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Inulin-enriched pasta improves intestinal permeability and modifies the circulating levels of zonulin and glucagon-like peptide 2 in healthy young volunteers

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ARTICLE INFO

Article history:
Received 27 December 2011
Revised 28 June 2012
Accepted 21 September 2012

Keywords:
Glucagon-like peptide 2
Intestinal permeability
Inulin
Randomized double-blind crossover study
Zonulin
Human

ABSTRACT

Apart from the intestinal environment, inulin induces physiological effects, which includes a reduction in glucose and lipid concentrations and modulation of gastrointestinal motility through the release of different peptides. We hypothesized that inulin-enriched pasta may also improve small intestine permeability in relation to zonulin and glucagon-like peptide 2 (GLP-2) levels in healthy young subjects. Twenty healthy, young male volunteers completed a randomized, double-blind crossover study consisting of a 2-week run-in period and two 5-week study periods (11% inulin-enriched or control pasta), with an 8-week washout period in between. The intestinal barrier function was assessed by lactulose-mannitol excretion in urine. Zonulin values and GLP-2 release were evaluated by enzyme-linked immunosorbent assay. In the inulin group, the urinary lactulose recovery was significantly lower than the other 2 groups. There were no significant differences in urinary mannitol levels between groups. Accordingly, the lactulose-mannitol excretion ratio was significantly decreased in the inulin-enriched pasta group compared with the other 2 groups. The inulin-enriched pasta group had significantly lower zonulin serum values and significantly higher GLP-2 basal values when compared with the baseline and control pasta groups. The dietary use of inulin-enriched pasta preserves intestinal mucosal barrier functioning and modulates circulating levels of zonulin and GLP-2, suggesting that prebiotics could be used in the prevention of gastrointestinal diseases and metabolic disorders.

Abbreviations: AUC, area under the curve; ELISA, enzyme-linked immunosorbent assay; GI, gastrointestinal; GLP-2, glucagon-like peptide 2; IP, intestinal permeability; La, lactulose; NAFLD, nonalcoholic fatty liver disease; Ma, mannitol.

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http://dx.doi.org/10.1016/j.nutres.2012.09.010

1. Introduction

Under normal conditions, gut physiology is formed by the interaction between intestinal microbiota and the host's gastrointestinal (GI) functions including motility, absorption and secretion, and the mucosal barrier [1]. The available data suggest that alterations in the integrity of the mucosal barrier might be actively involved in not only inflammatory and autoimmune diseases [2] but also the onset of different metabolic disorders such as obesity, diabetes, and metabolic syndrome [3–5]. The function of the intestinal barrier is a dynamic parameter, promptly responding to different stimuli that range from the dietary state and inflammatory mediators to neuronal or humoral signals.

The protein zonulin is able to modulate the mucosal barrier by disassembling the tight intercellular junctions that characterize the early phase of inflammatory states [6]. This protein is involved in the innate immunity of the gut and, when deregulated in genetically susceptible individuals, appears to play a key role in the pathogenesis of different diseases including celiac disease and type 1 diabetes [7].

Another important molecule in the control of intestinal barrier function is glucagon-like peptide 2 (GLP-2). This peptide is secreted from enteroendocrine L cells in the small and large intestine and is an intestinotrophic growth hormone involved in the regulation of cell proliferation in the crypt compartment. In addition, GLP-2 functions in the inhibition of enterocyte apoptosis [8], enhances mucosal thickness, promotes nutrient absorption [9], and rapidly enhances mucosal barrier functions [10].

The gut barrier function can be evaluated clinically by intestinal permeability (IP) tests that use nonabsorbable sugars such as lactulose (La) and mannitol (Ma). Lactulose is a disaccharide that reflects the permeability of large molecules (0.93 nm), whereas Ma is a monosaccharide that can be considered a marker of absorption of small molecules (0.65 nm) [11]. Enhancing the mucosal barrier function could represent a way to improve health, starting at a young age [12].

