opuntia Ficus* Indica) is a cactus plant that has its origin in Mexico and has been used as traditional medicine to prevent overweight and obesity. Since Nopal cladodes are rich in several bioactive compounds, it may be considered as a functional food. The objective of this study was to investigate the perceived effect of Nopal cladodes on appetite variables in healthy humans.

Method: Two test-products with two different fractions of Nopal cladodes flour (soluble Nopal fraction bread (SNB) and insoluble Nopal fraction bread (INB)) and a control product (white wheat bread (WB)) were studied in healthy young volunteers (n=17). The subjective appetite variables (hunger, satiety and desire to eat) were measured repeatedly by using a Visual Analog Scale (VAS) during a time perspective of three hours post consumption.

Results: The INB resulted in significantly improved response in appetite variables ($p < 0.05$) compared to the WB. After consumption of the INB, the feeling of hunger and desire to eat were reduced by 27% and 32% respectively, while the feeling of satiety was increased by 25% during the period between 15-180 min post consumption. Importantly, the INB test product also resulted in a significant reduction on hunger and desire to eat, and an increased feeling of satiety during the final hour (120-180 min), compared to the control WB.

Conclusion: The results indicate that the insoluble fraction of Nopal flour may beneficially affect appetite variables in healthy young adults. The results thus suggest that Nopal may help to modulate food intake and therefore contribute to antidiabetic effects previously observed with this edible plant.

Keywords: Nopal cladodes, appetite variables, dietary fiber, hydrolysis index, obesity

2. Introduction

2.1 Obesity and appetite regulation

Obesity is now a global epidemic and one of the major challenges to human health worldwide [1]. The amount of overweight and obese people has increased significantly and become common in every part of the world. Malnutrition tends to replace infection diseases and under-nutrition as the largest causes of illness [2]. According to the global burden of disease, in 2017 there were more than 4 million deaths each year related to overweight or obesity. The prevalence of obesity continues to rise in the world's population and it has increased more than four times (4% to 18%) since 1975. According to Worldometer, more than 750 million adults are obese in this moment [3]. WHO defined overweight and obesity as abnormal fat accumulation that presents a risk to health. Healthy body mass index (BMI) values range between 18.5 and 25 kg/m^2. A person with BMI under 18.5 kg/m^2 is considered to be underweight and possibly malnourished. If the BIM is in the range 25 to 30 kg/m^2, it is considered as overweight, and a BMI higher than 30 kg/m^2 is considered as obesity. The causes of obesity are complicated and are a combination of many factors such as diet, genetic susceptibility or personal lifestyles in modern society. In particular, obesity is associated with the metabolic syndrome (MetS). The MetS is defined as a condition with a cluster of metabolic abnormalities. It includes a group of metabolic risk factors that increases the risk of developing type 2 diabetes (T2D) and cardiovascular disease (CVD) [4]. Metabolic risk factors are abdominal obesity, high triglyceride level, low HDL level, high blood pressure or high fasting blood sugar [5]. Patient can have any one of these risk factors by itself, but they tend to occur together. To be diagnosed with MetS, patient must have at least three metabolic risk factors [5].

To slow down the epidemic of obesity, an appropriate diet is extremely important. It is known that a poor diet with high energy density food, high simple carbohydrates and low dietary fiber (DF) contents can increase the body weight and contribute to the development of obesity. This type of diet generally consists of high glycemic index (GI) foods which can be digested and absorbed easily in the gut. GI expresses how fast the carbohydrate content in a food is absorbed and contributes to the circulating glucose levels after the food consumption. High GI foods, e.g. refined grain breads or rice, induce a rapid and high rise in blood glucose levels, while foods with low GI, e.g. cooked legumes and pasta products, are slowly digested and results in a more limited blood glucose response [6].

DF is defined as carbohydrate polymers that are neither digested nor absorbed in the small intestine; DF includes cellulose, hemicellulose, pectin, gums, lignin and waxes [7]. DF passes through the digestive system before it reaches the colon where most of its components are fermented by the microbiota to a varying extent [8]. DF can thus be described also as the nondigestible part of plant foods which is resistant to the enzyme digestion in the small intestine but that the microorganisms in the colon will use as fermentation substrate leading to the synthesis of metabolites such as short chain fatty acids, which can favor the colonic health and also have positive effects on other organs' metabolism [9]. It is known that DF also includes attached bioactive compounds (e.g. antioxidants, mineral and vitamins), and that the intake of DF can decrease the consumption of fat [6]. Previous research has shown that a healthy diet with high level of DF has positive health effects that can be used for prevention of diet-related disease [10]. Therefore, food with low GI and high DF contents should be included in the daily diet to prevent obesity and illnesses related to MetS.

The transition in modern society has led to changes in eating habits among world population. Overeating has become more and more common as people consume more calories than they need for daily activities. Fast food with high content in calories and saturated fat, and low in DF is not only affordable but also easily accessible. The increasing in size of served portions further increases the energy intake from this type of food [6]. In addition, the amount of processed and unhealthy food (normally high in sugar, salt and saturated fat), that are marketed on television or social media both for adults and children is continuing to increase. Being exposed to these food trends leads people to an unhealthy diet, which can increase the risk of overweight and obesity. This unhealthy prevalence should be stopped by introducing healthier food options that can decrease the rate of obesity and the MetS.

As mentioned, over-eating is the main cause of obesity. By changing the characteristics of the food in order to increase its satiating power, it is possible to modulate food intake. DF is demonstrated to have beneficial effects related to obesity and MetS development [11, 12]. The intake of DF has been associated with increased satiety. One of its physico-chemical properties that has impact on subjective appetite variables is the viscosity. Viscosity is the resistance of a fluid to flow. High viscosity food has ability to slow down the digestion in the digestive system and thus prolong the satiety sensation for longer time [13]. Dhingra *et al.[9]* indicate that soluble viscous DF can limit the food digestion and absorption rate as it increase the viscosity in the lumen of the small intestine. In general, fibers with larger molecular weight and longer polymer chains give a higher viscosity solution in the gut [9]. Generally, DF lowers the food energy density as it is not digested by enzymes in the small intestine. Thus, satiety signals sent to the brain are maintained for longer time [14]. Additionally, viscous DF also increases stomach distension due to the amount of water it absorbs which can affect the fullness feeling. Previous studies also link delayed gastric emptying with the presence of viscous DF [15, 16]. However, the positive link between a healthy diet and appetite regulation needs to be further studied to provide more scientific evidences regarding effects of different food. Furthermore, different functional foods, which are foods that provide health benefits beyond those attributed to their nutritional value, have shown their abilities to enhance satiety and lower postprandial blood glucose levels for a longer period of time [17], i.e. wholegrain and fiber-rich foods [18]. Therefore, the impact of functional foods on appetite and its possible applications in the battle to control the obesity epidemic should be more focused in upcoming investigations.

Appetite regulation is believed to have impact on the development of obesity and MetS. The gut-brain axis is the bidirectional communication between the gastrointestinal tract and the brain. The gut hormones produced in the gastrointestinal tract communicate with the appetite relating regions in the central nervous system through the gut brain axis in order to regulate food intake and appetite [19]. The mechanism behind food intake regulation through gut brain axis is illustrated in **Figure 1**. Intake of food and nutrients released from digested food activate G-protein receptors in the gastrointestinal channel that regulate the release of gut hormones that influence the food intake [20]. Thus, the gut brain axis plays an important part in appetite regulation. Over 30 gastrointestinal peptide hormones are released from the gut. Many of these peptide hormones, such as, leptin, ghrelin, cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), oxyntomodulin (OXM) and peptide tyrosine-tyrosine (PYY), contribute to regulate the satiety, hunger and desire to eat. The interaction between these gut peptides and the gut brain axis affects the long term and short term responses on appetite variables [21].

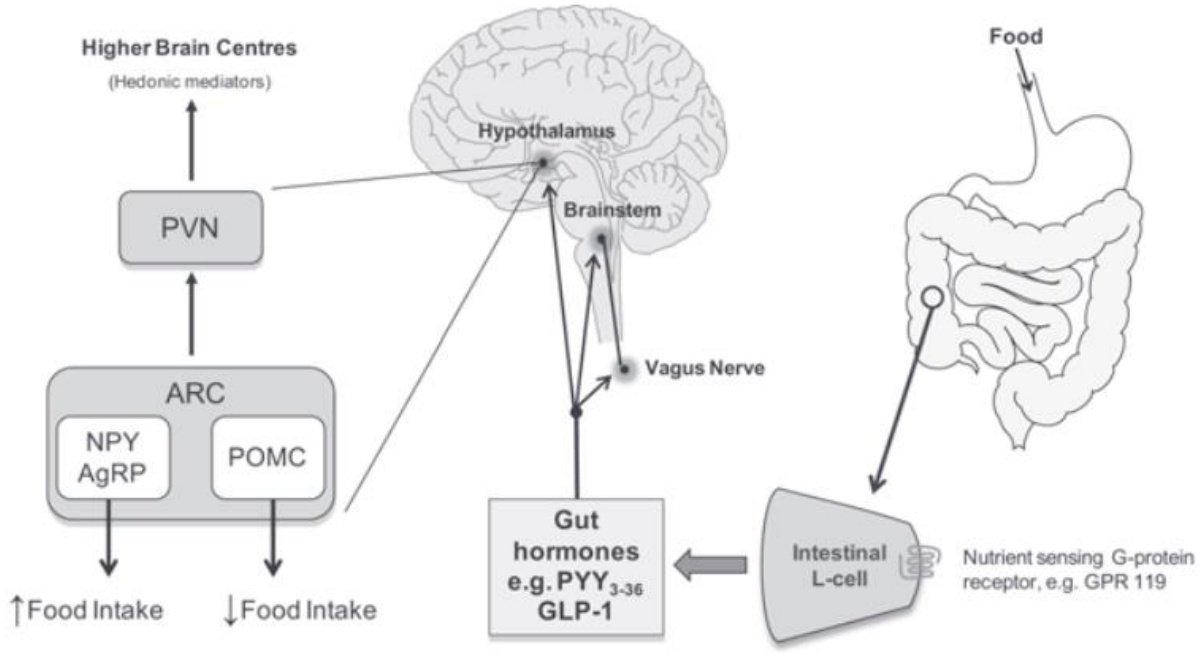

Figure 1. Schematic representation of principal mechanisms behind gut brain axis and food intake regulation [20]

2.2 Nopal

Nopal (Opuntia Ficus Indica) is a cactus plant originated from Mexico [22] and belongs to the *Cactaceae* family, which includes about 1500 species of cactus [23]. Nopal cladodes (i.e. the green parts or "leaves" of the plant) are rich in ascorbic acid, flavanols, carotenes, and flavonoids, which are bioactive compounds with antioxidant activity. Thus, Nopal cladodes are considered as a functional food option [22].The physicochemical and nutritional characteristics of Nopal are allowing its use in diverse food applications [24]. In addition, Nopal cladodes contain low level of calories (27kcal/100g) and high level of DF [25]. Fresh Nopal cladodes are used widely in Mexico for human consumption since it is believed that Nopal has potential health benefits. For instance, Nopal is used in traditional medicine for the prevention and treatment of obesity and overweight. Additionally, Nopal has been commonly used in the Mexican cuisine over the past decades. The thick leaves of the cactus plant are mainly consumed as an ingredient of salads (raw or steamed) or added as powder into maize flour as flour for preparing tortilla bread [26]. Besides, the Nopal cactus fruit is also used in the forms of juice, jam and tea, while oil is extracted from its seeds [27]. Although the scientific evidence supporting the potential beneficial effects on appetite variables (hunger, satiety and desire to eat) is limited, a diploma work was conducted at Lund University by Karin Mogard in 2019 [28] about postprandial appetite parameters following the intake of a Nopal-containing bread. Mogard's study indicated that bread containing Nopal cladode flour affects subjective appetite variables by reducing hunger, increasing satiety and reducing desire to eat. However, the mechanisms behind those mentioned effects of Nopal are unknown. Therefore, this project will take a first step in the investigation of such mechanisms.

