Diet Shapes the Ability of Human Intestinal Microbiota to Degrade Phytate - *In Vitro* Studies

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Running headline: Phytase activity of intestinal microbiota

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Abstract

Aims: Investigation of intestinal bacterial groups involved in phytate degradation and the impact of diets with different phytate contents on phytase activity.

Methods and Results: Faecal samples of adults on conventional (n=8) or vegetarian (n=8) diets and breastfed infants (n=6) were used as an inoculum for modified media

supplemented with phytate. Populations of Gram-positive anaerobes (GPA), lactic acid bacteria (LAB), Proteobacteria-*Bacteroides* (P-B), coliforms and anaerobes were studied. The PCR-DGGE analysis revealed a random distribution of DGGE profiles in the dendrograms of GPA, P-B and coliforms, and a partially diet-specific distribution in the DGGE dendrograms of LAB and anaerobes. The degradation of phytic acid (PA) was determined with HPLC method in supernatants of the cultures. Regardless of the diet, the Gram-positive anaerobes and LAB displayed the lowest ability to degrade phytate whereas the coliforms and P-B cultures produced higher amounts of intermediate *myo*-inositol phosphates. Bacterial populations grown in a non-selective medium were the most effective ones in phytate degradation. It was the vegetarians' microbiota that particularly degraded up to 100 % phytate to *myo*-inositol phosphate products lower than InsP_3.

Conclusions: A diet rich in phytate increases the potential of intestinal microbiota to degrade phytate. The co-operation of aerobic and anaerobic bacteria is essential for the complete phytate degradation.

Significance and Impact of the Study: This study provides insights on the effect of diet on specific metabolic activity of human intestinal microbiota.

Keywords: phytate degradation, human microbiota, *myo*-inositol phosphates, vegetarian diet, conventional diet, breast feeding

Introduction

A diet provides nutrients both for the human organism and the bacteria inhabiting the human gastrointestinal tract. All compounds of a diet are potentially biologically active and may have both beneficial and harmful effects on the organism and intestine health. It has been also proved that components of the diet (*e.g.* prebiotics, proteins and glycoproteins or fat) influence the composition and metabolic activity of intestinal microbiota (Bielecka et al. 2002; Brunser et al. 2006; Barbier de La Serre et al. 2010; Świątecka et al. 2010; Świątecka et al. 2011).

Phytic acid (PA) and its salts (phytates, *myo*-inositol hexakis phosphates or InsP_6) are components of cereal grains and legume seeds. Its chemical properties make it both an antinutrient (decreases protein digestion and bioavailability of nutritionally important cations such as Ca^{2+}, Mg^{2+}, $Fe^{2+}$and Zn^{2+}; Wodzinski and Ullah 1996) and a beneficial (strong antioxidant) food compound. A degradation of PA leads to a release of inorganic phosphate and formation of lower inositol phosphates: penta-, tetra-, tri-, di-, and mono-*myo*-inositol phosphates (InsP_5, InsP_4, InsP_3, InsP_2 and InsP_1) of which only InsP_5 displays the ability to decrease the availability of minerals (Sandberg, Carlsson and Svandberg, 1989). Other derivatives of PA have no antinutritional properties, moreover, InsP_3 and InsP_4 play an important role in the intra-cell signal transduction pathways (Suzuki et al. 2010, Suzuki and Hara, 2010).

A partial degradation of PA may occur during food processing (Afify et al. 2011) but most of the food phytate remains non-degraded and reaches the gastrointestinal tract. The human intestinal tissue expresses a very low phytase activity, which is insufficient to degrade all the dietary phytate and cannot contribute to any physiologically relevant degradation of inositol phosphates in the small intestine (Iqbal, Lewis and Cooper, 1994). Ingested phytate is subjected to a chemical (low pH in the stomach) or enzymatic

degradation carried out by the intrinsic food phytases (mainly 6-phytases of plant origin) or intestinal microbiota (3-phytases and 6-phytases) (Sandberg, 2002) and the efficacy of phytate degradation in the intestine is higher due to longer colon transit times (Jung et al. 2007).