Inulin is a carbohydrate that belongs to a class of compounds known as fructans. Inulin and oligofructose meet the definition of a prebiotic fiber, a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in GI microbiota [13]. In addition, it has been proven that this prebiotic has the ability to support intestinal barrier function [14]. Previous reports from studies on animal models demonstrated that inulin-type fructans induced changes in the intestinal mucosa that were characterized by higher villi, deeper crypts, and a thicker mucus layer on the colonic epithelium [15].

A series of research studies previously performed by our group in healthy young male volunteers demonstrated that inulin-enriched pasta caused a significant reduction in triglyceride and glucose levels and an increase in high-density lipoprotein cholesterol levels [16]. In addition, inulin caused a significant delay in gastric-emptying rates [17] and the release of some gut peptides involved in the control of GI motility (namely, neurotensin and somatostatin) [18]. Therefore, we hypothesized that the administration of inulin-enriched pasta to healthy young volunteers could affect small intestine permeability in relation to the circulatory and fecal levels of zonulin and the release of GLP-2.

2. Subjects and methods

2.1. *Experimental protocol*

The study featured a randomized double-blind crossover design consisting of a baseline assessment and two 5-week study periods (inulin-enriched pasta or control pasta diet: 100 g/d = 11.0 and 1.4 g/d of fructans, respectively) with a washout period (8 weeks) in between and a 2-week run-in period. The run-in period was included to bring all participants to similar levels with respect to baseline intake of insoluble and soluble fiber. Throughout the study (run-in and intervention periods), the subjects consumed a controlled diet and abstained from alcohol or heavy physical activity. All dietary instructions were provided by trained staff who interviewed each subject before the start of the study to obtain a report of their usual diet and calculate their energy requirements. The diet provided 50% of their total energy as carbohydrates (approximately 20% simple carbohydrate, mainly as fruit; approximately 30% complex carbohydrate, mainly as bread, potatoes, and pasta) and 35% as fats (approximately 12% each of saturated, monounsaturated, and polyunsaturated fatty acids) [19]. The saturated fatty acid sources were mainly butter, cheese, and meat. Edible plants with rich inulin contents were excluded. The subjects consumed 3 meals per day. Food and beverages were provided by the Institute's kitchen, and to ensure compliance, the refectory staff constantly verified the meals and food intake. Dietary surveys were conducted during the last week of each diet period. All subjects in the study wrote down any extra food consumed in their diets. The 2 diets only differed in terms of pasta composition.

2.2. *Subjects*

Students from the Istituto Tecnico Agrario "B. Caramia" in Locorotondo (BA), Italy, were enrolled in this study. Twenty healthy male volunteers were enrolled, and all completed the study. The average age was 18.8 ± 0.7 years. The average subject height was 176 ± 6 cm, and the average weight was 73.7±14.6 kg. The average body mass index was 22.8±2.3 kg/m^2 (means ± SD). The patients did not have dyspepsia or other GI diseases and were not taking any medication. Information on the health status of the subjects was obtained at the time of enrollment during an examination that consisted of an interview about their current diet, lifestyle, and medical history and a physical examination. Gastrointestinal symptoms were assessed using a specific questionnaire [20] and food records. The study exclusion criteria were as follows: body mass index outside the range of 20 to 25 kg/m^2, alcohol intake above 30 g/d, and smoking, hypertension, diabetes mellitus, and other pathologies (eg, systemic, endocrine, collagen-related diseases, familial hypercholesterolemia, and hypertriglyceridemia). The subjects had not consumed vitamins, minerals, nonsteroidal anti-inflammatory or

prokinetic drugs, antibiotics, bismuth, antacids, H2-receptor antagonists, omeprazole, sucralfate, or misoprostol within the 6 months before examination. The study participants had no history of gastric or duodenal ulcers or gastric surgery. The subjects were asked to complete a daily diary to evaluate symptoms (abdominal pain, abdominal bloating, and flatulence) on a 100-mm visual analog scale (VAS). The protocol was approved by the local ethics committee, and all subjects gave informed consent to take part in the study.