Figure 2. Nopal Cladodes

2.3 The aim of this study

The aim of this study was to investigate the effects of the water soluble and water insoluble fractions of Nopal cladodes on postprandial appetite-related variables (hunger, satiety and desire to eat) in young healthy humans, in a period of three hours post consumption. For this purpose, two designed test products (breads) supplemented with Nopal cladodes flour were prepared, and their postprandial impact on appetite was compared to those of an in-house baked control bread lacking Nopal ingredients. This study was part of a larger project with the objective to study effects of Nopal intake on a wider range of metabolic test variables.

2.4 Hypothesis

In the present study it was hypothesized that the water soluble and water insoluble fractions of Nopal, added to a bread meal, will have different impact on postprandial satiety, hunger and desire to eat.

3. Material and Method

This study included two main parts. The first part of the study was about the separation of Nopal cladodes flour into water soluble and insoluble fractions. From these fractions, the test products (breads) for the investigation were designed and produced. The second part of the study investigated the effects on postprandial appetite-related variables in healthy volunteers eating the breads added with Nopal fractions. Briefly, the subjects consumed two test products presented as bread containing either the soluble fraction or the insoluble one. The response on appetite variables to these preparations was compared to a control product lacking Nopal. After consumption of each test product or the control, postprandial appetite-related variables (hunger, satiety and desire to eat) were measured repeatedly during a period of time (180 minutes) using Visual Analog Scale (VAS).

3.1 Separation of Nopal cladodes Fractions

A dried Nopal cladodes flour was used which was provided by J. Manuel Orozco-Rodríguez, a doctoral student at the Department of Biology at Lund University. The flour was produced by VERALMEX, Sayula 117 Col. Mitras Sur Monterrey, N.L. Mexico C.P. 64020. The study required the separation of the water soluble and water insoluble fractions of the flour. For this purpose, different extraction temperatures (10^oC, 22^oC and 90^oC) and centrifugation times were tested to optimize the separation process. One (1) g of Nopal flour was mixed with 4g of water at the different temperatures, using manual stirring, and thereafter incubated for 15 minutes at room temperature (22^oC). The mixtures were then centrifuged at 4200 rpm and 10^oC for 0, 30, 60 or 90 minutes. It was found that the most effective water temperatures were 10^oC and 22^oC (room temperature), as shown in the **Appendix 1**. Therefore, extraction with room temperature water was chosen as standard condition. Centrifugation for 60 minutes was the most appropriate for separating the two Nopal fractions (**Appendix 1**). The supernatant corresponded to the soluble fraction and the precipitate represented the insoluble fraction.

3.2 Raw Material and Recipes for Test and Control Products

The test products which contained the above-described fractions from the flour from the Nopal cactus were consumed as breakfast bread by the test subjects [29].

The meal study included three different breads: one control bread that did not contain Nopal (white wheat bread-WB) and two different breads containing soluble Nopal fraction and insoluble fraction. From the previous study by Karin Mogard [28], it was known that the test product containing 25% (w/w, dry basis) Nopal flour can modulate appetite variables in healthy young adults. In this study, the soluble and insoluble fractions obtained from the same amount of Nopal flour used in the previous investigation were separately incorporated into the test breads (SNB and INB, respectively)

The WB, SNB and INB were baked with the same procedure and the final recipes for each bread are shown in **Table 1**. The recipes of the three test products were based on previous study by Karin Mogart, using a Sage household bread baking machine [30].

For WB making, the dry ingredients i.e. 530g wheat flour with 12% protein (Vetemjöl Special, Kungsörnen, Stockholm), 4.6g dry yeast (KronJäst, Jästbolaget AB, Sollentuna), 4.6g NaCl (Falksalt, Ab Hanson & Möhring c/o Salinity AB, Gothenburg), were mixed together in a bowl. Then, 360g water was added into the bread making machine. Finally, the dry ingredients were added to the water.

For SNB and INB, the water soluble and insoluble cladode fractions were freshly obtained. Based on the preliminary separation trials (see **Section 3.1**), 134 gram of Nopal flour were separated into two centrifuge bottles equally. Thereafter, 300g water (room temperature) was poured into each bottle, mixed and left at room temperature for 15 minutes. The bottles were then centrifuged for 1h at 4200 rpm at 10^oC. After centrifugation, the water soluble (the supernatant) and insoluble fractions (the pellet) were separated and water was added to each fraction until reaching the total amount of liquid required in the recipe. Each diluted fraction was mixed with dry ingredients in the bread making machine to follow the same procedure described for WB making. The bread loaves were made using the basic setting of the machine's program for white bread: light crust and 1kg mode were chosen (including kneading for 26 min, resting for 40 min, punching down for 10 sec, rising for 25 min, shaping for 15 second, rising for 50 min, baking for 40 minute at 140^oC min and finally kept warm for 1 hour). The total process time was 3

hours and 1 min. The insoluble fraction was mixed with dry ingredients manually before loading into the baking machine to support the kneading process in the machine. After baking, each bread loaf was wrapped in a towel and cooled to room temperature until it was cooled down completely (2h). After that, the bread crusts were removed and the crumb was sliced into portions (based on 50g available starch) wrapped in aluminum foil, put in plastic bags and stored in a freezer (-18°C) until further use. The amount of bread loaves needed for the trial was determined based on the portion size of each bread (see section 3.3.2). One bread loaf of each type of bread was estimated to provide maximum four portions. As 17 test subjects participated in the project, five bread loaves per product were needed. Therefore, 15 bread loaves of WB, SNB and INB were baked in total.

Table 1. Final recipes for the breads that were used in the meal study

Test product	White wheat flour (g)	Nopal flour(g)	Liquid* (g)	Salt (g)	Dry yeast (g)
Control (WB)	**530**	**0**	**360**	**4.6**	**4.6**
SNB	**530**	**Soluble fraction from 134g**	**360**	**4.6**	**4.6**
INB	**396**	**Insoluble fraction from 134g**	**360**	**4.6**	**4.6**

**Liquid is the amount of water or the Nopal fraction suspended in water after centrifugation.*

3.3 Chemical Analysis of the Test and Control Products

The bread portions contained 50g available starch. The dry substance and the available starch contents in each type of breads were determined to calculate test portion sizes.

3.3.1 Determination of dry substances

The bread samples were taken out from the freezer and thawed at room temperature overnight, remaining in their foil and plastic bags. Two aluminum cups per bread types were weighted. About 3–5 g of the bread were crumbled into the aluminum cups and then weighed again. The weights of products are presented in the Appendix 2, Table 1A. The bread samples were dried overnight at 105°C while the rest of the bread were crumbled down on a metal tray and placed in a fume hood at room temperature to dry overnight. After taking the dry samples out from the oven, they were placed in a desiccator for at least 1 hour to avoid moisture reabsorption when cooling. The aluminum cups with dry samples were then weighed to calculate the absolute dry substance in fresh bread (dry substance 1 - DS1). The calculation of DS1 is presented in the Appendix 2, Table 1A. The air-dried bread samples in a room temperature hood were grounded to a powder. About 1.00 g of this powder was weighted in an aluminum cup and then put in an oven overnight at 105°C to get absolute dry. After drying overnight in the oven, the sample rested in a desiccator for at least 1 hour, and was then weighed again to calculate the dry matter in dry bread that had dried in room temperature overnight in the fume hood (dry substance 2 – DS2). The calculation of DS

is presented in the **Appendix 2**. DS1 and DS2 are needed to calculate starch content in fresh bread and starch based on dry matter. The starch analysis was performed on the air-dried (ambient dry) flour.

3.3.2 Determination of Available Starch

The method for starch analysis was performed according to Holm *et al.* (1986). The principle of the method is to hydrolyze the starch molecules to glucose by the combined action of Termamyl (α-amylase) and amyloglucosidase and then determine the released glucose with glucose oxidase/peroxidase color reagent. The procedure was done in duplicate for each bread [31].

An amount of 500 mg of ambient dried and ground sample of each final bread was weighed into a 50 ml beaker with thick wall. 10 ml phosphate buffer was added into the beaker. The beakers were then stirred gently until the solutions were homogenous, and another 10 ml phosphate buffer was added. Thereafter, 40 µl Termamyl was added under stirring and the beakers were placed in a boiling water bath for 20 minutes. The beakers were stirred after every 5 minutes during the incubation. The solutions were then transferred to 50 ml volumetric flasks and diluted with water to the total volume of 50ml. 1ml sample, 1ml water, 1 ml 0.3M NaAc-buffer and 50 µl amyloglycosidase were pipetted down into a simple set of tubes. The tubes were mixed and then incubated in 60°C for 30 minutes, stirring every 5 minutes. The solutions were then transferred to 100 ml conical flasks and diluted with water to the 100ml mark level. Preparation of glucose standards are presented in the **Appendix 2**. 1 ml of the samples or glucose standards and 1 ml water were added to a set of test tubes. 4 ml Glox-reagens was added to all tubes, and after mixing the tubes were incubated for 60 minutes at room temperature. After incubation, the tubes were mixed 20 times and then centrifuged for 5 minutes at 3000 rpm. The absorbance was measured at 450 nm [32]. The raw data from the absorbance measurement are presented in **Appendix 2**.

A glucose standard curve was created from a glucose standard solution to determine the released glucose from different bread samples after enzyme degradation. These values were used to calculate the available starch content in the samples. Each portion of test products is based on 50g of digestible starch to ensure the same amounts of glycaemic carbohydrates. The available starch content in each bread and the final weights for each portion for the trial are presented in **Table 2**.

Table 2. The available amount of starch for each bread and the final weight for test portions.

Test product	Available Starch* (%)	Portion size** (g)
WB (Control)	74.27	117.1
SNB	73.24	116.66
INB	58.14	158.78

** Available starch content expressed in dry matter basis*

*** Portion size is the amount of bread that contains 50g available starch ("as eaten" basis)*

Data are presented as means of duplicates. The values are rounded to two decimals

3.3.3 Determination of Dietary Fiber

Fiber analysis

The dietary fiber content was determined according to the method by Asp *et al*. (1983). The principle of this method is based on human digestion mimicking. Starch and protein are broken down by the enzymes into small molecules, and then the digested starch and protein are removed from the dietary fiber through the filtration process [33]. The procedure of this method included three main steps. In the first step, α-(1-4) bonds of α-linked polysaccharides in starch are broken by Termamyl (α-amylase) during gelatinization by incubation in boiling water. Then, protein was degraded by pepsin into small peptides. The last step is incubation with pancreatin. Pancreatin solution is a mixture of the different pancreatic enzymes which are proteinases, lipase and amylase [34]. During pancreatin incubation, the undigested protein and polypeptide after incubation with pepsin is continued to be degraded into small peptides and amino acids by proteolytic enzymes (trypsin, chymotrypsin, carboxypeptidases and elastase) [35] while fat contents (mainly triglycerides) was degraded by lipase to release fatty acids and monoglycerides. Lastly, the remaining starch is hydrolyzed into a mixture of alpha-dextrins, maltose and glucose by amylase.