Studies on the ability of particular groups of intestinal microbiota to decompose phytates are scarce. In fact, phytases are produced by *Klebsiella* and *Escherichia coli* (Gammaproteobacteria) (Bohm et al. 2010, Lim et al. 2000). Haros et al. (2005, 2007) have proved that some *Bifidobacterium* strains of the human intestinal origin manifest the phytase activity and the phytases from *Bifidobacterium pseudocatenulatum* and *Bifidobacterium longum* subsp. *infantis* have been recently characterized (Tamayo-Ramos et al. 2012). However, studies of Steer et al. (2004) conducted in the chemostat model indicated that this bacterial group is not involved in the phytate degradation.

This study aimed at examining which bacterial groups participate in the hydrolysis of PA and whether diets containing different amount of phytates (conventional, vegetarian and breastfeeding) influence the specific metabolic activity (phytate-degrading ability) of the intestinal microbiota.

Materials and Methods

Volunteers

Adult volunteers on the conventional (C) diet (6 women and 3 men, average age 35.5 years, average BMI 22.9 (min. 19.5, max. 28), adult vegetarians and vegans (V; 3 women and 5 men, average age 26 years, average BMI 21.1 (min. 18.6, max. 24.1) and breastfed infants (BF; 2 girls and 4 boys, average age 17.5 weeks) were included in the study. The adult volunteers declared that products containing live bacteria (*i.e.* fermented milk or fermented vegetable products and bacterial preparations) had been

97 excluded from their diets at least one week before the sampling. The conventional diet
98 was a western-type diet without any restrictions other than the aforementioned. The diet
99 of the vegetarians contained no meat, fish and seafood (4 volunteers) and, additionally,
100 no animal-originating food (*i.e.* milk and milk product and eggs; 4 volunteers).
101
102 Sampling
103 Fresh faecal samples of the adults were transported in a sterile tube to the laboratory in
104 anaerobic conditions (AnaeroGen System, Oxoid) within one hour and immediately
105 subjected to the analyses. Faecal samples of the infants were transferred to a sterile tube
106 and frozen at -20°C, transported to the laboratory within 2 days and subjected to the
107 analyses. The experimental design is presented in Figure 1.
108
109 Microbiological Analyses
110 The faecal samples were transferred to the Anaerobic Workstation (MG500,
111 DonWhitley Scientific, USA; atmosphere composition: 80% N_2, 10% CO_2, 10% H_2).
112 All the media and reagents were incubated under anaerobic conditions for at least 24 h
113 before their use. In order to investigate selected groups of intestinal bacteria, the
114 following modified liquid media were used: Garche's medium for *Bifidobacterium*
115 (Haros et al. 2005), de Man Rogosa medium (MRS) for the lactic acid bacteria (LAB),
116 the MacConkey medium for *Enterobacteriaceae*, the neomycin brilliant green
117 taurocholic acid medium with blood (NBGT) for *Bacteroides* (Yasui et al. 1979) and
118 the non-selective Wilkins-Chalgren medium with blood for the anaerobes. All the media
119 were modified by a supplementation of 1 mmol l^{-1} sodium phytate (Sigma), an
120 elimination of inorganic phosphorus and lowering the concentration of protein
121 components and a yeast extract in order to generate low-phosphate conditions, which

promote a synthesis of the enzymes responsible for the phytate degradation (Haros et al. 2005). For the detailed composition of the microbiological media see the Supporting Information. The modified media without the inoculum were incubated and processed in the same way as the cultures of the faecal bacteria and were taken as controls for the HPLC analysis.

The faecal samples (1 g) were diluted in 1 % peptone water (49 ml) and used for the media inoculation. Fifteen millilitres of medium were inoculated with approximately 10^7 cells, estimated with DAPI, and incubated at 37°C either in the anaerobic conditions (Garche's medium, NBGT and anaerobes; 40 h) or in aerobic ones (MacConkey, 24 h; MRS, 40 h). After the incubation, the bacterial cell number was assessed on appropriate non-modified media using the plate-count method. Results of the cell counts were expressed as $\log_{10}$ CFU ml^{-1}.