2.3. *Pasta making and pasta characteristics*

The formulation of the inulin-enriched pasta has been reported elsewhere [17,18]. Briefly, it contained 11% inulin from chicory (Raftline HPGel), which was supplied by Orafti (Tienen, Belgium). The average degree of polymerization was more than 23 units of fructose or glucose.

The standard pasta was composed of 100% durum wheat semolina. Each pasta formulation was made up into long pasta (spaghetti) and short pasta (rigatoni) in a pilot pasta-making plant (Namad, Rome, Italy). Soluble, insoluble, and total dietary fiber was quantified by the enzymatic gravimetric procedure [21]. The caloric energy was calculated as kcal and kJ: proteins, 4 kcal/g (17 kJ/g); carbohydrates, kcal/g (17 kJ/g); lipids, 9 kcal/g (37 kJ/g); and dietary fiber, 2 kcal/g (8 kJ/g) [22]. The chemical composition (in percent weight) and energy contents of the control pasta and the inulin-enriched pasta are reported in Table 1.

2.4. *Sugar absorption tests*

For the IP tests, a test solution was made containing 10 g La and 5 g Ma and dissolved in 100 mL of water. The subjects drank the test solution in the morning after an overnight fasting, and all urine was collected for the subsequent 5 hours. Urine samples were stored at –20°C until analysis.

The detection and measurement of 2 sugar probes, La and Ma, in urine were performed by chromatographic analysis, as described by Generoso et al [23], with a minor modification. Briefly, high-performance anion-exchange chromatography, coupled with pulsed amperometric detection, was performed on a Dionex Model ICS-5000 with a gold working electrode and a 25-μL peek sample loop (Dionex Corp, Sunnyvale, CA, USA). The carbohydrate separation was performed by a Carbopac PA-10 pellicular anion-exchange resin connected to a Carbopac PA-10 guard column at 30°C. The samples were eluted with 50 mM NaOH at a flow rate of 1 mL/min. The percentage of ingested La (La%) and Ma (Ma%) in urine was evaluated, and the ratio (La/Ma) was calculated for each sample.

Table 1 – Proximate analysis composition [a] and energy content of control pasta and inulin-enriched pasta [b]

	Control pasta	Inulin-enriched pasta
Moisture	12.5	11.8
Ash	0.90	0.59
Proteins	12.5	11.5
Fats	1.9	2.0
Dietary fibers		
Insoluble	2.3	3.1
Soluble	0.8	1.7
Total	3.1	4.8
Carbohydrates	67.7	58.3
Fructans	1.4	11.0
Energy per 100 g		
kcal	346	323
kJ	1468	1368

[a] Amounts are expressed as percentage of the weight.
[b] Pasta enriched with 11% of inulin.

2.5. *Zonulin evaluation*

Serum and fecal levels of zonulin were assayed using the zonulin enzyme-linked immunosorbent assay (ELISA) kit (Immunodiagnostik, Bensheim, Germany) [24]. The fecal samples were collected in plastic containers and immediately stored at –20°C. The raw stool samples were mechanically homogenized by an inoculation loop, and then a commercially available stool sample preparation system (Stool Sample Application System; Immunodiagnostik, Bensheim, Germany) was used to obtain a defined stool sample (approximately 15 mg in stool buffer solution) for further zonulin assays.

2.6. *GLP-2 evaluation*

After overnight fasting, basal blood samples were obtained from the subjects in the study. Sequential blood samples were obtained at 15, 30, 60, 120, and 180 minutes after the supply of a mixed solid/liquid standard meal consisting of 55% carbohydrates, 30% protein, and 15% fat (caloric content 513 kcal). This meal was consumed within 15 minutes.

Blood samples were collected in ice-chilled tubes containing 500 KIU/mL of aprotinin (100,000 KIU-MP Biomedicals, LLC, OH, USA) and 1.0 mg EDTA/mL blood. The samples were centrifuged at 1600 × *g* for 15 minutes at 4°C, and the separated plasma was stored at –70 °C until assayed. The plasma levels of total GLP-2 were measured by ELISA using a commercial kit (Millipore Co, Billerica, MA, USA) [22]. The total integrated response was calculated as the area under the curve (AUC) to give an approximation of the total GLP-2 release over the observation period.

