Inulin and fructoligoosaccharide affect in vitro calcium uptake and absorption from calcium-enriched gluten-free bread.

Journal:	*Food & Function*
Manuscript ID	FO-ART-02-2016-000140.R1
Article Type:	Paper
Date Submitted by the Author:	19-Feb-2016
Complete List of Authors:	Krupa-Kozak, Urszula; Institute of Animal Reproduction and Food Research of Polish Academy of Scienses, Department of Chemistry and Biodynamics of Food Świątecka, Dominika; Institute of Animal Reproduction and Food Research of Polish Academy of Scienses, Department of Immunology and Microbiology of Food Bączek, Natalia; Institute of Animal Reproduction and Food Research of Polish Academy of Scienses, Department of Chemistry and Biodynamics of Food Brzóska, Małgorzata; Medical University of Białystok, Department of Toxycology

Journal Name

ARTICLE

Inulin and fructoligoosaccharide affect *in vitro* calcium uptake and absorption from calcium-enriched gluten-free bread

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

U. Krupa-Kozak, *[a] D. Świątecka, [b] N. Bączek [a] and M.M. Brzóska [c]

Compromised intestinal calcium absorption affecting a deterioration of bone state is a sign of coeliac disease. Experimental calcium-fortified gluten-free bread (GFB) of improved calcium bioavailability could increase calcium content in coeliac patients' diet, allowing them to obtain the amount of calcium they need for the therapeutic use. Prebiotics, including inulin-type fructans (IFs) have a beneficial effect on calcium bioavailability. In the present study, the *in vitro* model composed of the intestinal-like Caco-2 cells and the human intestinal bacteria (*Lactobacillus*, *Enterococcus* and *Enterobacteriaceae)* was used to analyse the effect of inulin and fructooligosaccharide (FOS) of different chain length, on calcium uptake and absorption from experimental GFB. Analysed IFs, especialy short-chain FOS , significantly (p<0.05) increased a cellular calcium uptake from GFB digest and stimulated the intestinal bacteria applied in the cultures to the intensive synthesis of organic acids. In particular, the concentration of butyric, valeric and lactic acids increased significantly. Similarly, in the calcium absorption experiment, IFs increased the cellular calcium retention but concomitantly reduced its content in basolateral filtrates. Results obtained suggest that applied IFs affected differentially calcium uptake and absorption from the experimental calcium-enriched GFB, therefore a further study is needed to assess whether these observations made *in vitro* contribute to IFs effects on calcium absorption from experimental GFB *in vivo*.

1 Introduction

In coeliac disease (CD), which is an immune-mediated systemic disorder elicited by gluten in genetically-susceptible individuals, the autoimmune response is mainly targeted at the intestinal mucosa, therefore the rearrangement of the duodenal mucosa is a key feature of CD and the gastrointestinal symptoms are observed. However the clinical presentation of CD varies considerably. Calcium deficiency and metabolic bone diseases are a frequent co-morbidity of CD. They are mainly due to the impaired calcium and vitamin D absorption resulting principally from the chronic inflammation and loss of villous cells in the proximal intestine, where calcium is most actively absorbed. Steatorrhoea, alterations in the calcium-transport mechanisms, and lack of vitamin D are the additional factors that lead to calcium malabsorption. Dietary vitamin D is absorbed through the small intestine as a fat-soluble vitamin along with dietary fat and is incorporated into chylomicrons. The primary etiology of vitamin D deficiency in CD is malabsorption, however, an intestinal mucosal lesion is also a decisive issue of hypovitaminosis D. Moreover, the inflammatory diseases, including CD, are characterised by the chronic release of pro-inflammatory cytokines by immunologically competent cells. Osteotropic cytokines are involved in both normal and abnormal bone remodelling, and enhanced cytokine production in chronic inflammatory diseases is associated with increased bone loss. Approximately 75% of the newly diagnosed CD patients have reduced bone mineral density (BMD)[1], therefore, osteopenia and osteoporosis need be considered as signs of atypical CD presentation. Strict and lifelong adherence to a gluten-free diet (GFD) is still the only proven treatment for CD. GFD can help children and adolescents to recover normal bone mineral density;[2] however, it rarely normalizes bone density in adulthood.[3,4] Despite long-term strict adherence to GFD, 74% of CD patients displayed low BMD; among these 24% showed osteoporosis and 76% osteopenia.[5] Additionally, gluten-free formulas and baked products are poor in minerals, including calcium.[6] Therefore, the consumption of calcium-fortified gluten-free products, including calcium-enriched bread could increase dietary calcium intake and potentially increase calcium content in a daily diet of CD patients, allowing them to obtain the amount of calcium that they need for prophylactic or therapeutic use.

[a] *Department of Chemistry and Biodynamics of Food, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima 10 St., 10-748 Olsztyn, Poland. E-mail: u.krupa-kozak@pan.olsztyn.pl; Fax: +48 89 5240124; Tel: +48 89 5234618*
[b] *Department of Immunology and Food Microbiology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland*
[c] *Department of Toxicology, Medical University of Białystok, Białystok, Poland*

Calcium is an essential mineral that plays the important structural (in the skeleton and teeth) and regulatory (muscle contraction, blood clotting, nerve conduction, enzyme cofactor) functions. Certain dietary factors impact intestine calcium absorption and bioavailability. Soluble fibres may have negative or positive effects on calcium absorption. Supplementation of dairy infant formulas with locust bean gum and high esterified pectin reduced calcium availability, but inulin supplementation increased it by 30 %.[7] The ability of inulin-type fructans (IFs) to improve calcium absorption from the diet as well as to increase bone mineral density and bone structure is well documented both in animal and human studies.[8-10]. Notwithstanding, the exact mechanism by which IFs stimulated the intestinal calcium absorption is uncertain.