Insoluble fiber components are filtered off with glass filter crucibles with Celite as the filter aid. From the filtrate after first filtration, soluble fiber is precipitated out with ethanol and then filtered off with filter crucibles and Celite in the same way as insoluble dietary fiber. Dietary fiber residues are then dried and weighed. After that, the ash content and residual protein in the fiber fractions will be determined and subtracted from fiber fractions. The total fiber value content is the sum of soluble and insoluble fiber components.

1g of milled bread sample was weighed in a 500ml beaker, and then 25ml 0.1 N sodium phosphate buffers (pH 6.0) was added and mixed well. 40µl Termamyl was added to each beaker and incubated with boiling water for 20 minutes without stirring during the incubation. After that, 20ml HCl 0.2 N was added and then adjusted pH to 1.5 ± 0.1. One ml of pepsin was added, and then the mixtures were incubated at 40^{o}C for 60 minutes in shaking water bath. These steps were to mimic the condition of protein digestion in the stomach. When pepsin incubation was finished, 5ml 1N NaOH (1M) was added to each sample to adjust pH to 6.8 ± 0.1 to simulation the condition in the intestine which favor pancreatin activity. Thereafter, 1ml pancreatin was added and incubated at 40^{o}C for 60 minutes in shaking water bath. Finally, 4-5ml 0.5 N HCl was added to adjust pH to 4.5 ± 0.1 to stop the enzyme incubation. After the insoluble fiber was filtered off, the filter cakes were rinsed with 2 x 10 ml water, 2 x 10 m ethanol 95 %, and finally with ethanol 99%. Then the crucibles that contain the insoluble fiber were put in the oven at 105^{o}C overnight. Filtrates were diluted to 100 ml with water and then precipitated with 4 volumes of warm ethanol 95% (60^{o}C) for 1 hour. Thereafter, soluble fiber was filtered with a same procedure as insoluble fiber and rinsed filter cakes with ethanol 78%, ethanol 95% and ethanol 99% respectively. The crucibles with soluble fibers were also dried overnight in the oven at 105^{o}C until the weight was constant. All breads were analyzed in duplicate. After taking out from the oven, all crucibles (with insoluble and soluble fiber content) were put in desiccator at least 1 hour for cooling down before weighting. Weights of crucibles and Celite were subtracted to calculate the weight of insoluble and insoluble residues. As the analysis was duplicated for each product, one of sample was incinerated to identify the ash content and another sample was used in protein analysis [36].

Some minerals and protein are in complex forms with plant cell wall component [36]. Thus, the incineration of dietary fiber for ash correction and the analysis of protein in dietary fiber residues are needed.

Ash analysis

The soluble and insoluble residues were analyzed for ash content in a chamber furnace (Carbolite machine). The crucibles were loaded in an incinerator and were incinerated at 550^{o}C overnight. With high temperature, water and other volatile materials are vaporized and organic substances are burned in the presence of the oxygen in air to CO_2, H_2O and N_2. Most minerals are converted to oxides, sulfates, phosphates, chlorides or silicates. After the incineration, the crucibles were taken out and cooled down before weighing to calculate the ash content.

Protein analysis for dietary fiber determination

The protein analysis was performed using the FlashEA® 1112 N/Protein Analyzer where the sample is completely combusted at 900^{o}C with air in the presence of oxygen and then filtered to remove water and carbon dioxide by passing the gasses over adsorption filters. Nitrogenous gases are reduced to nitrogen and detected by protein analyzer. The preparation consisted of packing the sample in a capsule made of fine thin foil. The calibration of this method is done with a pure material (aspartic acid; known nitrogen content). The nitrogen content is converted to protein crude content using a conversion factor i.e. 6.5 (Jones Factor).

3.3.4 Determination of Hydrolysis Index (In vitro starch Hydrolysis Index)

The chewing/digestion method proposed by *Granfeldt et al.* (1994) was used to determine Hydrolysis Index (HI) of the products. This method measure in-vitro starch digestion rate of chewed products, by stimulating human digestion process [37, 38]. Each bread sample (WB, SNB and INB) was chewed by six participants on different days (1 day per each bread sample). The participants were asked not to eat anything nor brush their teeth for 1.5-2h before the experiment.

A portion of the products containing 1 g of available starch was chewed by each participant. The sample should be chewed 15 times, then spitted out into a beaker containing 1 ml pepsin, 5 ml of amylase buffer or "chew" phosphate buffer (0.022 M, pH 6.9) and 10 drops of HCI (2M). After that, they rinsed their mouth with 5 ml buffer during 1 minute and then spit it into the mixture. This procedure aimed to mimic the physiological first step of digestion, i.e. chewing and mixing with the amylase-containing saliva. In the next step, the pepsin added simulates the physiological conditions of digestion in the stomach. HCl (2 M) were used to adjust the medium pH to the same prevailing in stomach. Then the samples were incubated at 37^{o}C for 30 minutes in order to allow for the enzymatic breakdown of proteins.

After the incubation, samples were neutralised to pH 6.9 by adding 2M NaOH. Then each sample was transferred into a syringe and 1 ml of α-amylase (SIGMA A6255) was added. Phosphate buffer was added to fill the syringe and then the content in the syringe was transferred into a dialysis tube. Each dialysis tube was placed into a 1L beaker containing phosphate buffer. The beakers were placed in a water bath at 37^{o}C with constant magnetic stirring. Aliquots (1 ml) of the dialysate were collected from the beakers every 30 minutes during 3 hours and transferred into glass tubes containing 1 ml of DNS

solution. DNS reacts with reducing sugars, as those released by starch hydrolysis (mainly maltose) and produces a change in the colour of the solution (from yellow to red), which can be quantified colorimetrically.

The samples and maltose standards were incubated in boiling water in 10 minutes. Maltose standards were prepared in duplicate and are presented in the **Appendix 2**. After boiling, test tubes were cooled down in cold water, then diluted with deionised water. The absorbance (530nm) of the samples and the maltose standards were measured to calculate the HI values. Data were expressed as maltose concentrations and were plotted as degree of starch hydrolysis (%) versus incubation time, in order to measure the area under the curve (AUC). The HI calculation was based on the formula bellow:

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$$HI = \frac{AUC\ for\ the\ test\ product}{AUC\ for\ the\ reference\ product} \times 100$$

In this experiment, the reference product was the WB. The HI value for each test product was calculated based on the mean of six replicates (i.e. the six participants).

3.4 Visual Analog Scale for appetite evaluation

VAS are commonly used to measure appetite sensations [29]. In this study, subjective appetite-related variables were determined using a 100 mm VAS. Each appetite variable (hunger, satiety and desire to eat) was evaluated using separate scales by answering these questions "How hungry are you right now?", "How full are you right now?" and "How much do you desire to eat right now?". **Figure 3** shows the scales of satiety, hunger and desire to eat which were used to evaluate the subjective appetite variables in the study. The test subjects marked their appetite sensations on each scale repeatedly in the postprandial period after intake of the test and control products. The scales were black 100 mm lines on a white paper. Each scale had marks at 0mm (left end of the scale), 50 mm (the middle) and 100 mm, to simplify the response from the subject. The test subjects were carefully instructed on how to use the scales at the beginning of the trial. The mark at 50mm indicates an answer represented that their appetite sensation not was directed to any of the sides (e.g neither "hungry" nor "not hungry"). The mark at 0 mm or 100 mm represented extremes of the sensations e.g. not hungry at all and can't be hungrier, respectively.

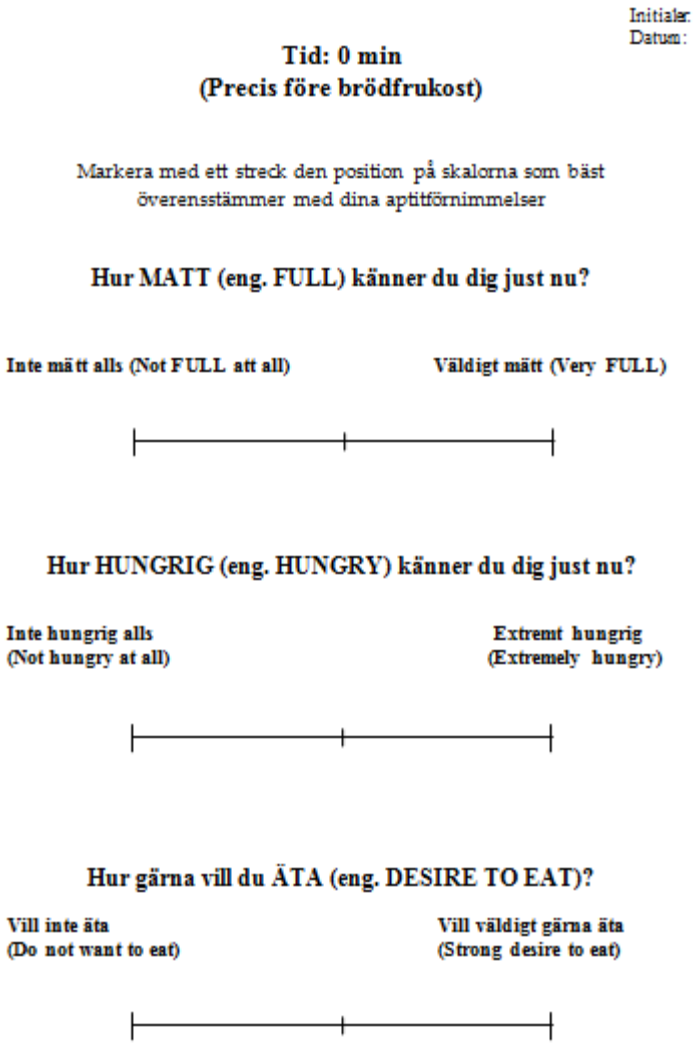


Figure 3. The scales of satiety, hunger and desire to eat which were used to evaluate the subjective appetite variables

3.5 Study design

Twenty-four healthy human volunteers registered for the study to investigate the effects of soluble and insoluble fractions of Nopal cladodes on subjective appetite variables. However, only 17 completed the whole study. The dropouts were due to treatment with antibiotics (n=1), difficulties to eat the test products with Nopal (n=2), did not match the criteria (n=3), and did not manage to come on the scheduled times. (n=1). The inclusion criteria for participating in this study were healthy people aged 20 – 40 years, BMI between 18.5 – 25.0 kg/m^2 and diet habits that resembles the pattern suggested by the Nordic Nutrition Recommendations. Exclusion criteria were known conditions that affect the metabolism (diabetes, lactose intolerance or gluten intolerance), depression disorders, food allergies/sensitivities, and vegetarianism or smoking habits. In addition, people who declared snuffing, taking antibiotics, probiotic or supplements during the last month or suffering any other conditions or receiving medication that may affect the results of the study were not allowed to participate. The 17 healthy subjects that completed the study (9 men and 8 women) were between the ages of 20 and 40 years (mean ± SD: 22.5 ±. 2.3 years)

with normal BMI (mean ± SD: 22.5 ± 3.2 kg/m^2). The test subjects were all given instructions to maintain their daily lifestyle, not change in exercise habits or diet, except for changes in accordance with the study protocol.