2.7. *Statistical analyses*

The sample size calculations were based on the data from our previous research [16–18]. There were 20 patients enrolled and analyzed in this study. This number of patients was greater than necessary because the probability that the study would detect a treatment difference with a 2-sided .05 significance level equal to 80% required enrolling only 17 subjects. This calculation was based on the assumption that the true difference between the treatments was 20% of urinary recovery of La, and the standard deviation of the difference was 27%.

To avoid the assumption of normal distribution, the data were expressed as medians and interquartile ranges, unless otherwise specified. The nonparametric tests were performed using a specific software package (StataCorp 2005; Stata Statistical Software: Release 9, College Station, TX, USA). The Wilcoxon matched pairs test was used to analyze the

Table 2 – Daily macronutrient intake in 20 healthy subjects during the 2 diet periods [a]

	Control pasta [b]	Inulin-enriched pasta [b]	P [c,d]
Energy intake(kcal/d)	2860 ± 685.0	2840 ± 851.5	NS
Proteins (% of energy)	15.1% ± 1.9	15.3% ± 2.3	NS
Carbohydrates (% of energy)	48.6 ± 7.6	49.6 ± 8.7	NS
Fats (% of energy)	30.9 ± 5.9	32.7 ± 6.9	NS
Fibers (g/d)	32.1 ± 7.4	30.2 ± 6.4	NS

NS, not significant.
[a] All values are presented as mean ± SD.
[b] The dietary intakes as calculated from the dietary surveys conducted during the last week of each diet period of a randomized crossover study design. During the study periods, food plants with rich inulin contents were excluded from diet.
[c] P value for the effect of treatment between the 2 diets.
[d] P value for Wilcoxon matched pairs test.

differences in the daily dietary intake during the 2 diet periods. The Friedman repeated-measures analysis of variance on ranks was used to compare the effects of different dietary treatments. When a difference was found, nonparametric multiple comparisons were performed (Dunn post hoc test) to determine the groups that had significant differences between them. The total integrated response was calculated as the AUC to obtain overall values for the GLP-2 peptide over time. The correlations were investigated using the Spearman correlation test. All differences were considered significant at the 5% level.

3. Results

3.1. Dietary intake

The dietary intake of the subjects, as calculated from the dietary surveys conducted during the last week of each diet period, is reported in Table 2. There were no significant differences between the 2 diets (Wilcoxon matched pairs test). All subjects followed the diets without any major exceptions. According to the questionnaires on GI symptoms, no subject complained of adverse effects related to inulin administration, such as flatulence, meteorism, or postprandial fullness. There were no changes in bowel habits recorded during the study.

3.2. Sugar urinary excretion tests

There were statistically significant differences found in the small intestine permeability (medians and the range) between the baseline (0.71% and 0.65%-1.20%), control pasta (0.75%; 0.58%-1.05%), and inulin-enriched pasta (0.45%; 0.36%-0.77%) diets in the percentage of urinary La ($P = .0001$, data analyzed by Friedman repeated-measures analysis of variance). All paired multiple comparison post hoc tests assessed the significance of differences between the inulin-enriched pasta diet versus baseline and control groups ($P < .05$, Dunn post hoc test; Fig. 1A). There was no significant difference found in the percentages of urinary Ma between the 3 groups (Fig. 1B). Accordingly, the La/Ma excretion ratio significantly differed between the baseline (0.05; 0.02-0.10), control pasta (0.05; 0.04-0.09), and inulin-enriched pasta (0.03; 0.02-0.05) diets ($P = .0012$, data analyzed by Friedman repeated-measures analysis of variance). In addition, all paired multiple comparison post hoc tests assessed the significance of differences between the inulin-enriched pasta diet versus the baseline and control groups ($P < .05$, Dunn post hoc test; Fig. 1C).