Inulin and FOS (fructooligosaccharide) have gained increasing attention in the nutrition field due to their beneficial health effects. They belong to prebiotics, defined as selectively fermented ingredients that allow specific changes in the composition and/or balance of the microbiota.[11] Prebiotics act as a carbon and energy sources for selected bacteria in the large bowel, where they are fermented to short chain fatty acids (SCFAs) and other organic acids (e.g., lactic acid), leading to the increase in mineral solubility in the luminal phase, which can then be readily absorbed from the gut.[12] In addition, via indirect stimulation of the production of butyrate and/or certain polyamines, prebiotics might induce histological and functional changes in the intestinal epithelium. Their effect is accompanied by modifications of the architecture of the intestinal mucosa that arise as a consequence of the increase in cellularity and the number of crypts or of mechanisms that could contribute to an increase in the absorptive surface for minerals uptake.[12]

Inulin is a fructose polymer consisting of 8 to 10 or more fructose units, while chains of fructose with 2 to 10 units, produced by hydrolysis of inulin, are categorized as FOS. Inulin and FOS are resistant to human alimentary digestive enzymes. In general, they show also a good stability during the usual food processes nevertheless, the 1,2-β-glycosidic bonds are sensitive towards treatment with acid or heat. The use of inulin and FOS in gluten-free bakery products is reported but very limited information exists on their stability during thermal treatment, like baking.[13] Physiological effects of fructans are determined by their structure thus, the differences in fermentability, and transit time have been influenced by chain length.[14,15] Therefore, the aim of the present study was to analyse the effect of two unprocessed IFs of different chain length, inulin and FOS, or their mixture on calcium uptake and absorption from the experimental calcium-enriched gluten-free bread (GFB). To conduct both uptake and absorption experiments, Caco-2 cell line derived from human colon adenocarcinoma was chosen because it is capable of enterocytic differentiation[16] and is commonly accepted as the *in vitro* model for calcium bioavailability assessments.[17] As physiological effects of IFs are microbiota-mediated, therefore in both experiments a Caco-2 cells monolayer was apically treated with the suspension of the human intestinal bacteria (enterobacteria, lactobacilli and enterococci). We hypothesised that the addition of inulin and/or FOS to the experimental calcium-enriched GFB digest will stimulate the intestinal bacteria applied in the culture to the intense production of organic acids which will facilitate calcium absorption and transport through the model epithelial barrier.

2 Materials and methods

2.1 Gluten-free bread enriched in calcium

Calcium-enriched experimental gluten-free bread (GFB) was prepared under laboratory conditions according to the previously published recipe[18] with slight modification. Briefly, potato starch (160 g) and pectin (40 g) were added successively to corn starch (660 g) and mixed together with calcium supplements: 25 g of calcium citrate (E 333(iii), Hortimex, Konin, Poland) and 175 g of calcium caseinate (PHZ SM Lacpol, Murowana Goślina, Poland). Subsequently, sugar (50 g), fresh yeast (50 g) and salt (14 g), previously dissolved separately in deionised water (1000 mL), were added to the dry mixture together with oil (26 g). After mixing, the resulting batter was proofed for 20 min (35°C/ 70% relative humidity). Then, the batter was divided into 200-g samples, placed in baking tins and proofed for another 20 min under the same conditions. Baking was carried out for 20 min at 220 ºC in a laboratory oven (Sveba Dahlen AB, Sweden) integrated with a proofing chamber. GFB baking was conducted three times. Each time the obtained GFBs (9 loaves) were cooled to room temperature (2 h), then all bread loaves were crumbled and dried (at RT). Dry GFB crumbs were combined, disintegrated, and a 100-g homogenous sample was freeze-dried (Labconco, USA), milled and sieved (mesh 0.40 mm) to obtain a powder.

2.2 Enzymatic *in vitro* digestion

All chemicals and digestive enzymes were obtained from Sigma-Aldrich (Poznań, Poland). Prior to each experiment, the GFB was subjected to *in vitro* digestion.[19] To prepare a pepsin solution, 1.6 g of pepsin (porcine) was suspended in 10 mL of 0.1 N HCl; whereas for the pancreatin/bile extract solution, 0.2 g of pancreatin (porcine) and 1.25 g of bile extract (porcine) were dissolved in 50 mL of 0.1 M $NaHCO_3$. The working enzyme solutions were prepared immediately before use. All glassware was washed with detergent and concentrated nitric acid (24h), then rinsed with distilled deionised water before use.

Enzymatic *in vitro* digestion was conducted according to the procedure described by Frontela et al.[19]; briefly, deionized water (80 mL) was added to 10 g of freeze-dried GFB powder. To initiate the gastric digestion step, the pH of the water suspension of GFB was adjusted to 2.0 with 6 N HCl, and 3 mL of a freshly prepared pepsin solution were added. The sample was filled to 100 g with deionised water and incubated in a shaking water bath at 37 °C for 2 h. To stop the pepsin hydrolysis, the gastric digest was placed in ice for 10 minutes. Prior to the intestinal digestion step, the pH of the gastric digest was raised to 5 by dropwise addition of 1 M $NaHCO_3$.

Then, 25 mL of the pancreatin/bile extract mixture was added and the incubation was continued for additional 2 h. To stop the intestinal digestion, the sample was maintained on ice. After 10 min of cooling, the pH of the digest was adjusted to 7.2 by the dropwise addition of 0.5 M NaOH. Then, the digest was heated for 4 min at 100 °C to inhibit proteases, and immersed in an ice bath to cool. Samples were transferred to polypropylene centrifuge tubes (100 mL) and centrifuged at 3500 × *g* and 4 °C for 1 h (MPW-350R, NAPMED, Warsaw, Poland). The obtained supernatant of GFB digest was decanted. A 20-mL parts of GFB digest were mixed with the appropriate amount of inulin (Frutafit HD, Hortimex, Poland), or FOS (Orafti^R P95, Hortimex, Poland), or their mixture (1:1, w/w) respectively, to obtain a 7 % solution (v/w) of IFs, whereas one part, the control, contained no IFs. Control, GFB digest with inulin (I), GFB digest with FOS (FOS), and GFB digest with inulin and FOS (I/FOS) were applied in the Caco-2 calcium uptake and absorption experiments described below (Section 2.4), additionally a 2-mL portion of each digest was kept frozen (- 80 ºC) until calcium content determination (Section 2.6).