DNA Extraction, PCR-DGGE Analysis and Band Sequencing

The DNA from 1.5 ml of the faecal bacteria cultures was isolated using the GeneMATRIX Bacterial and Yeast Genomic DNA Purification Kit (Eurx, Poland) applying the protocol delivered by the producer. The amplification of the V6-V8 fragment of 16S rRNA gene was carried out using the extracted DNA as a template and universal primers 1404-r (cggtgtgtacaagaccc) and 968-GC-f (GC-aacgcgaagaacctta) (Satokari et al. 2002). The reaction mixtures (30 µl) comprised of 3 µl of a reaction buffer, 1 U of *Taq* DNA polymerase (Fermentas, Lithuania), 5 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} each: dATP, dCTP, dGTP and dTTP, the template DNA (2 µl), sterile deionized water for filling up to 30 µl. The reactions were run in a MasterGradient Cycler (Eppendorf) using the following program: 95°C for 5 min, 35 cycles at 95°C for 20 s, 58°C for 20 s, 72°C for 20 s, and the final 72°C for 20 min. The PCR product (20

147 µl) was separated in a polyacrylamide gel (8%, acrylamide:bisacrylamide 37.5:1) with
148 denaturing gradient ranging from 25 to 60%. The 100% concentration of denaturants
149 corresponded to 40% (v/v) of formamide and 7 mol l^{-1} urea (Satokari et al. 2002). The
150 electrophoresis was carried out in the 0.5× TAE buffer at 60°C, the steps of pre-
151 electrophoresis (10 min, 200 V) and separating electrophoresis (18 h, 85 V) were
152 applied. The gels were stained with SybrGreen I dye (Sigma) in the 1× TAE buffer
153 according to the supplier's recommendations.
154 Selected DGGE bands were cut out from a gel with a sterile scalpel, incubated
155 overnight in 40 µl of the TE buffer at 4°C. Two microliters of the aliquots were used as
156 a template in the re-amplification reactions conducted in the conditions described above.
157 The re-amplified DNA was purified using the GeneMATRIX PCR/DNA Clean-Up
158 Purification Kit (Eurx, Poland). The DNA was commercially sequenced at Genomed
159 (Warsaw, Poland). The obtained sequences were identified using a blastn tool and
160 deposited in the GenBank database (accession numbers: KC108628-KC108644,
161 KC137555-KC137570, KC147729-KC147781).
162
163 Determination of *Myo*-Inositol Phosphates by HPLC
164 Five millilitres of the faecal bacterial cultures were centrifuged (15 min, 10000 ×*g*,
165 10°C) and the supernatant was mixed with 10 ml of 0.025 mmol l^{-1} HCl. The samples
166 were transferred to plastic mini-columns filled with the Dowex AG 1-X8 resin, from
167 which inositol phosphates were eluted using 2 mmol l^{-1} HCl (5×2 ml). After
168 desolventising by evaporation, the dry residue was dissolved in a mixture of
169 methanol/0.05 mmol l^{-1} formic acid and 15 ml l^{-1} TBA-OH (tertrabutylammonium
170 hydroxide) and analysed by HPLC according to the methods by Sandberg and
171 Ahderinne (1986) and Sandberg, Carlsson and Svandberg (1989) using a Shimadzu

chromatograph (LC-10 AD pump, refractometric detector RID-6A, CTO 6A column oven) and the Nova-Pak C_{18} column. The mobile phase was: methanol/0.05 mmol l^{-1} formic acid (51:49 v/v) and 15 ml l^{-1} TBA-OH. The flow rate was 0.7 ml min^{-1}. Sodium phytate (Sigma) was the external standard and the injections were made with a 20 µl loop. The concentrations of InsP_3, InsP_4, InsP_5 (intermediate hydrolysis product; INT-Ins*P*s) and InsP_6 determined in the supernatant were expressed as percentage of the initial amount of phytic acid in the media. The amount of the lower derivatives (InsP_1, InsP_2 and *myo*-inositol; LD) was calculated as a difference between the initial content of phytate and the sum of InsP_6 and INT-Ins*P*s).

Data Analysis

The DGGE banding profiles were processed using the BioNumerics software (Applied Maths, Belgium). The gels were normalized to one sample (as an external standard) run for the each gel set. Similarities of the profiles were calculated using the Pearson correlation moment coefficient and the dendrograms were constructed using the UPGMA method. The average similarity of a composite dataset and a dendrogram made of similarity of profiles of the bacteria grown in Garche's, MRS, MacConkey and NBGT media was also calculated.