3.3. Zonulin

As reported in Fig. 2A, the zonulin serum values (medians and the range) were lower in the inulin-enriched pasta group (3.63 ng/mL; 2.69-4.39 ng/mL) compared with the baseline (5.24 ng/mL; 4.47-6.75 ng/mL) and the control pasta groups (5.59 ng/mL; 4.99-7.23 ng/mL) ($P = .013$; data analyzed by Friedman repeated-measures analysis of variance). A significant difference was found between the inulin-enriched pasta, baseline,

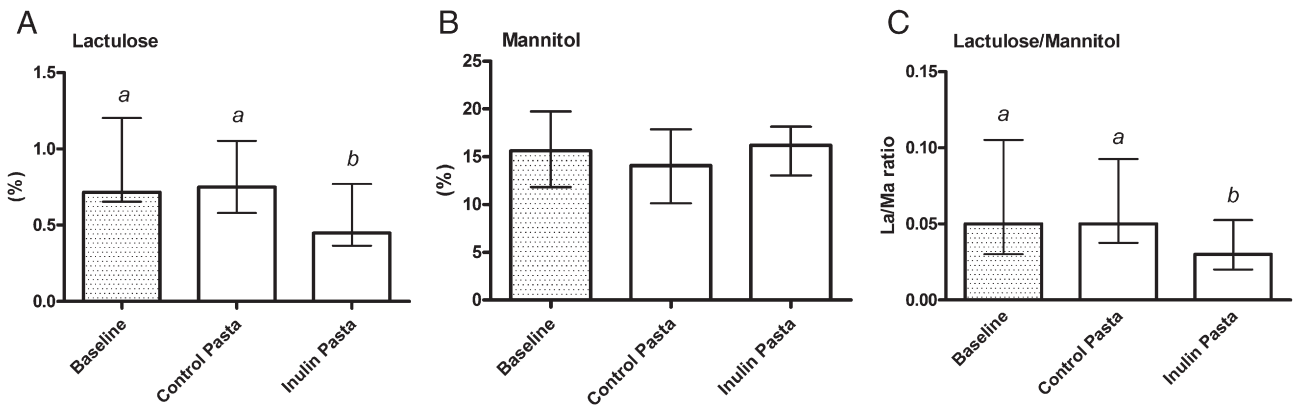

Fig. 1 – Improved IP in the inulin pasta group. Small IP was probed by measuring the urinary cumulative 5-hour amount of La (percentage of ingested), Ma, and the La/Ma ratio in 20 healthy young volunteers. All values are medians and the range. Bars showing a different letter differ significantly ($P < .05$; Friedman repeated-measures analysis of variance with Dunn post hoc test).

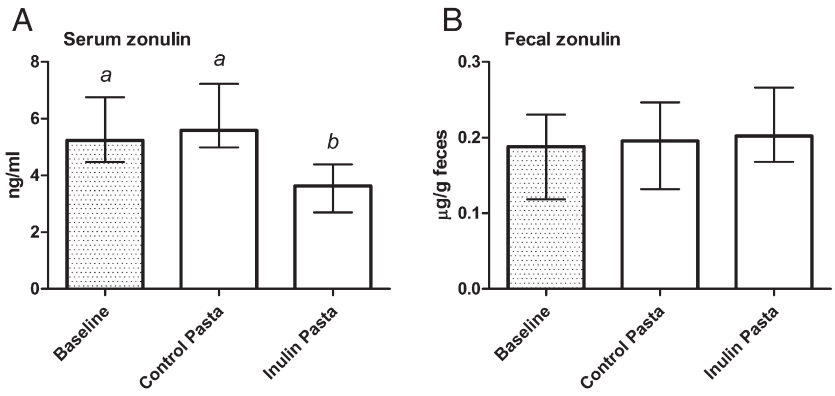

Fig. 2 – Change from baseline in serum and fecal zonulin levels in response to control pasta and inulin-enriched pasta in 20 healthy young volunteers. All values are medians and the range. Bars showing a different letter differ significantly ($P < .05$; Friedman repeated-measures analysis of variance with Dunn post hoc test).

and control pasta groups ($P < .05$; Dunn post hoc test). In the fecal samples (Fig. 2B), the zonulin values in the inulin-enriched pasta group (0.20 μg/g feces; 0.17-0.27 μg/g feces) were not different from either the control pasta group (0.19 μg/g feces; 0.13-0.25 μg/g feces) or the baseline group (0.19 μg/g feces; 0.12-0.23 μg/g feces).