2.3 Preparation of human intestinal bacteria suspension

According to the opinion of the Local Ethical Commission, the experimental design of the studies is not invasive to a human and does not bear either the characteristics of medical experiment or clinical trial and, therefore, the studies did not require formal approval of the Commission. All adult, healthy volunteers were informed about the aim of investigations and provided written consent to participate in the study.

Fresh stool samples were collected anonymously from five healthy adult volunteers and frozen at -20 ºC until analysed. In the experiment, stool samples were used as a source of bacteria: *Lactobacillus*, *Enterococcus* and Enterobacteriaceae, as the main bacterial representatives colonising the small intestine region.[20] The stool samples were transferred into the anaerobic workstation (MG500 Don Whitley Scientific, Shipley, West Yorkshire, UK) where they were suspended in sterile peptone water (1:10 w/v) and homogenised for 2 minutes with glass beads. One-hundred microlitres of a particular stool suspension were transferred onto the solid media to obtain single colonies. Various solid media were used in the experiment to obtain particular bacterial isolates: Rogosa medium – for the isolation of *Lactobacillus*; MacConkey medium – for the isolation of *Enterobacteriaceae*; and KEA (kanamycin esculin agar) medium – for the isolation of *Enterococcus*. Plates were incubated at a temperature of 37 °C aerobically or anaerobically, depending on the bacterial oxidative requirements. After incubation, single colonies (approximately 5-15 colonies), belonging to a particular bacterial group: lactobacilli, enterococci and enterobacteria, chosen on a macro- and microscopic basis, were harvested and suspended in 1 mL of sterile, anaerobic phosphate-buffered saline (PBS). The cultures were centrifuged (5804R, Eppendorf, Warsaw, Poland,) at 9500 x *g* for 5 min, and each obtained pellet was re-suspended in 1 mL of Minimum Essential Eagle's Medium (MEM) (Sigma-Aldrich) and agitated for 1 min. Then, the appropriate volume of each of those MEM-bacterial solutions was mixed together to obtain an intestinal bacterial suspension of a final concentration of 10^7 cells/mL. Subsequently, the intestinal bacterial suspension was used in the Caco-2 calcium uptake and availability studies.

2.4 The *in vitro* calcium uptake and absorption experiments

The Caco-2 cell line was obtained from the American Type Culture Collection (Sigma-Aldrich, Poznan, Poland) and used as a model of small intestinal enterocytes.[21] Cells were cultured in 75 cm^2 plastic flasks (Becton Dickinson, Poland) in the MEM medium supplemented with 20 % (v/v) inactivated foetal bovine serum (FBS) (Gibco, Life Technologies, Warsaw, Poland), 1 % (v/v) non-essential amino acid mixture (Gibco,), 1 % (v/v) L-glutamine (Sigma-Aldrich), and 0.5 % (v/v) of penicillin/ streptomycin (Gibco). The incubation was conducted at 37 °C in 5 % CO_2/ 95 % air, with 95 % relative humidity. For cells maintenance, the medium was changed every 2 days.

Uptake buffer (130 mM NaCl, 10 mM KCl, 1 mM $MgSO_4$, 5 mM glucose, and 50 mM HEPES; pH 7) and a calcium stock solution, containing aqueous 10 mM $CaCl_2$ in 1 mM HCl, were freshly prepared.[19] The appropriate volume of the calcium stock solution was added to the uptake buffer to obtain 25 mL of a calcium standard solution of 5 mM calcium concentration. The uptake buffer and calcium standard solution were incubated at 37°C until the beginning of the experiments.

For the uptake experiment, Caco-2 cells (at passage 67) were seeded at the concentration of 5.0×10^4 cells/well on 12-well plates (Becton Dickinson, Warsaw, Poland) (Fig. 1). Based on microscopic examination (CKX41, Olympus, Warsaw, Poland), the cells formed a confluent monolayer after 3-4 days and were allowed to grow and differentiate for another 19 days.[22] The cells were imaged by an inverted Nikon Eclipse TE300 microscope. At least 24h before the calcium uptake experiment, MEM with antibiotics was replaced by MEM without antibiotics. At the day of uptake experiment, the medium was removed and cell monolayers were washed with sterile PBS (37 ºC), next with calcium and a magnesium-free Hank's balanced salt solution. Then, Caco-2 cell monolayers were treated apically with the human intestinal bacterial suspension (0.5 mL/well) and co-incubated at 37 ºC for 2 h. Afterwards, the appropriate volumes (0.8 mL) of the uptake buffer used as a blank, or a calcium standard solution, or GFB digest without IFs used as the control, or GFB digests with IFs (I, FOS or I/FOS), respectively were added to apical chambers. Calcium cellular uptake was analysed after 2h-incubation at 37 ºC. Media surrounding the Caco-2 cultures from apical chambers were collected in order to analyse the content of SCFAs. Then, cell monolayers were washed firstly with a sterile PBS (37 ºC), followed by washing with an ice-cold uptake

buffer. Then, the cells were lysed and harvested by adding 1 mL of 2 % (w/v) sodium dodecyl sulphate (SDS) and kept frozen (-80 °C) until the calcium content determination.

Calcium uptake was estimated as follows:

$$\text{Ca uptake (\%)} = 100 \times ((C2-C1)/S)$$

where:
C1 - calcium content of cultures incubated with uptake buffer (blank),
C2 - calcium content of cultures incubated with samples (Control, I, FOS or I/FOS),
S - soluble calcium added onto the cell monolayers.