Recruitments were made through social media i.e. student groups within Facebook. All volunteers were given all information needed about study protocols both orally and written. Additionally, written consent was also obtained from every participant. Each volunteer was received an economic compensation for participating in this study, and they had the right to leave the study whenever if it was needed or desired.

The meal study had a randomized, controlled crossover design with approximately one week washout period between each product. The effects of the products containing different factions of Nopal were compared to the effects of the control bread and the Nopal test products were compared to each other to investigate fraction effect.

The study was approved by the Swedish Ethical Review Authority (Etikprövningsmyndigheten) in Uppsala (Dnr 2019-00980), and registered at ClinicalTrials.gov (NCT04439630).

3.6 Standardized Meal

The day before each experiment day should be as similar to each other as possible regarding food intake, physical activity and other things that can affect the physiological parameters that was to be measured. The evening before a trial day, food with low fiber content should be eaten, as high DF foods such as beans and peas can affect test results. Additionally, high physical activities and alcoholic beverages in the evening before the trial should be avoided. Before each trial day, participants were instructed to consume the same dinner at the same time to standardize their metabolic status. At 21.00 the day before the experiments, all volunteers consumed a standardized meal consisted of one to two slices of white wheat bread (*Jättefranska, Pågen AB, Malmö, Sweden)* with a glass of water, coffee or tea, without milk or sugar. Each subject decided the amount of bread and drink to consume, and then maintained the same composition before each trial. After this standardized evening meal, the subjects were fasting without eating or drinking anything before the test products were served on the trial day morning. If they were thirsty in the morning, they were allowed to drink a half glass of water before arrival at the trials, on an otherwise empty stomach. However, they needed to do the same procedure each morning before the trial if they chose to drink water.

3.7 Study Outline

Portions of test meals were stored in a freezer (-18°C). The day before a trial day, portions to be consumed were put in room temperature to thaw overnight, remaining in foil and plastic bags. The subjects arrived in the morning at 07.30 and rested seated at least 10 minutes before starting the experiment. After obtaining the fasting samples, one of the test meals was served. The test subjects were instructed to eat the whole portion within 12 minutes, together with 250 mL tap water. The appetite variables were measured by VAS before the subjects started eating (time 0) and 15, 30, 45, 60, 90,120, 150, 180 minutes after the start of the test meal.

3.8 Statistical analysis

The order of the test products given to test subjects was randomized by using "RANDOM" formula in Microsoft Excel. The number of participants in the study is based on a desired power of > 80%, with a significance level > 95%.

The data collected between 15-180 minutes are presented as Mean ± SEM. The mean values were used for the statistical evaluations for each test subject and test-product using the program MiniTab Statistical Software (release 17; Minitab, Minitab Inc, State Collage, PA, USA). A subset of data corresponding to the test variables assessed during the 120-180 min interval (late postprandial phase) was also analyzed. The results from the statistical analyses were transferred to the program GraphPad Prism (version 8.1.1, GraphPad Software, San Diego, CA, USA) for graph plotting. ANOVA general linear model was applied to analyze potential differences in subjective appetite variables depending on test-products. When ANOVA showed significant differences, a Post hoc test (Tukey's pairwise multiple comparisons) was applied to explore the differences between the test products. In addition, Dunnett test were applied to perform comparison between the test-products and the reference product. The significance level was set to $P < 0.05$.

4. Results

4.1 Appetite variables

The results for postprandial appetite variables (hunger, satiety and desire to eat) are shown in **Table 3** and **Figure 4**. **Table 3** presents the average rating on VAS for the trial population at these time points: 0 min (fasting value), mean values 15-180 min and mean value 120-180 min. **Figure 4** shows the average ratings of VAS appetite variables in the postprandial period after the consumption of the test meal.

For all appetite variables, there was no significantly difference at fasting time (0 minute) before consumption of the test products.

The INB test product reduced the hunger feelings significantly during the period of 15-180 minute (-27%, $p<0.05$) when compared with control WB. In addition, the fullness feeling increased significantly after the INB was consumed (25%, $p<0.05$) during the period of 15-180 minute. In the same period of time, the subjective desire to eat decreased significantly (-32%, $p<0.05$) compared to the reference.

The SNB test product had less effect on the appetite variables. However, the feeling of hunger declined slightly but without reaching significance (-4%, $p>0.05$) compared to WB. Additionally, also a non-significant ($p>0.05$) increased subjective feeling of satiety (7%) and reduced feeling of desire to eat (-5%) were observed over the period of 30-180 minutes, compared to the control.

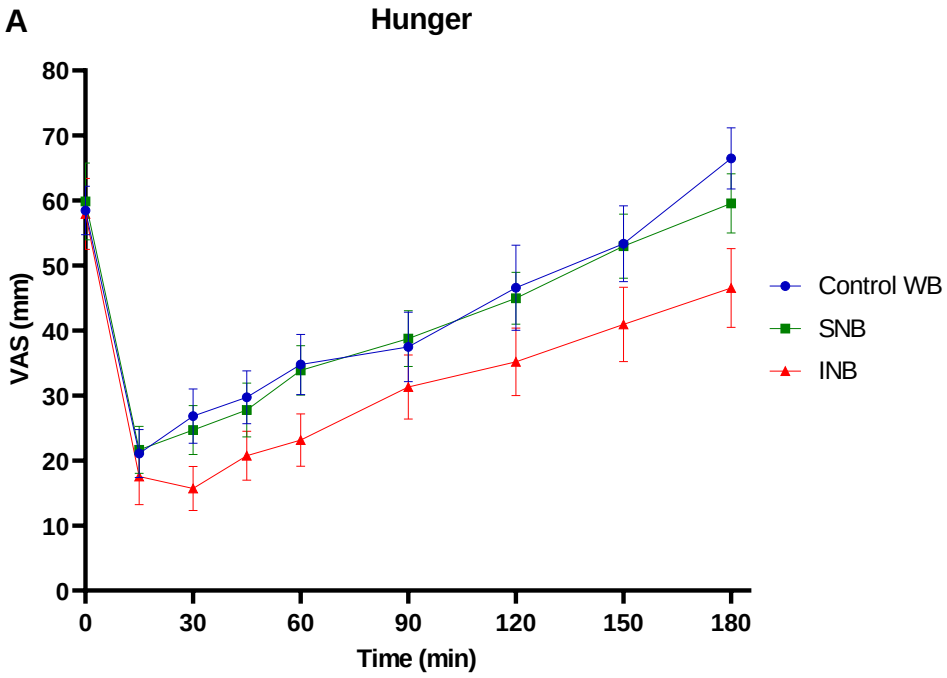
Between 120-180 min after consumption, the consumption of INB test product resulted in a considerably drop of hunger and desire to eat feeling, -26% and -25% respectively ($p<0.05$), while the feeling of satiety increased to 60% ($p<0.05$) compared to the control. During the same time, the SNB test product affected the appetite variables to a lesser extent than the INB test product. SNB did not result in significant changes compared to the control bread.

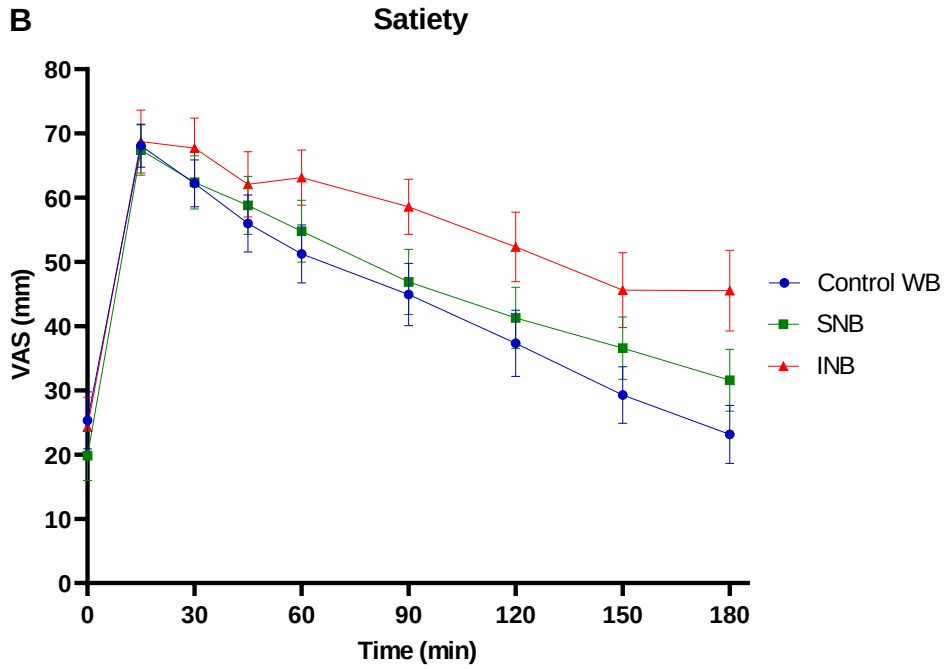
Besides, also when comparing with the SNB, the INB test product showed its ability to reduce hunger and desire to eat significantly, 24% and 30% respectively ($p<0.05$), and increase the satiety sensation (16%, $p<0.05$) during 3 hours post consumption. A similar pattern could also be observed during the last hour of the investigated time period.

Table 3. Subjective appetite variables (hunger, satiety and desire to eat) after consumption of test- and control products (WB, SNB and INB), rated with a VAS scale (mm)

Test variables	Control (WB)	SNB	%	INB	%	
			Compare to control WB		Compare to control WB	Compare to SNB
Hunger						
0 min, fasting (mm)	58.47 ± 3.72	57.94 ± 5.47		59.8 ± 5.92		
15-180 min	39.54 ± 4.25	38.03 ± 3.62	-4	28.89 ± 3.91*	-27	-24
120-180 min	55.47 ± 5.84	52.50 ± 4.54	-5	40.89 ± 5.66*	-26	-22
Satiety						
0 min, fasting (mm)	25.32 ± 4.43	19.85 ± 3.87		24.29 ± 4.67		
15-180 min	46.53 ± 3.89	49.96 ± 4.52	+7	57.97 ± 3.94*	+25	+16
120-180 min	29.92 ± 4.79	36.47 ± 5.00	+22	47.82 ± 5.96*	+60	+31
Desire to eat						
0 min, fasting (mm)	66.82 ± 3.20	68.47 ± 4.68		66.12 ± 3.43		
15-180 min	45.51 ± 4.94	43.24 ± 4.64	-5	31.17 ± 4.20*	-32	-30
120-180 min	61.20 ± 5.56	57.30 ± 5.05	-6	46.08 ± 6.14*	-25	-20

**Difference from Control (WB) and SNB, $p < 0.05$ (ANOVA, followed by Dunnett test).*
The percentage change is calculated as the difference from Control (WB). n=17.
All data values are presented as Mean ± SEM. The values are rounded to two decimals
Control (WB), white wheat bread; SNB, Nopal bread with water soluble fraction; INB, Nopal bread with water insoluble fraction.