3.4. *Glucagon-like peptide 2*

The total GLP-2 content (median and the range) in blood samples obtained after overnight fasting was higher in the inulin-enriched pasta group (5.10 ng/mL; 4.38-8.05 ng/mL) compared with the baseline (4.85 ng/mL; 3.95-6.40 ng/mL) and the control pasta groups (4.99 ng/mL; 3.97-6.65 ng/mL) ($P = .0044$; data analyzed by Friedman repeated-measures analysis of variance). The difference was significant between the inulin-enriched pasta, baseline, and control pasta groups ($P < .05$, Dunn post hoc test; Fig. 3A).

Fig. 3B shows the AUC values of GLP-2, representing the total release of the peptide over the observation period, after a standard meal in the subjects at baseline and after the 2 dietary periods (control pasta and inulin-enriched pasta) of the study. The AUC of the GLP-2 levels was not significantly different in the inulin-enriched pasta group compared with the other 2 groups. Finally, no significant correlation was found between zonulin levels, GLP-2 release, and IP, as evaluated by the urinary excretion tests (data not shown).

4. Discussion

In this controlled crossover study, we demonstrated that the administration of inulin-enriched pasta to healthy young volunteers improved small intestine permeability in relation to changes in the circulatory levels of zonulin and the release of GLP-2. Our previous work has demonstrated that the administration of pasta enriched with this prebiotic induces a significant reduction in triglyceride and glucose levels along with a significant delay in gastric-emptying rates [16–18]. The current study indicates an effect in regulating intestinal barrier functioning so that inulin could have multilevel mechanisms in protection from the metabolic syndrome [25]. Adding inulin to pasta may be a suitable strategy because it would represent a minimal change in the diet of the Western population and could be maintained for a long period.

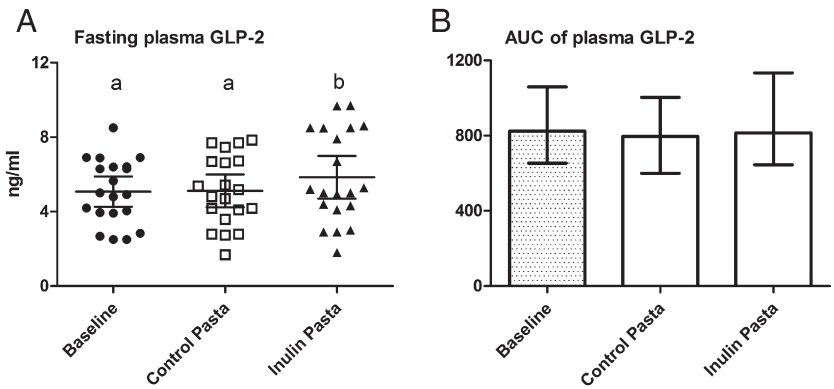

Fig. 3 – Changes from baseline in basal values obtained after an overnight fasting and total release of the GLP-2 over the time of observation as the AUC after a standard meal (B) in response to control pasta and inulin-enriched pasta in 20 healthy young volunteers. All values are medians and the range. Bars showing a different letter differ significantly ($P < .05$; Friedman repeated-measures analysis of variance with Dunn post hoc test).

The permeability of the small intestine was assessed by the use of nonabsorbable sugars such as La and Ma. These sugars represent ideal compounds for measuring differential sugar absorption because they have a negligible affinity for the monosaccharide transport system, and they are passively absorbed and not metabolized before urine excretion [26]. In the presence of mucosal damage, La and Ma can cross the epithelium of the small intestine and eventually appear in the urine [27]. In some pathological conditions (eg, Crohn disease), La IP is increased, whereas the permeability of Ma is unchanged or even decreased [28].