For transepithelial transport experiment, Caco-2 cells were seeded (5.0×10^4 cells/well) on hanging inserts (12 mm well diameter, 113 mm^2 membrane area, 0.4 µm mean pore size; Millipore, Merck, Poland) placed at the 12-well plates (Figure 1). The apical and basolateral compartments contained 0.5 mL and 1.5 mL of MEM medium, respectively. As previously, the confluent Caco-2 monolayers were allowed to grow and differentiate (19 days). One day before the transport experiment, MEM without antibiotics were replaced by MEM with antibiotics. On the day of the experiment, cell monolayers were washed with a sterile PBS (37 °C) and a calcium and magnesium free Hank's balanced salt solution, then incubated with the human intestinal bacterial suspension (0.5 mL/ apical chamber) at 37 °C for 2 h. Like before, 0.8 mL of blank, or a calcium standard solution, or control, or GFB digests with IFs (I, FOS or I/FOS), respectively was added to the monolayers and incubated for another 2h at 37 °C. Afterwards, the medium was removed and Caco-2 monolayers were washed with a sterile PBS (37 °C), next with an ice-cold uptake buffer. Both, the cells (harvested with 2 % SDS) as well as the basolateral filtrates were secured (-80 °C) for further AAS analysis. Calcium absorption was calculated as follows:

$$\text{Ca absorption (\%)} = 100 \times ((C2-C1)+(T2-T1)) / S$$

where:
C1 – calcium content of cultures incubated with uptake buffer (blank),
C2 – calcium content of cultures incubated with samples (Control, I, FOS or I/FOS),
T1 – calcium content in basolateral filtrate of culture incubated with blank,
T2 – calcium content in basolateral filtrates of cultures incubated with Control, I, FOS or I/FOS,
S – soluble calcium added onto the cell monolayers.

In both experiments, at least three wells were examined per treatment. Cells viability was assessed by a trypan blue exclusion test (Sigma-Aldrich) after application of both a bacterial suspension or GFB digests, and was typically 80-95 %. Cells were recounted using a Millipore scepter (Scepter™ 2.0 Cell Counter, Merck Millipore). To assess the integrity of cell culture grown on hanging inserts Caco-2 cells monolayer was imaged by an inverted microscope (Nikon Eclipse TE300) and a monolayer transepithelial electrical resistance (TEER) was measured (Millicell ERS-2 Volt-Ohm Meter, Merck Millipore, Poland) at room temperature before and upon the 2-h incubation with the human intestinal bacterial suspension and uptake buffer (blank culture). Only the inserts whose TEER value reminded unchanged and higher than 600 Ω/cm^2 were used for the calcium availability study.

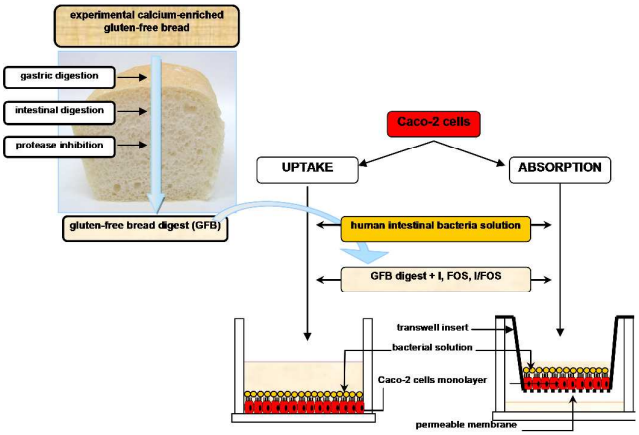

Fig. 1 The design of the in vitro calcium uptake and absorption experiments.

2.5 Short chain fatty acids

The reverse phase high performance liquid chromatography (RP-HPLC-PDA-UV) was applied to determine the composition and content of SCFAs in the media surrounding the Caco-2 cultures collected from apical chambers after the calcium uptake experiment (Section 2.4). The separation was conducted using a Shimadzu HPLC system (Model LC-8A, Japan) equipped with a Phenomenex C18 300 Å Jupiter column (5 µm; 4.6 x 250 mm), a binary pump, an in-line degasser, a photodiode array (PDA), and a variable wavelength absorbance detector set at 210 nm for SCFA, equipped with the Lab Solutions Software for data analysis. The analysis was conducted according to the procedure previously described by De Baere et al.[23] with minor modifications. Briefly, aliquots were preserved in 0.1% trifluoroacetic acid (1:1, v/v). Prior to the analysis, the aliquots were mixed with 1 M HCL (1:1, v/v) and then shaken and filtered through Whatman 0.22-µm filter paper. The separation was performed using elution phases consisting of (A) 20 mM phosphate buffer adjusted to a pH of 2.2 with phosphoric acid, and (B) acetonitrile (8149/J.T.Baker, USA). Twenty microlitres of the samples were injected in each analysis. The gradient elution was used at a 1.5 mL min^{-1} flow rate. The gradient program started with 100 % of solvent A, and after 7 min, solvent B was increased linearly to reach 7 % by 12 min. Seven percents of solvent B were maintained till 19 min. Afterwards, the rate was changed to the starting

conditions to equilibrate the column for 15 min. The chromatographic signals obtained during the analysis were compared with the signals of commercially-available standards of SCFA (Sigma-Aldrich, Poland) under the same conditions. Identification of the signal was performed by matching the retention times of the peaks from analyses.

2.6 Determination of calcium content using atomic absorption spectroscopy

Calcium content was determined in all GFB digests (control, I, FOs and I/FOS), Caco-2 cells, and basolateral filtrates by flame (air – acetylene burner) atomic absorption spectrometry method (AAS) using an atomic absorption spectrophotometer (model Z-5000; Hitachi, Tokyo, Japan) equipped in an autosampler and the cathode lamp of calcium (Photron, Narre Warren, Australia) operated at the resonance line of this bioelement of 422.7 nm. Before analysis, the samples were wet-digested with a mixture (9 : 1; v/v) of concentrated nitric acid (65 % HNO3; Merck, Darmstadt, Germany) and hydrochloric acid (30 % HCl; Merck.) using a microwave system (Multiwave, Anton Paar GmbH, Graz, Austria). Calcium concentration in these digests, after their dilution with ultra-pure water (received from two-way water purification MAXIMA system; ELGA, Bucks, UK) with an addition of lanthanum(III) chloride hydrate ($LaCl_3$ x H_2O/l; Sigma-Aldrich, St. Louis, MO, USA; 5 g/L) was automatically read from a calibration curve (range 0.2 – 2.0 µg/mL) prepared using a stock of AAS standard solution of this element (Sigma, St. Louis, MO, USA). The analytical quality of the measurements was checked by repeating analysis (n = 3). The limit of detection was 0.2 µg/mL and the recovery reached 97.6 %.