A
Hunger
VAS (mm)
80
70
60
50
40
30
20
10
0
0
30
60
90
120
150
180
Time (min)
Control WB
SNB
INB


B
Satiety
VAS (mm)
80
70
60
50
40
30
20
10
0
0
30
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90
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Time (min)
Control WB
SNB
INB

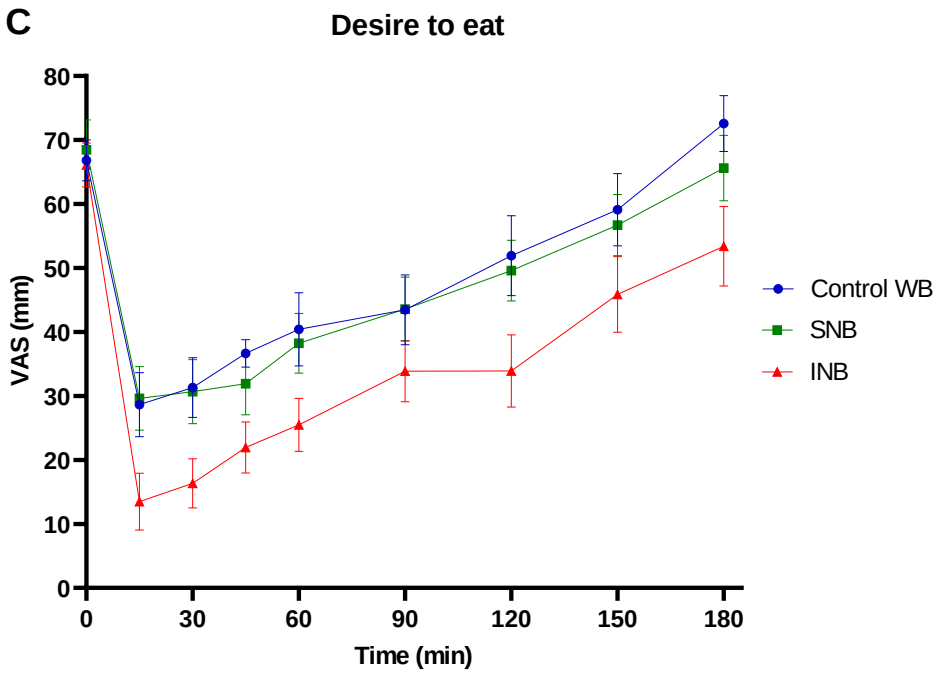

Figure 4. Postprandial responses of appetite variables (hunger, satiety and desire to eat)
All values are presented as Mean ± SEM. Means of subject appetite ratings (VAS) of hunger (A), satiety (B) and desire to eat (C) over the period of 0-180 minutes. Control (WB), white wheat flour bread. SNB, Nopal bread with water soluble fraction. INB, Nopal bread with water insoluble fraction. VAS, Visual Analog Scale.

4.2 Dietary fiber analysis

The total, soluble and insoluble dietary fiber contents based on dry matter basis in WB, SNB and INB are presented in **Table 4 and Figure 5**. WB had the lowest total DF content (1.08g/100g) while INB showed the highest level (10.03g/100g). The level of soluble DF in INB was higher (4.54g/100g) than in WB and SNB (1.08g/100g and 2.94g/100g, respectively). INB also contains the highest soluble DF level (5.49g/100g) while WB had no detectable insoluble DF.

Table 4. Dietary fraction of test products

Product	Soluble DF		Insoluble DF		Total DF	
	% dry matter	g/test meal	% dry matter	g/test meal	% dry matter	g/test meal
Control WB	1.08 ± 0.11	0.67 ± 0.07	0 ± 0.13	0 ± 0.13	1.08 ± 0.11	0.7 ± 0.11
SNB	2.94 ± 0.73	1.88 ± 0.47	1.65 ± 0.16	1.7 ± 0.2	4.59 ± 1.19	2.94 ± 0.76
INB	4.54 ± 1.31	3.58 ± 0.7	5.49 ± 0.18	4.33 ± 0.2	10.03 ± 3.40	7.90 ± 0.7

Data are presented as mean ± SEM
The values are rounded to two decimals

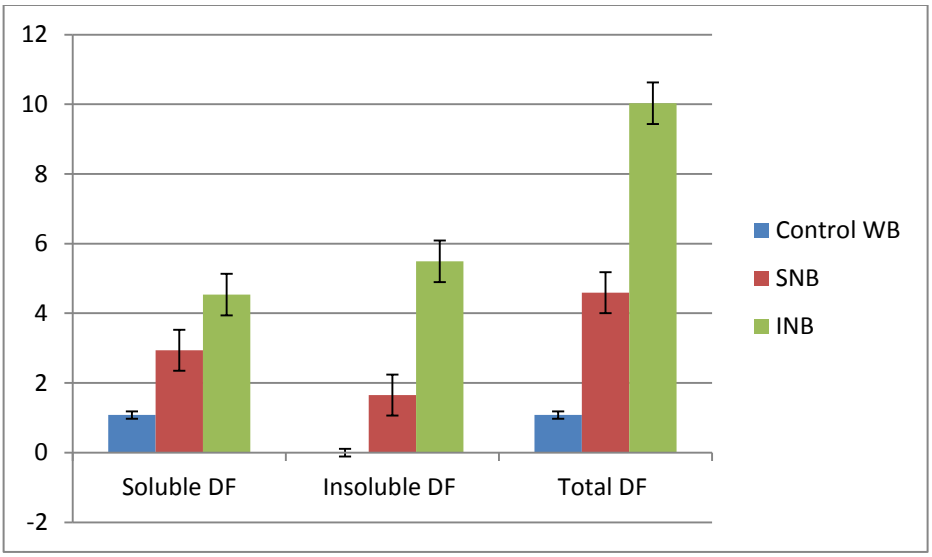

Figure 5. Total dietary fiber, Soluble dietary fiber and Insoluble dietary fiber content in WWB, SNB and INB (dry matter basis)

4.3 Determination of Hydrolysis Index

The results for HI are shown in **Figure 6** and **Table 5 below**. In **Figure 6**, the curves of starch hydrolysis for WB, SNB and INB are shown. The lowest rate of hydrolysis, expressed as HI, was observed in INB (**Table 5**) with significant lower values compared with WB (-63%) and SNB (-45%) ($p<0.05$). SNB resulted in a non-significant reduction of HI value compared to WB (-14%, $p>0.05$). The detailed calculations for HI are presented in **Apendix 2**.

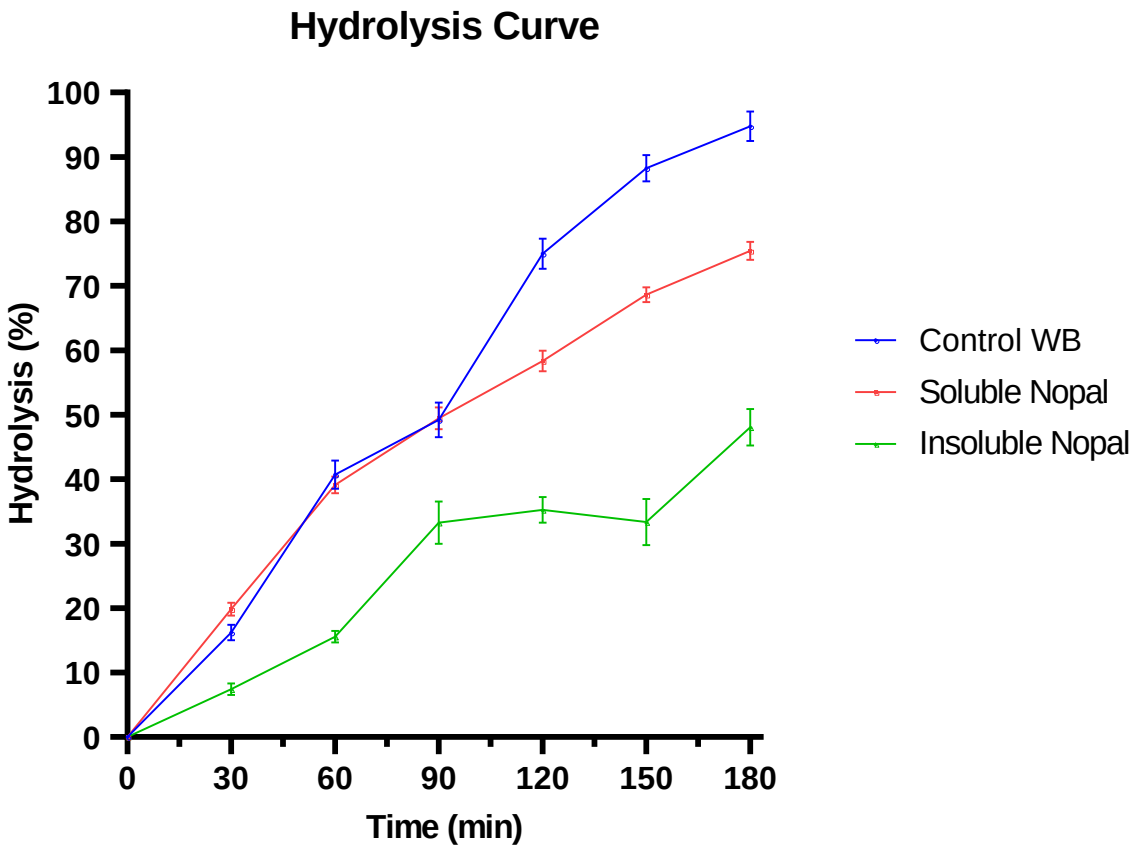

Figure 6. Starch hydrolysis curve of the products during 3 hours of incubation with α-amylase. Data are presented as mean; bars represent SEM

Table 5. Starch Hydrolysis Index (HI) values

Product	HI (%)
Control WB	100
SNB	86.2 ± 4.1
INB	47 ± 3.9*

The hydrolysis index (HI) was referred WB
Values are mean of six subjects chewing/dialysis replicates
Data values are presented as Mean ± SEM.
** Significantly different from WB and SNB ($p < 0.05$)*

5.0 Discussion

The purpose of the present study was to examine the influence of water soluble and insoluble fractions of Nopal flour on postprandial appetite variables following a breakfast including the different fractions. Therefore, the separated fractions were combined with wheat flour and served as breakfast breads to evaluate their effects on postprandial appetite variables in healthy subjects. Characterization of Nopal fractions with respect to dietary fiber content and the impact on starch hydrolysis index were also investigated, as a first approach to understand the mechanisms governing the effects of Nopal on postprandial appetite variables.

5.1 Separation of Nopal cladodes Fractions

A quick comparison of the two Nopal fractions collected after centrifugation indicated that the water soluble fraction had dark brown color with bitter taste and was slightly viscous. This fraction may contain phytochemical compounds that soluble in water, e.g. polyphenol, phenolic acids, organic acids [4, 27, 39, 40]. The viscosity might come from viscous soluble DF in Nopal [9, 41]. The insoluble fraction had dark green colour showing slightly sandiness and gel-like texture. The bitter taste of insoluble fraction was less intense compared to the soluble fraction. The insoluble fraction may consist of insoluble DF and other organic compounds that are not soluble in water. In addition, Nopal cladodes contain appreciable amounts of oxalic acid, which forms insoluble complexes with divalent cations, such as Ca^{2+} [42]. The presence of calcium oxalate may be the cause for the sandiness of the insoluble fraction.