The significant reduction in the La/Ma ratio in the inulin group, relative to the baseline and control pasta groups, appeared to be exclusively determined by reduced La concentrations. This result is consistent with the involvement of processes regulating macromolecular passage through the paracellular pathway.

Furthermore, the addition of inulin-enriched pasta to a normal diet caused a significant reduction in the circulating levels of zonulin compared with baseline and control pasta levels. Where permeability is increased, a common pathophysiological event is the up-regulation of zonulin secretion from a lamina propria source into the lumen with inappropriate activation of this pathway. The result is increased paracellular permeability. Therefore, some intestinal bacterial strains selected by prebiotic administration could improve IP by increasing the intercellular integrity of tight apical junctions by preventing tight junction protein redistribution [25] [29]. In addition, intestinal bacteria could also affect other molecules involved in tight junction function, such as Zo-1 [30], thereby stopping the passage of molecules into the lamina propria. Conversely, no modifications were found in fecal zonulin after prebiotic administration. It is possible the active zonulin that has permeating activity is cleaved into an inactive form in the gut lumen; therefore, our ELISA assay was not able to detect the signal [31].

The circulating levels of total GLP-2 were significantly increased in the inulin pasta group compared with the baseline and control pasta groups. However, the release of this peptide did not change enough to induce a significant modification in the AUC values after meal stimulation. This result is most likely because the release of the gut peptide is very fast after meal stimulation, reaching its peak within the first 15 minutes [32]. The present results from our study cannot be compared with other results in the literature; therefore, we have to be very cautious in the interpretation of the actual role of GLP-2 in humans after prebiotic administration.

Studies in vitro and on animal models have previously connected an altered microbiota composition with the development of obesity, insulin resistance, and diabetes in the host. There are several possible mechanisms for this result, including the modulation of intestinal barrier integrity by GLP-2 secretions [33].

Recently, Cani et al [34] assessed the effect of oligofructose on gut microbiota, IP, and hepatic and systemic inflammation in ob/ob mice. The prebiotic enriched diet increased intestinal lactobacilli and bifidobacteria and preserved intestinal barrier function. These effects were related to increased production of intestinal GLP-2. This result suggests that GLP-2 might mediate the benefits of prebiotics.

Our study was performed on healthy young volunteers. As reported elsewhere [18], the choice of this cohort of subjects was initially supported by the evidence that metabolic disorders can start at a young age. Therefore, early control of metabolic variables should be recommended. Growing evidence suggests that the initial years of life could have a substantial impact on an individual's gut microbiota composition [35]. The microbiota might subsequently affect the future response of overweight individuals in dieting for weight loss [36]. Cumulatively, these findings suggest that knowledge of the factors that modulate gut microbiota composition early in life may have therapeutic or prophylactic implications for adult metabolic disorders.

There are some limitations to our study. It should be emphasized that only a small number of healthy young male subjects were studied over a relatively short period. However, regarding sample size, this study was a crossover design so the power was adequate to detect treatment differences. In addition, we evaluated only the small intestine permeability by means of La and Ma administration. We did not use other probes such as sucralose, which is indicated for evaluating colonic permeability. This limitation, in conjunction with our lack of fecal short-chain fatty acid data, prevents us from drawing firm conclusions on the modifications of intestinal microbiota composition and end results.

However, our data also has several strengths. The data from the present study are consistent with data from other studies and our previous results. Our results suggest further consideration of using inulin-enriched pasta as a possible tool for treating metabolic disorders. At the chosen dose of inulin (11%), no adverse effects or GI symptoms were recorded, and compliance to the study scheme was very high. The dietary intervention in the present study was negligible because a normal diet including the daily intake of pasta was maintained. The dietary use of inulin-enriched pasta could be a promising approach for improving and supporting epithelial barrier function, through its prebiotic actions. This may offer the host protection against the invasion and translocation of pathogens (endogenous and/or exogenous) and prevention of not only GI diseases but also metabolic disorders.

Acknowledgment

This work was supported by a grant from the Italian Ministry of Health (No. 132). The authors thank MS Benedetta D'Attoma for her precious technical assistance.

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