2.7 Statistic analysis

Results were presented as the mean values ± SD. One-way analysis of variance (ANOVA) was performed on the data, and Fisher's least significant difference test (LSD) was applied to define significant differences ($p < 0.05$) between mean values. The statistical analyses were carried out using a Statistica 7.1 (StatSoft, Kraków, Poland).

3 Results

3.1 Effect of inulin and/or FOS on calcium uptake from the experimental GFB

The influence of inulin-type fructans (IFs), inulin, FOS or their mixture, on calcium uptake from the experimental GFB digest was analysed in the *in vitro* model composed of fully differentiated Caco-2 cells exposed to the solution of the human intestinal bacteria (Table 1).

In the present study, enterobacteria, lactobacilli and enterococci were used as the main bacterial representatives colonising the small intestine region, contributing, along with enterocytes (represented by Caco-2 cells), to the intestinal barrier structure and degradation processes. Their metabolic activities resulted in the synthesis of organic acids (Table 2).

The calcium uptake (%) determined in the Caco-2/intestinal bacteria culture incubated with the control GFB digest reached 0.87% and was four times higher then in the culture incubated with the calcium standard solution (Table 1). In the cultures incubated with GFB digest with IFs addition, a significant ($p<0.05$) increase of calcium uptake was observed (Table 1), comparing with the control. Inulin of longer chain length increased the cellular calcium uptake by about 30 %, whereas the addition of short-chain FOS as well as the mixture of FOS and inulin doubled it. In the culture incubated with the uptake buffer (blank culture), calcium content reached 2.14µg/mL.

GFB digest was a convenient nutrients source for the intestinal bacteria applied in the present study. In the control Caco-2/intestinal bacteria culture incubated with GFB digest, lactic and butyric acids were predominantly produced, whereas the other SCFAs were synthesised in lower amounts (Table 2). Independently on the chain length, the addition of IFs to the GFB digest stimulated the applied bacteria to the intensive production of metabolites. Compared to the control culture, a significant ($p<0.05$) increase in butyric, valeric and lactic acids concentration was noticed (Table 2), affecting the increase in the sum of organic acids. Lactate and butyrate were also the primary metabolites of bacterial fermentation in the culture incubated with the calcium standard solution.

3.2 The effect of inulin and/or FOS on calcium absorption from the experimental GFB

Table 1 Effect of inulin and FOS on calcium uptake (%) from experimental calcium-enriched GFB

	Standard	Control	I	FOS	I/FOS
Soluble calcium added[&] (µg/mL)	460.05 ± 11.12	459.45 ±15.25	467.06 ± 17.35	465.54 ± 19.66	468.75 ± 15.46
Calcium cellular uptake[¶](µg/mL)	2.97 ± 0.13	6.13[c] ± 0.27	7.38[b] ± 0.45	12.08[a] ± 0.31	11.84[a] ± 0.20
Calcium uptake (%)	0.18	0.87	1.12	2.14	2.07

[a] Values followed by different superscript uppercase letters indicated significant differences ($p < 0.05$) within row by Fisher's least significant difference test (without Standard); [&] Amount of soluble calcium added onto cultures; [¶] Amount of calcium absorbed into cells incubated with Standard, Control, I, FOS or I/FOS.

Table 2 The effect of inulin and FOS on SCFAs (mM/mL) and the lactic acid (mM/mL) content in media surrounding the Caco-2 cells/ human intestinal bacteria culture

	Standard	Control	I	FOS	I/FOS
Acetic	13.966 ± 0.485	24.615^{ab} ± 0.958	25.450^{a} ± 2.040	24.439^{ab} ±1.107	22.833^{b} ± 1.285
Propionic	35.861 ± 2.536	97.99^{a} ± 6.539	96.810^{ab} ± 7.193	83.466^{c} ± 2.759	93.118^{b} ± 4.282
Butyric	65.783 ± 4.202	160.146^{c} ± 11.569	180.976^{b} ± 6.357	186.540^{b} ± 19.517	229.990^{a} ± 13.163
Valeric	24.672 ± 1.045	35.825^{c} ± 1.724	42.316^{b} ± 2.503	34.965^{c} ± 4.054	47.080^{a} ± 2.898
Lactic	200.614 ± 18.525	457.560^{b} ± 22.364	519.049^{a} ± 24.211	502.516^{a} ± 40.013	516.100^{a} ± 15.731
Total acids	340.896	776.136	864.601	831.926	909.121

[a] Values followed by different superscript uppercase letters indicated significant differences ($p < 0.05$) within row by Fisher's least significant difference test (without Standard).

The measurement of the amount of calcium retained in cells and calcium amount in the basolateral filtrates allowed analysing calcium absorption from GFB digest, which was generally considered as the sum of these both elements. Cellular calcium retention determined in the control Caco-2/intestinal bacteria culture incubated with GFB digest reached 6.59 µg/mL (Table 3).

Relative to the control culture, the addition of IFs to the GFB digest, especially the mixture of inulin and FOS, significantly increased (p<0.05) the amount of calcium retained in cells (Table 3). Nevertheless, this increased calcium retention affected by IFs was not simply translated into the general increase of calcium absorption. This was a consequence of 30 % decrease of the amount of calcium transported to the filtrate of basolateral compartment of cultures incubated with GFB digest and IFs (Table 3), in comparison with its amount in the filtrate of the control culture. The results obtained (Table 3) indicated that inulin and/or FOS enhanced cellular calcium retention, alongside with a drop of calcium transport to the basolateral filtrate, that resulted in a reduction of the calcium absorption from GFB digest. Cellular calcium uptake in the culture incubated with the uptake buffer (blank uptake) reached 1.04 ± 0.06 µg/mL, whereas in the filtrate of this culture calcium content was at 53.05 ± 2.01 µg/mL (transport blank).