The colour of the soluble fraction might come from flavonoids, while the bitter taste might come from tannins. Tannins (such as tannic acid) are water soluble polyphenols that are thought to have beneficial health effects [43]. A study conducted by Marely et.al [40] revealed that tannins have the ability to inhibit α-amylase and α-glucosidases activity, an effect that may result in the lowering of postprandial glycemic responses. On the whole, the health effects of Nopal have been suggested to associate with several phytochemical compounds, preventing the development of MetS associated with diet-induced obesity [44, 45].

The separation of Nopal cladodes fractions in this study is a critical point that needs to be considered. The results of dietary fiber analysis aided in concluding that the level of soluble DF in INB is still high compared to WB and SNB. This result might be due to an insufficient separation process, which may have failed to separate completely water soluble and insoluble factions of Nopal flour, resulting in that part of soluble fraction still remained in the isolated insoluble portion. Thereby, further investigation needs to be performed to improve the separation process. The maximum rate for the centrifuge machine in the department was used for Nopal flour separation (4200rpm, as mentioned in **Section 3.1**). Therefore, increasing the centrifuge force might give a better separation of the soluble constituents of the Nopal flour (such as soluble DF). On the other hand, an additional washing of the pellet in order to remove remnants of the soluble materials may help to a more efficient extraction of soluble components. That would imply increasing the final total volume of the soluble fraction solution, but that can be solved by freeze-drying or water evaporation under vacuum condition.

5.2 Appetite variables

The subjective appetite variables, i.e. hunger, satiety and desire to eat were measured by using a VAS, which often is used to assess appetite sensations. Previous studies about the reproducibility and validness of VAS has pointed out that VAS is reliable for appetite research in healthy volunteers [29]. Therefore, VAS can be considered as an appropriate tool concerning the determination of perceived appetite sensations.

The previous study by Karin Mogard (2019) gave supportive evidence about the beneficial effect of Nopal cladodeson postprandial appetite variables: hunger, satiety and desire to eat [28]. Here we followed up the conclusion of that study, indicating that a bread with 25% supplementation of Nopal flour (% DM) reduces huger and desire to eat while increasing fullness. The purpose of this study was to evaluate which fraction of Nopal cladodes flour gives the main effects on appetite variables.

Consequently, the results show that the water insoluble fraction of Nopal cladodes exerted more striking beneficial effects on appetite variables compared to water soluble fraction. The consumption of INB test product increased remarkably the rating in postprandial satiety compared to the control bread and SNB. Furthermore, INB reduced the feeling of hunger and desire to eat significantly compared with WB and SNB. SNB also exhibited tendencies in the same direction as compared to INB, but the impact did not reach statistical significance. From the results, it can be concluded that a meal with INB makes people feel full for a longer period of time compared to the control and SNB, especially during the final period assessed (120 -180 min after the meal). Consequently, it can be stated that the water insoluble fraction exerts a significant beneficial effect on appetite sensations. If a meal can reduce the hunger and desire to eat while increasing the fullness for a longer period of time, it may lower the amount of food eaten on a daily basis.

The original idea was to incorporate each Nopal fraction to bread in the same proportion they were in the 25% Nopal flour-containing bread tested in the study by Mogard [28]. This was achieved for INB. However, the amount of the soluble fraction in SNB could not reach the desired level due to the difficulties experienced during the extraction. As mentioned in **Section 3.2**, a relatively large amount (600g) of water was added to 134g Nopal flour to allow for mixing and separation. A lower amount of water in the extraction mixture would have resulted in a less efficient separation. Therefore, with the amount of liquid extracted from the centrifugation (i.e. the portion containing the soluble Nopal fraction), the amount of white wheat flour used for preparing SNB had to be increased as compared with INB, which made the soluble fraction in SNB more diluted with wheat flour compared to the insoluble fraction in INB. This dilution results in that the SNB test portion had a reduced relative content of soluble fraction compared to the insoluble content in INB, which could be at least part of the reason why SNB had lesser effects on subjective appetite variables than INB. Thereby, if the amount of the soluble fraction in SNB is increased in future experiments, it might exert stronger effects on appetite variables.

As INB show significant effects on appetite variables, the components responsible for those effects of Nopal cladodes appear in this study to be present mainly in this fraction. The identification of such components was not an objective of this work, but it should be researched further, in the future parts of the further project that this study belongs to. It has to be noted though, that the amounts of soluble fractions in the SNB was lower than planned, and did not correspond to the intended portion 25% Nopal flour, thus, it is difficult to draw conclusions regarding effects on appetite variables of the soluble

fraction. However, the mechanisms behind the effects of Nopal on appetite variables might be related to DF. Previous studies showed that intake of DF has beneficial effects on treatment and prevention of obesity, T2D, cancer and CVD [46-48]. One of the main factors that lead to obesity is an increased energy intake. Thus, a diet containing high amounts of DF can reduce energy intake as DF contributes to improved appetite regulation and lower caloric density of food, which may result in improved weight management. In addition, in a slightly longer time perspective when water soluble fibers are fermented in the large intestine, glucagon-like peptide 1 (GLP-1) and peptide tyrosine-tyrosine (PYY) are increasingly released [6]. These gut hormones play an important role on derived satiety signals from the brain through the gut brain axis [6]. DF has the ability to delay the absorption of carbohydrates due to its viscous and gel forming properties in the intestine, and its presence thus results in a longer digestion time [49]. It can be suggested that also the delayed digestion and absorption may result in increased release of satiety hormones. Therefore, the fullness feeling can last longer and reduce the hunger and desire to eat. Although both soluble and insoluble dietary fiber have ability to help in the reduction of body weight, a study showed that in a high fat diet, insoluble fiber may have stronger effect on weight loss compared to soluble fiber [6]. Furthermore, insoluble fiber with more complex structure and high molecular weight increases stomach distension and stimulate the satiety signals by activating gut hormones release [6]. Nopal is a plant rich in DF and bioactive components [39]. It has been reported that Nopal leaves have 18 g soluble fiber and 33 g insoluble fiber content (per 100 g, dry matter) [50]. From the results obtained in this study, Nopal has shown health effects on postprandial appetite variables, and may therefore have application in the prevention and/or treatment of MetS, and the effect may be associated especially to the insoluble fiber.

The development of obesity is highly related to the energy balance. However, the mechanism for obesity development has not been fully understood. The gut brain axis is believed to play an important role in food intake regulation. The gut brain axis is the two way extrinsic communication between gastrointestinal tract and the central nervous system. Gut hormones produced in the gastrointestinal tract interact with specific areas of the brain to regulate the metabolism and appetite behavior. These effects on appetite regulation can be short term or long term [32] . Ghrelin, leptin, cholecystokinin (CCK), GLP-1, oxyntomodulin (OXM) and PYY are gastrointestinal peptide hormones that regulate the satiety, hunger and desire to eat. Leptin, GLP-1, PYY, OXM are gut hormones that result in satiety feeling while ghrelin is known as a “hunger hormone” [51]. GLP-1, PYY, OXM are synthesized and released from the endocrine intestinal cells. Investigations about the effects of GLP-1, PYY, and OXM indicated the reduction in food intake after the secretion of these peptide hormones [19]. Ghrelin is an orexigenic peptide produced in the stomach and regulates meal initiation as it signals the brain that you are hungry [52]. Ghrelin plays a major role in appetite, hunger and food intake as it increases appetite and food consumption [53]. Diet manipulation of these gut hormones in a beneficial direction may represent an important strategy in the prevention of obesity. To understand more about the obesity development, these peptide hormones can be used as biomarkers in studies about appetite sensations in further studies with Nopal flour [21].

There are many factors that contribute to the development of obesity. These factors are related to genetics, eating habits, physical activities, daily life-style and also social factors that interact in different level to promote overweight development [54]. Therefore, the prevention and treatment of obesity also focus on these factors [55]. Among them, the diet is considered of major importance. WHO develops guidelines and recommendations about food choice and food intake to achieve a healthy diet [56]. According to

WHO, the consumption of fruits and vegetables should be increased while reducing the content of free and added sugar, saturated fatty acids or the level of salt added to food (especially prepared or processed food). In this case, functional foods, which are food that offer health benefits beyond their nutrition value, can be considered as potential food choices for obesity prevention. This study suggests that foods including Nopal (especially the insoluble fraction) can be considered as potential functional food since the INB product showed significant beneficial effects on appetite variables. Additionally, a more active lifestyle is also recommended to reduce the risk of obesity. The treatments of obesity include pharmacological treatments. In drug therapy, anti-obesity drugs (orlistat and sibutramine) are applied to obese people. These drugs can decrease body weight slightly but are sometimes not effective or suitable for some patients [51]. Another treatment method is surgical intervention. Treatment of obesity seems to be ineffective and difficult, therefore the focus on the prevention of obesity with the consumption of functional foods, i.e. development of foods including Nopal may be an appropriate approach to control obesity pandemic in the future. Many studies suggest that obesity is causally associated with features of the metabolic syndrome (MetS), such as hyperglycemia, dyslipidemia and hypertension, which can lead to stroke, cardiovascular disease (CVD), and type 2 diabetes (T2D) which is caused by insulin resistance [52]. The rapid increase in the prevalence of obesity worldwide over the past decades has set an emergency for finding effective obesity prevention strategies, in which a healthy diet is one of the key factors.

5.3 Dietary fiber determination

As mentioned on Section 5.2, DF is an important component that plays a role in affecting appetite variables. From the result of dietary fiber analysis, INB contains highest total DF, soluble DF and insoluble DF. The soluble fiber in Nopal flour contains mainly mucilages, gums and pectin, while insoluble fraction is composed of lignin, cellulose and insoluble hemicelluloses [57].

A study revealed that the content of total DF, soluble DF and insoluble DF differ depending on the maturity stages of the Nopal [58]. The total DF and insoluble DF in Nopal flour increases with the plant age, while soluble DF decreases overtime. It is suggested that older Nopal cladodes are a better source of insoluble DF compared to younger ones. Insoluble DF content in Nopal cladodes increased from 40 to 135 days of plant age while the soluble DF decreased in the same period [58]. It has been suggested that for optimal calcium content, Nopal cladodes should be harvested and consumed at maturation stage (135 days old) [58]. With this in mind, it seems important to study the optimal harvest time aiming to get the most beneficial effects on postprandial appetite variables.

5.3 Hydrolysis Index (HI)

An *in-vitro* experiment was also performed to identify whether the effects of Nopal on the rate of starch digestion in the test products correlates with the results for appetite variables in the human trial. The results in the HI analysis show that the control WB is most rapidly digested after consumption, while the lower HI of INB suggest that Nopal slows down α-amylase cleaving rate on starch. Thus, the digestion of INB (and perhaps to some extent also SNB) is slower than in the case of control WB. INB has the lowest HI, indicating that the insoluble fiber has more marked effects on delaying the starch digestion and absorption in this model. Since the INB also contained considerable amounts of soluble DF it can also be speculated if a combination of soluble and insoluble DF may result in more efficient reduction of the HI.

Low GI food can exhibit a relatively slow digestion and nutrient absorption rates which have impact on appetite sensations, as it can extend the satiety and reduce the feeling of hunger and desire to eat. The texture and particle size of the test products may also affect the digestive process [59], factors that may be considered in further studies on the effects of Nopal. The high contents of DF in INB and SNB resulted in a more complex matrix structure compared to the WB. This complex matrix may have contributed to reduce the digestion rate in the small intestine which was simulated in the in vitro model with the inclusion of a dialysis tube.

5.4 Limitations and further perspectives

The small trial population (n=17) is a one of the limitations of this study. Therefore, a larger study population should be considered in further studies. Another limitation can be the differences in the appearance and flavor of the test products, which allows the test subjects to distinguish them (see **Figure 7**). This can affect the perception on appetite of the participants. When the subject notices that the test products contain special compounds, they may assume that the product will be different and this will affect their response on VAS. Another limitation is that the extraction process of the soluble materials present in the Nopal flour was not optimized. The compounds that are responsible for the effect of Nopal in the insoluble fraction need to be investigated in further studies as IBN has shown effect on postprandial appetite variables. The lower level of Nopal flour fraction in SNB is also a limitation of this study. New studies may look at the possibility of increasing the concentration of the soluble fraction in the test bread in order to determine if that may give a more clear effect on appetite variables.

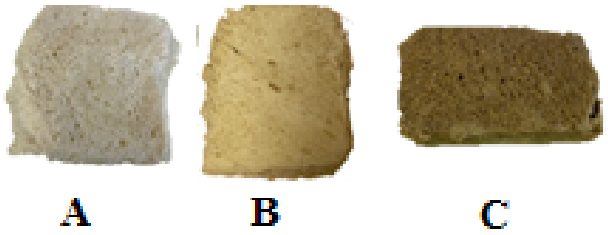

Figure 7. Investigated products in the study
WB (A), SNB (B), INB (C). WB, white bread. SNB, Nopal bread with water soluble fraction. INB, Nopal bread with water insoluble fraction.

6. Conclusion

In conclusion, the results of this study indicate that a bread containing the water insoluble fraction of Nopal cladodes had beneficial effects on postprandial appetite variables. In specific, it led to the reduction in hunger and desire to eat, while satiety feeling increased. Thus, the intake of food during a day can be reduced by including the water insoluble fraction of Nopal cladodes in a meal, since it has ability to make a person feel full for a longer period of time after the meal. The water insoluble DF of Nopal flour and other potentially bioactive substances associated to it emerge as good candidates behind the effects of Nopal cladodes on postprandial appetite variables. Thereby, this fraction may be included in dietary strategies for prevention and treatment of obesity in the future. Finally, no firm conclusions can be drawn regarding the effects of the soluble fraction on appetite variables, due to the relatively reduced amounts of soluble Nopal fraction in the portion of SNB tested.

7. Popular Scientific Summary

Obesity is now a global epidemic and one of the major challenges to human health worldwide. The amount of overweight and obese people has increased significantly and become common in every part of the world. According to Worldometer, more than 750 million adults are obese in this moment. Obesity is associated with the metabolic syndrome and increases the risk of developing type 2 diabetes and cardiovascular disease.

The transition in modern society has led to changes in eating habits among world population. Overeating has become more and more common. The amount of processed and unhealthy food that is marketed on television or social media both for adults and children is continuing to increase. Being exposed to these food trends leads people to an unhealthy diet, which can increase the risk of overweight and obesity.

Appetite regulation is believed to have impact on the development of obesity and metabolic syndrome. The gut hormones produced in the gastrointestinal tract communicate with the appetite relating regions in the central nervous system through the gut brain axis in order to regulate food intake and appetite

To slow down the epidemic of obesity, an appropriate diet is extremely important. Furthermore, by changing the characteristics of the food to increase its satiating power would be another way to modulate food intake. Therefore, the research on food options that can be used in daily meals to prevent the development of obesity and improve appetite regulation is important.

Nopal is a cactus plant originating from Mexico. Nopal cladodes are except of dietary fiber rich in ascorbic acid, flavanols, carotenes, and flavonoids, which are bioactive compounds with antioxidant activity. Nopal is used in traditional medicine for the prevention and treatment of obesity and overweight.

A diploma work was conducted at Lund University in 2019 about postprandial appetite parameters following the intake of a Nopal-containing bread indicated that bread containing Nopal cladode flour affected subjective appetite variables by reducing hunger, increasing satiety and reducing desire to eat. The purpose of this study was to investigate the effects of the water soluble and water insoluble fractions of Nopal cladodes on postprandial appetite-related variables (hunger, satiety and desire to eat) in young healthy humans, in a period of three hours post consumption. Two designed breads supplemented with Nopal cladodes flour were prepared, and their postprandial impact on appetite was compared with a baked control bread lacking Nopal ingredients. The test persons rated their appetite by the use of a questionnaire, called Visual Analog Scale. There were three scales with different questions, “How hungry do you feel right now?”, “How full do you feel right now?” and “How much do you desire to eat right now?”. They answered these questions on a 100 mm line before they started eating (time 0) and 15, 30, 45, 60, 90,120, 150, 180 minutes after the start of the test meal by marking their current feeling of appetite from 0 - 100.

This study showed that a bread containing the water insoluble fraction of Nopal cladodes had beneficial effects on postprandial appetite variables. It led to the reduction of hunger and desire to eat, while the satiety feeling increased. Thus, the intake of food during a day can be reduced by including the water insoluble fraction of Nopal cladodes in a meal, since it has the ability to make a person feel full for a longer period of time after the meal. Thereby, this fraction may be included in dietary strategies for prevention and treatment of obesity in the future.

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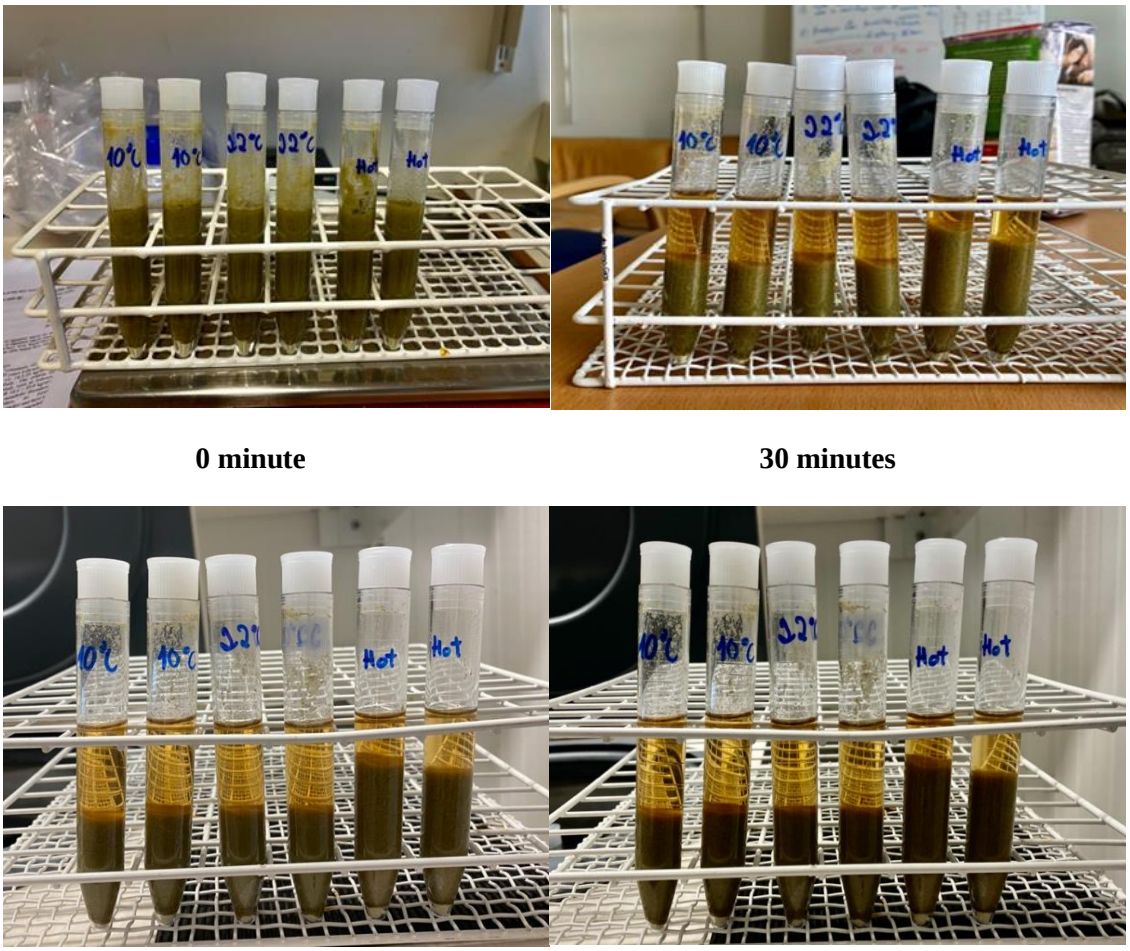
Appendix

Apendix 1

Separation of Nopal fractions (Soluble and Insoluble fraction)



0 minute **30 minutes**

60 minute **90 minutes**

Figure 1A. The separation of soluble and insoluble fraction at 0, 30, 60 and 90 minutes in different water temperatures (10ºC, 22ºC, and 90ºC)

Appendix 2

Chemical analysis

Table 1A. Data needed for determination of dry substance 1

Product	Aluminum cup (g)	Sample (g)	Aluminum cup + dry sample	DS1	DS1 mean value
WB 1	1.8731	4.3963	4.184	0.5256	0.5258
WB 2	1.879	4.7064	4.3547	0.5260	
SNB 1	1.8488	4.3705	4.2496	0.5493	0.5489
SNB 2	1.8652	4.6302	4.4045	0.5484	
INB 1	1.8746	4.3568	4.0424	0.4976	0.5489
INB 2	1.8583	4.2532	3.9638	0.4950	

Weights of petri dish, fresh samples and (aluminum cup + dry sample) measured in grams and dry substance (DS1) for each product, WB, SNB and INB, in duplicates.

Calculations for dry substance 1

$$\frac{(Aluminum\ cup + dry\ sample\ (g)) - (Aluminum\ cup(g))}{\text{Fresh sample (g)}}$$

WB1: $\frac{4.184-1.8731}{4.3963} = 0.5256$

WB2: $\frac{4.3547-1.879}{4.7064} = 0.5260$

DS1 WB (Mean) = $\frac{0.5256+0.5260}{2} = \mathbf{0.5258}$

SNB 1: $\frac{4.2496-1.8488}{4.3705} = 0.5493$

SNB 2: $\frac{4.4045-1.8652}{4.6302} = 0.5484$

DS1 SNB (Mean) = $\frac{0.5493+0.5484}{2} = \mathbf{0.5489}$

INB 1: $\frac{4.0424-1.8746}{4.3568} = 0.4976$

INB 2: $\frac{3.9638-1.8583}{4.2532} = 0.4950$

DS1 INB (Mean) = $\frac{0.4976+0.4950}{2} = \mathbf{0.4963}$

Table 2A. Data for the determination of dry substance 2

Product	Aluminum cup (g)	Sample (g)	Aluminum cup + dry sample	DS2	DS2 mean value
WB 1	1.8541	1.0275	2.7943	0.9150	0.9146
WB 2	1.8699	1.0131	2.7961	0.9142	
SNB 1	1.8601	1.0362	2.8111	0.9178	0.9166
SNB 2	1.8809	1.0092	2.8048	0.9155	
INB 1	1.9008	1.0114	2.8273	0.9161	0.9164
INB 2	1.8887	1.012	2.8164	0.9167	

Weights of aluminum tins, samples and aluminum cup + sample in grams, for the determination of dry substance 2. Dry substance 2 (DS2) for each product, WB, SNB and INB.