Table 3 The effect of inulin and FOS on calcium absorption (%) from experimental calcium-enriched GFB

	Standard	Control	I	FOS	I/FOS
Soluble calcium added[&] (µg/mL)	460.05 ±11.12	459.45 ±15.25	467.06 ± 17.35	475.54 ± 19.66	468.75 ± 15.46
Calcium retained by cells[¶] (µg/mL)	2.69 ± 0.25	6.59^{c} ± 0.05	7.10^{b} ± 0.14	7.09^{b} ± 0.27	7.43^{a} ± 0.28
Calcium transported by cells[#] (µg/mL)	58.7 ± 2.66	94.81^{a} ± 1.18	83.37^{b} ± 1.99	84.44^{b} ± 1.01	79.50^{c} ± 1.22
Absorption (%)	1.59	10.30	7.80	7.87	7.00

[a] Values followed by different superscript uppercase letters indicated significant differences ($p < 0.05$) within row by Fisher's least significant difference test (without Standard); [&] Amount of soluble calcium added onto culture; [¶] Amount of calcium absorbed into cells incubated with Standard, Control, I, FOS or I/FOS; [#] Amount of calcium transported to basolateral liquid by cells incubated with Standard, Control, I, FOS or I/FOS.

4 Discussion

Calcium bioavailability from the diet depends on the chemical form of calcium compound and several factors affecting its solubility. Low pH, basic amino acids, lactose, organic acids, bile salts and adequate calcium/phosphorus ratio increase calcium bioavailability, whereas higher pH, non-soluble dietary fibre, phytates and oxalates greatly reduce calcium absorption. Literature data indicate that prebiotics, including IFs, influence positively the calcium availability from foods.[8-10] In the present *in vitro* study, conducted on the fully differentiated Caco-2 cells exposed to the solution of the human intestinal bacteria, we investigated the influence of IFs of different chain length on calcium uptake and absorption from the experimental calcium-enriched GFB. The results of our uptake experiment indicated that application of IFs, especially FOS, increased the cellular calcium uptake. Similarly, Bosscher *et al.*[7] showed that inulin increased calcium availability from dairy infant formulas. Other study demonstrated a significant increase of calcium solubility in the presence of inulin in a biscuit recipe suggesting that inulin could be an important promoter of calcium bioavailability.[24]

The mechanism by which an intensified intestinal calcium absorption is observed in the presence of IFs in a diet is uncertain however, it has been well documented that IFs are utilised as substrates by some groups of intestinal bacteria, usually bifidobacteria and lactobacilli.[25,26] The length of a fructan chain is also an important factor of fermentation patterns.

FOS were more rapidly fermentable than long-chain inulin[15] therefore, under the physiological conditions a rapid fermentation of short-chain fructans occurred in the proximal colon, whereas the long-chain fructans were more steadily fermented and their fermentation occurred over a longer

section of the colon. In the present study, the addition of inulin and FOS to a GFB digest triggered the expected increase in SCFAs production by the human intestinal bacteria applied in the culture. Bacterial fermentation in the cultures containing additional substrates (IFs) was more intensive than that observed in the culture with GFB digest alone. This may be due to the fact that the rate and amount of SCFA depend, apart from the species and amounts of intestinal microbiota present, also on substrates availability.[27] Digested in the *in vitro* conditions GFB was a source of readily and quickly fermented degradation products, whereas inulin and FOS, chains of fructose moieties linked by β-(2→1)-glycosidic bonds, added to the culture were the more efficiently and profitably metabolised substrates. The physiological effects of IFs are microbiota-mediated therefore, a longer exposure to the bacterial suspension applied in this study could potentially multiply the effect of inulin and FOS observed in this experiment.

SCFAs are the main end-products of bacterial fermentation. By contributing to a reduction of the luminal pH, SCFAs may increase the amount of soluble calcium and stimulate its absorption.[28] The stimulated by IFs addition intensive production of organic acids, especially of lactate and butyrate, observed in the surroundings of Caco-2 cells in the uptake experiment, accelerate calcium uptake from the digest of GFB. It is possible that moderate environment acidification (resulting from stimulated organic acids production) could facilitate solubility of calcium salts, thus promoting calcium uptake. Increased lactic acid synthesis, resulting from the pyruvate transformation, is an indicator of the fermentative activity of bacteria from the genera of *Lactobacillus* and *Enterococcus*,[29] considered as microbes beneficial for intestinal health. Dietary fermentable carbohydrates have the potential to produce butyrate, however, not all of them are butyrogenic. Their chemical and physical properties determine the course of fermentation by specific bacteria and metabolite profiles. Oligofructose is fermented by mainly acetate and lactate-producing bacteria rather than butyrate-producing bacteria.[30] In the present study, despite the absence of typical butyrate producers - bifidobacteria, this acid predominated among SCFAs synthesised by the intestinal bacteria in the cultures. Khan and Edwards reported an increased butyrate production from oligofructose by human faecal bacteria *in vitro*.[31] This high butyric acid concentration could probably be explained by the biochemical interconversions between SCFAs. The previous studies with sheep have demonstrated that up to 60 % of butyrate is synthesised directly from extracellular acetate through interconversion reactions.[32] More recently, Duncan *et al*. showed that acetate was further metabolised by the human colonic bacteria to produce butyrate.[33] There are two distinct pathways of intracellular butyrate production in bacteria, via butyrate kinase or butyryl CoA:acetyl CoA transferase. [34,35] FOS is speculated to play a role in the induction of the butyryl CoA:acetyl CoA transferase system. Morrison *et al*. reported that the addition of oligofructose to faecal batch cultures significantly enhanced butyrate production.[36] These authors stated that the majority of the butyrate synthesised from oligofructose fermentation was derived from interconversion of extracellular acetate and lactate, with acetate being quantitatively more significant and that butyryl CoA:acetyl CoA transferase was the predominant butyrate-producing pathway.

To assess the influence of IFs on calcium absorption, cellular calcium retention and calcium transport were determined. Calcium transport is mediated by a complex array of transport processes that are regulated by hormonal, developmental, and physiological factors. Its movement across the intestinal epithelium is based on two mechanisms: passive (paracellular) and active (transcellular) one. The transcellular calcium transport is a three-step metabolically-energized process, consisting of the apical uptake, cytoplasmic translocation, and the basolateral extrusion. In contrast, the paracellular, passive calcium transport is dependent on calcium gradient and is regulated by the permeability of the tight junction, constituted by several charge-selective proteins, arranged in the arrays of channel-like paracellular pores.[37,38] To determine if the transport occurs due to modulation of calcium transport mechanisms, it is important to verify the monolayer integrity. As it was indicated in the Materials and methods section (section 2.4), only inserts of the integral monolayer whose TEER value was unaffected by the treatment with a solution of human intestinal bacteria were used in the calcium absorption experiment. Additionally, it was documented that the application of non-digestible saccharides in the Caco-2 cell monolayers is not accompanied by cell membrane damage.[39] Although the mechanism of calcium transport was not investigated in this study, however, some assumptions could be made in the view of the literature data. In the present calcium absorption experiment, IFs application stimulated calcium retention and simultaneously decreased calcium transport to the basolateral filtrate, causing the reduction of calcium absorption (sum of calcium retention and transport). The results obtained allowed as to hypothesise that the analysed IFs facilitated only calcium entrance into the Caco-2 cells, which is the first step of the transepithelial calcium transport, without the effect on its extrusion to the basolateral filtrate. Additionally, the significant decrease in calcium concentration in the basolateral filtrate suggested that the applied IFs affected negatively the passive calcium transport. Results obtained are therefore contrary to the results of Suzuki and Hara who showed that the presence of FOS at the apical side of Caco-2 cell monolayers significantly increased the paracellular calcium transport and decreased transepithelial resistance.[39] In the present Caco-2 experiment, however, the mixture of intestinal bacteria was applied at the apical chamber, therefore both the bacteria and their metabolites could potentially affect the intestinal epithelial calcium transport. Gilman and Cashman compared the effect of commensal (*E.coli*) and probiotic bacteria (*Lactobacillus salivarius* UCC 11 8 and *Bifidobacterium infantis* UCC 35624) on calcium uptake and transepithelial calcium transport in a Caco-2 cell culture.[40] They demonstrated that the exposure of Caco-2 cell monolayers to probiotic bacteria had no effect on transepithelial calcium transport in Caco-2 cells but calcium uptake was significantly higher in the cells exposed to

Lactobacillus salivarius, and tended to be increased by *E. coli*. While, the other research on Caco-2 cell indicated that bacterial metabolites, especially butyric acid, in physiological concentrations, can enhance functions of the intestinal barrier.[41] Molecular mechanisms underlying this effect remain unclear, however it has been speculated that SCFAs may be involved in the regulation of the expression of tight junction-associated proteins.[42] Recently, Peng *et al*. proved, however, that the treatment with butyrate did not alter the expression of tight junction proteins, including occludin, claudin-1, claudin-4, and ZO-1, instead the authors demonstrated that the butyrate treatment increased the AMP-activated protein kinase activity and accelerated the assembly of tight junctions as shown by the reorganization of tight junction proteins, as well as the development of TEER.[43]

Summarising, the present *in vitro* studies on Caco-2 cells treated with the human intestinal bacteria suspension investigated whether inulin and/or FOS influence calcium uptake and absorption from the experimental GFB. Results obtained in the first calcium uptake experiment indicated that the addition of IFs, especially short-chain FOS to the GFB digest enhanced cellular calcium uptake, probably via the alteration of the rate and amount of organic acids produced by the human intestinal microbiota applied in the cultures. In the calcium absorption experiment, IFs stimulated cellular calcium retention but concomitantly reduced its transport. , The overall adverse effect of the applied IFs on calcium absorption from the experimental GFB digest was observed. Although the direct comparison of results of both conducted experiments is not possible we suppose that this effect, partly inconsistent with literature data, may be due to a few limitations of conducted experiments. First of all, the differences in experiments methodology, like incomparable dimension of the effective growth area; additionally, the duration of the absorption experiment may not be sufficient to observe the intracellular calcium transport by cytosolic calcium-binding proteins and/or calcium extrusion across the basolateral membrane; finally, the intestinal bacteria solution was prepared from stools of five volunteers which may not adequately represent the diversity of human small intestine microflora. .

5 Conclusions

Based on the *in vitro* experiments, it could be concluded that the applied IFs of different chain length affected differentially calcium uptake and absorption from the experimental calcium-enriched GFB, therefore a further study is needed to assess whether these observations made *in vitro* contribute to IFs effects on calcium bioaccessibility from experimental GFB *in vivo*.

Acknowledgements

We are grateful to Dr Małgorzata Gałażyn-Sidorczuk from the Medical University of Białystok, Poland, for AAS analysis and to Anna Szyc from the Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland, for SCFA analysis. The research work and cost of publication as open access were supported by a grant 2011/01/D/NZ9/02692 from the National Science Centre, Poland.

Conflict of interest

No conflicts of interest are declared for any of the authors.