Calculations of dry substance 2

$$\frac{(Aluminum\ cup + dry\ powder\ sample\ (g)) - (Aluminum\ cup(g))}{\text{Powder sample (g)}}$$

WB1:$\frac{2.7943-1.8541}{1.0275} = 0.9150$

WB2: $\frac{2.7961-1.8699}{1.0131} = 0.9142$

DS2 WB (Mean) = $\frac{0.9150+0.9142}{2} = \mathbf{0.9146}$

SNB 1: $\frac{2.8111-1.8601}{1.0362} = 0.9178$

SNB 2: $\frac{2.8048-1.8809}{1.0092} = 0.9155$

DS2 SNB (Mean) = $\frac{0.9178+0.9155}{2} = \mathbf{0.9166}$

INB 1: $\frac{2.8273-1.9008}{1.0114} = 0.9161$

INB 2: $\frac{2.8164-1.8887}{1.012} = 0.9167$

DS2 INB (Mean) = $\frac{0.9161+0.9167}{2} = \mathbf{0.9164}$

Table 3A. Glucose standards concentrations.

Standard	D-Glucose (mL)	Water (mL)
0 %	0.0	2.0
25%	0.5	1.5
50%	1.0	1.0
100%	2.0	0.0

Table 4A. Raw data for samples in absorbance (450nm) measurements and glucose standards.

Sample	Well Row	Well col	Absorbance	Average based on Raw data (450)	Linear regression fit based in Raw Data in µg (450)
Control WB	A	9	0.651	0.6535	74.145
	A	10	0.656	0.6535	74.775
	A	11	0.646	0.65045	73.514
	A	12	0.6549	0.65045	74.636
SNB	B	1	0.6299	0.63635	71.485
	B	2	0.6428	0.63635	73.111
	B	3	0.6418	0.6428	72.985
	B	4	0.6438	0.6428	73.237
INB	C	5	0.5429	0.5323	60.517
	C	6	0.5217	0.5323	57.844
	C	7	0.5183	0.51575	57.416
	C	8	0.5132	0.51575	56.773
Glucose Standard 1 (0 µg) (Blank)	A	1	0.0708	0.07225	1.001
	A	2	0.0737	0.07225	1.367
Glucose Standard 2 (25 µg)	A	3	0.2535	0.2526	24.033
	A	4	0.2517	0.2526	23.806

Glucose Standard 3 (50 µg)	A	5	0.4563	0.45355	49.599
	A	6	0.4508	0.45355	48.906
Glucose Standard 4 (100 µg)	A	7	0.8656	0.8612	101.198
	A	8	0.8568	0.8612	100.089

Table 5A. Mean values of digestible starch, dry substance 1 and dry substance 2

Product	Digestible Starch (%)	DS2 (%)	DS1(%)
Control (WB)	74.2675	91.46	52.58
SNB	73.237	91.66	54.89
INB	58.1375	91.64	49.63

Calculation of digestible starch:

$$\left(\frac{\text{Mean Starch for sample}}{\text{Mean DS2}}\right) \times \text{Mean DS1}$$

Control WB: $\left(\frac{74.2675}{91.46}\right) \times 52.58 = 42.70\%$

SNB: $\left(\frac{73.237}{91.66}\right) \times 54.89 = 43.86\ \%$

INB: $\left(\frac{58.1375}{91.64}\right) \times 49.63 = 31.49\%$

Calculation of portion sizes for each product, WB, SNB, INB:

$$\frac{50g}{Digestible\ starch}$$

WB: $\frac{50g}{0.4270} = 117.096g$

SNB: $\frac{50g}{0.4386} = 116.659\text{g}$

INB: $\frac{50g}{0.3149} = 158.780\text{g}$

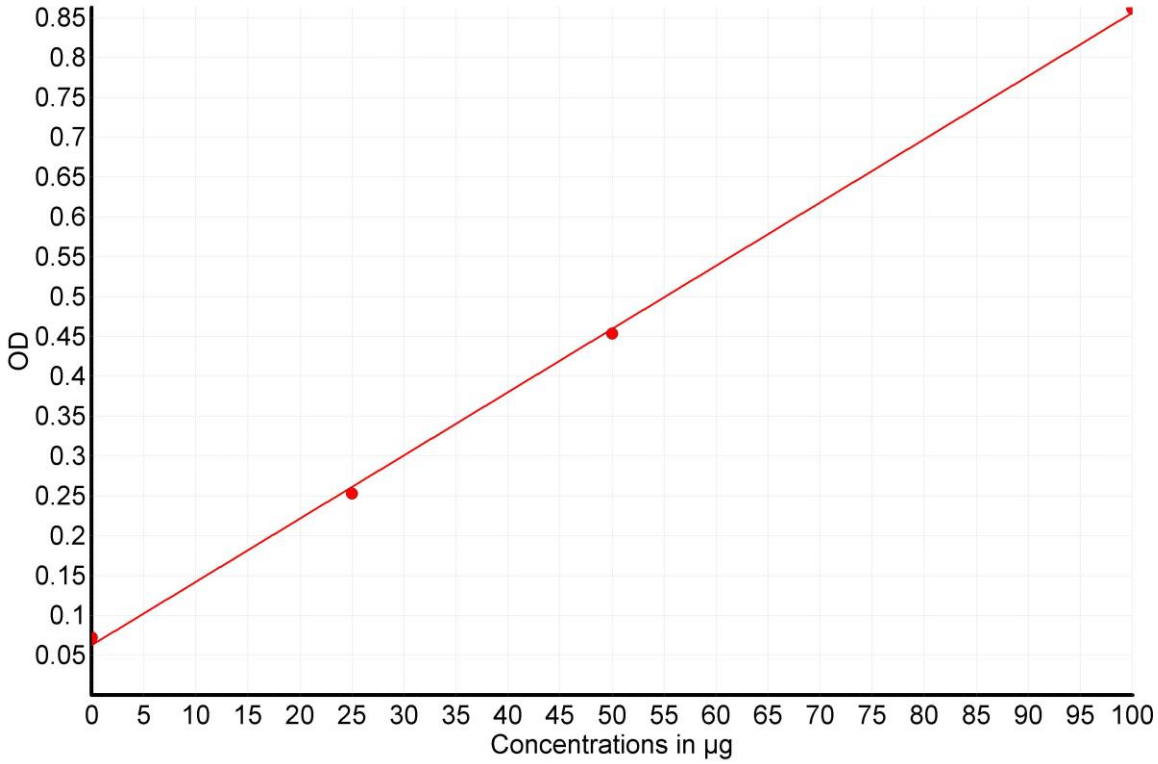


Figure 2A. Linear regression of glucose standard measurements. y= 0,00793x+0,0286. OD, Optical density.

Table 6A. Data for the determination HI

Person	Area Under the Curve (AUC)			Hydrolysis Index (HI)	
	WB As Reference	**SNB**	**INB**	**SNB**	**INB**
x1	7846	8428	3821	107.42	48.70
x2	9690	7263	4043	74.95	41.72
x3	9559	8201	3635	85.79	38.03
x4	10368	8988	4675	86.69	45.09
x5	9686	8174	5321	84.39	54.93
x6	9862	8111	5301	82.24	53.75
Mean	9502	8194	4466	86.23	47.00

Calculation of HI values of WB, SNB and INB:

$$HI = \frac{AUC\ for\ the\ test\ product}{AUC\ for\ the\ refernce\ product} \times 100$$

Appendix 3

Chemical solutions

Starch analysis

- 0.30 M NaAc – buffer, pH 4.75: 24.6 NaAc (40.8 NaAc x 3 water) is solved in 900 water. pH is set with acetic acid to 4.75. Solved to a volume of 1000 ml.
- Glox: Frozen Glox is solved in 1000 ml. Tris-buffer.
- 0.50 M Tris-buffer (for Glox-reagens): 61.0 g Tris is solved in 900 ml H_2O. pH is set to 7.0 with 80 – 90 ml. 5M HCI. Solved to a volume of 1000ml.
- Glucose standard: 50 mg D-glucose (anhydrous) is solved in 1000 ml of H_2O
- 4.0 M KOH: 56.1 g dry KOH is solved in 250 ml . H_2O
- 5.0 M HCL: 207 ml concentrated HCI (37%) is solved in H_2O to volume of 500 ml.
- Termamyl 300 L (Novo A/S Köpenhame)
- Amyloglucosidase (3500U/25 ml): 3500U is solved in 25 ml water is frozen in container.
- 0 – 1 M phosphate-buffer, pH 6.0: 12.1 g NaH_2HP0_4 x 2 H_2O is solved in 900 ml H_2O. pH is set to 6.0 and then solved to a volume of 1000 ml.

Fiber analysis

- Aspartic acid
- Termamyl 120 L (300) (Novo A/S, Köpenhame)
- Pepsin (100 mg/ml, 2000 FIP U/g, Merk, Darmstadt, Germany, Art. No. 1.07190.0100)
- Pancreatic enzymes (50 mg/ml, Activity equivalent to 8* USP specifications, sigma, ST. Louis, USA, Art No. P-7545)
- Sodium phosphate buffer (0.1 M, pH 6.0)
- HCI (0.2 N, 0.5 N) to adjust pH
- NaOH (5 N) to adjust pH
- ethanol (78%, 95% and 99%)

Hydrolysis index analysis

- 0.022 M Amylase buffer (tuff – buffer) Dissolve 15.15 g KH_2PO_4, 19.8 g Na_2HPO_4 and 2.0 g NaCl in 4,000 ml. H_2O. Adjust the pH to 6.9 and add water up to 5000 ml. make 10 1/time. Keep in +4 - +8 ºC

- Pepsis – solution (2000 FIB – U/g; MERCK, Darmstadt, Tyskland): Dissolve 5.0 g in 00 ml amylase buffer. Freeze in 7 ml-batches

- α-amylase (SIGMA A – 6255) Make new each analysis time, 30 min before the chewing.

- DNS – Solution: Dissolve 10 g 2 – hydroxy – 3, 5 – dinitrobensoesyra (MERCK 10846) and 300 g K – Na – tartrat – tetrahydrate ($C_4H_4KNaO_6$ x 4 H_2O) in 800 ml water, 16 g NaOH and water up to 1000 ml.

- 2 M NaOH

- 2 M HCI : Dilute 82.3 ml Concentrated HCI to 500 ml.

- Maltose – Standard (1 mg/ml): Dissolve 1,000 g maltos (dry) of 1,050 g maltose (wet) in 1000 ml buffer. Freeze in 10 ml – batches.