References

1 S. Pantaleoni, M. Luchino, A. Adriani, R. Pellicano, D. Stradella, D.G. Ribaldone, N. Sapone, G.C. Isaia, M. Di Stefano and M. Astegiano, *Scientific World J.,* 2014*,* **2014**, 173-182.
2 U. Kavak, A. Yüce, N. Koçak, H. Demir, I.N. Saltik, F. Gürakan and H. Ozen, *J. Pediatr. Gastroenterol. Nutr.*, 2003, 37, 434–436.
3 V.D. Capriles, L.A. Martini and J.A.G. Arêas, *Nutr. Rev.*, 2009, **67**, 599–606.
4 U. Krupa-Kozak, *Nutrition*, 2014, **30**, 16-24.
5 T. Larussa, E. Suraci, I. Nazionale, I. Leone, T. Montalcini, L. Abenavoli, M. Imeneo, A. Pujia and F. Luzza, *World J. Gastroenterol.* 2012, **18**, 1622-1627,
6 U. Krupa-Kozak, M. Wronkowska and M. Soral-Śmietana, *Czech J. Food Sci.,* 2011, **29**, 103–108.
7 D. Bosscher, M. Van Caillie-Bertrand, R. Van Cauwenbergh and H. Deelstra, *Nutrition*, 2003, **19,** 641–645.
8 L. Raschka and H. Daniel, *Eur. J. Nutr.,* 2005, **44**, 360-364.
9 S.A. Abrams, I.J. Griffin, K.M. Hawthorne, L. Liang, S.K. Gunn, G. Darlington and K.J. Ellis, *Am. J. Clin. Nutr.,* 2005, **82**, 471-476.
10 S.A. Abrams, I.J. Griffin and K.M. Hawthorne, *J. Nutr*., 2007, **137**, 2524-2526.
11 M. Roberfroid, G.R. Gibson, L. Hoyles, A.L. McCartney, R. Rastall, I. Rowland, D. Wolvers, B. Watztl, H. Szajewska, B. Stahl, F. Guarner, F. Respondek, K. Whelan, V. Coxam, M. Davicco, L. Léotoing, Y. Wittrant, N. Delzenne, P. Cani, A. Neyrinck and A. Meheust, *Brit. J. Nutr.,* 2010*,* **104**(S2), 1-63.
12 E. Scholz-Ahrens and J. Schrezenmeir, *Brit. J. Nutr.,* 2002, **87**, 179–186.
13 J. Korus, K. Grzelak, K. Achremowicz and R. Sabat, *Food Sci. Technol. Int.*, 2006, **12**, 489-495.
14 M. Velazquez, C. Davies, R. Marett, J.L. Slavin and J.M. Feirtag, *Anaerobe*, 2000, 6,87-92.
15 M.L. Stewart, D.A. Timm and J.L. Slavin, *Nutr. Res*., 2008, **28**, 329–334.
16 P. Etcheverry, M.A. Grusak and L.E. Fleige, *Front Physiol.,* 2012; **3**17, 1-22.
17 J.C. Fleet, F. Eksir, K.W. Hance and R.J. Wood, *Am. J. Physiol.,* 2002, **283**, 618–625.
18 U. Krupa-Kozak, A. Troszyńska, N. Bączek and M. Soral-Śmietana, *Eur. Food Res. Technol.,* 2011, **232**, 497–508.
19 C. Frontela, G. Ros and C. Martinez, *Eur. Food Res. Technol.,* 2009, **228**, 789–797.
20 D. Świątecka, M. Iwan, A. Świątecki, H. Kostyra and E. Kostyra, *Food Res. Int.,* 2010, **43**, 1566–1576.
21 M. Pinto, S. Robine-Leon, M.D. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh and A. Zweibaum, *Biol. Cell.*, 1983, **47**, 323-330.
22 M.D. Peterson and M.S. Mooseker, *Cell Sci.,* 1993, **105**, 445-460.

23 S. De Baere, V. Eeckhaut, M. Steppe, C. De Maesschalck, P. De Backer, F. Van Immerseel and S. Croubels, *J. Pharm. Biomed. Anal.,* 2013, **80**, 107-115.
24 D. Vitali, M. Radic, B. Cetina-Cizmek and V. Dragojevic, Acta Alim., 2011, **40**, 480–489.
25 J.H. Cummings, G.T. Macfarlane and H.N. Englyst, *Am. J. Clin. Nutr.,* 2001, **73**, 415-420.
26 L. Raschka and H. Daniel, *Bone,* 2005, **37**, 728–735.
27 J.M. Wong, R. de Souza, C.W. Kendall, A. Emam and D.J.A. Jenkins, *J. Clin. Gastroenterol.*, 2006, **40**, 235-243.
28 K.E. Scholz-Ahrens, G. Schaafsma, E.G.H.M. van den Heuvel and J. Schrezenmeir, *Am. J. Clin. Nutr.,* 2001, **73**, 459-464.
29 G.T. Macfarlane, H. Steed and S. Macfarlane, *J. Appl. Microbiol.,* 2008, **104**, 305-344.
30 S. Kolida, T. Tuohy and G.R. Gibson, *Br. J. Nutr.,* 2002, **87**, 193–197.
31 K.M. Khan and C.A. Edwards, *Eur. J. Nutr.,* 2006, **44**, 371–376.
32 M. Linington, J. Meyer and J. van der Walt, *South Afr. J. Anim. Sci.,* 1998, **28**, 82–98.
33 S.H. Duncan, G. Holtrop, G.E. Lobley, A.G. Calder, C.S. Stewart and H.J. Flint, *Br. J. Nutr.,* 2004, **91**, 915–923.
34 T.L. Miller and S.E. Jenesel, *J. Bacteriol.,* 1979, **138**, 99–104.
35 S.H. Duncan, A. Barcenilla, C.S. Stewart, S.E. Pryde and H.J. Flint, *Appl. Environ. Microbiol.,* 2002, **68**, 5186–5190.
36 D.J. Morrison, W.G. Mackay, C.A. Edwards, T. Preston, B. Dodson and L.T. Weaver, *Br. J. Nutr.,* 2006, **96**, 570-577.
37 F. Bronner, *J. Exp. Zool. A Comp. Exp. Biol.,* 2003, **300**, 47–52.
38 J.G. Hoenderop, B. Nilius and R.J. Bindels, *Physiol. Rev.,* 2005, **85**, 373–422.
39 T. Suzuki and H. Hara, *J. Nutr*., 2004, **134**, 1935-1941.
40 J. Gilman and K.D. Cashman, *Curr. Issues Intest. Microbiol*., 2006, **7**, 1-5.
41 L. Peng, Z.J. He, W. Chen and I.R. Holzman, J. Lin, *Pediatr. Res*., 2007, **61**, 37–41.
42 J.M. Mariadason, D.H. Barkla and P.R. Gibson, *Am. J. Physiol*., 1997, **272**, 705–712.
43 L. Peng, Z-R. Li, R.S. Green, I.R. Holzman and J. Lin, *J. Nutr.,* 2009, **139**, 1619–1625.

Inulin-type fructans enhance cellular calcium uptake and retention from calcium-enriched gluten-free bread digest affecting calcium absorption contrary.