The differences in the bacterial cell numbers of *in vitro* cultures and the concentrations of *myo*-inositol phosphates were analysed with the non-parametric Kruskal-Wallis test (for non-uniformly distributed variables) or the variance analysis (F-test; for variables with normal distribution and equal variances; Statistica, Statsoft). The means (medians) of inositol phosphate percentage distribution for all the samples were calculated to compare the potential of investigated bacterial groups to degrade phytate. The relative proportions of LD, INT-Ins*P*s and non-degraded phytic acid (NDPA) were calculated

on the basis of inositol phosphate percentage distribution. To numerically characterize the ability of bacterial cultures to degrade phytate a factor (*F*) was introduced. A general aptitude to degrade phytate (to release at least one phosphate group from InsP_6) was defined as F_{total} ((initial amount of phytate – NDPA) × (LD + INT-Ins*P*s))/100. Ability of bacterial cultures to generate intermediate inositol phosphates ($F_{INT\text{-}InsPs}$) was defined as ((initial amount of phytate – NDPA) × INT-Ins*P*s)/100. Ability of bacterial cultures to hydrolyse phytate to the lowest inositol phosphates (F_{LD}) was defined as ((initial amount of phytate – NDPA) × LD)/100. Values of LD, INT-Ins*P*s and NDPA were medians of inositol phosphate percentage distribution, whereas the initial amount of phytate was 100%. The maximum value for each *F* factor (corresponding to the possible highest ability to degrade phytate or generate particular group of inositol phosphates) was 100.

Results

Counts of Faecal Bacteria Cultures

The mean for the faecal bacteria culture counts reached the levels ranging from 6.9 in the NBGT medium for infants' microbiota to 9.1 log CFU ml^{-1} in the cultures of the anaerobes from the vegetarians (Fig. 1). Due to a high intra group variability, in most cases no differences in the *in vitro* growth of investigated groups of microbiota were observed when compared to the both tested media and the volunteer groups counts (Fig. 1). Significantly lower bacterial number of infant's microbiota grown solely in NBGT medium comparing to both adult's groups ($P<0.05$) was observed.

Composition of Faecal Bacteria Cultures

In order to determine the composition of bacterial cultures in the tested media, a PCR-DGGE assay (with primers universal for the bacteria) combined with band sequencing was applied. This also made it possible to confirm the selectivity of the particular medium (targeted bacteria) and to identify the growth of the other bacteria.

The analysis of the DGGE profiles for the faecal bacterial cultures grown in modified Garche's, MacConkey, and NBGT media provided dendrograms in which the profiles were distributed randomly and characterised by their relatively high similarities (Supporting Information). The DGGE profiles of the cultures grown in the modified MRS medium (LAB) and in the modified Wilkins-Chalgren (anaerobes) medium were partially clustered in a diet-dependent manner. Particularly, the DGGE profiles of the MRS cultures were grouped into three clusters in which the profiles of vegetarians (cluster V), omnivores (cluster O) and infants (cluster I) predominated (Supporting Information). Also, the advanced cluster analysis of a composite including comparisons of the DGGE profiles for all the examined bacterial groups did not demonstrate a relation between the diet and the similarity of the DGGE patterns in the cultures. The analysis resulted in a dendrogram consisting of two clusters of relatively high similarity (60%), cluster I-V (comprising microbiota the profiles for the infants and vegetarians) and cluster V-C (comprising the profiles for vegetarians and all the profiles for the conventional diet group microbiota) (Fig. 2).

Sequencing of the DGGE bands showed that apart from the targeted bacteria, the growth of which improved in the particular medium, other bacterial groups were also detected (Table 1). The DGGE profiles of the cultures grown in Garche's medium consisted of bands representing mostly the Gram-positive anaerobic bacteria of Clostridia and Actinobacteria and some representing microaerophilic Gram-positive bacteria (Lactobacillales) (Table 1). Therefore, although Garche's medium is usually

dedicated for the *Bifidobacterium* cultivation, in the conditions applied in this study (liquid cultures of complex microbiota), that medium was also appropriate for the Gram-positive anaerobes and was described as the GPA. In the cultures grown in the modified MRS medium, apart from the lactic acid bacteria (genera: *Lactobacillus*, *Lactococcus*, *Streptococcus*) that constituted the majority of the identified bands, there were also two bands identified as *E. coli* detected. In the modified MacConkey medium only Gammaproteobacteria (genera: *Enterobacter*, *Escherichia*, *Klebsiella*, *Citrobacter*) were detected. In the NBGT medium, dedicated for *Bacteroides,* one sequence was classified as *Bacteroides vulgatus* species, whereas the rest of the bands represented Proteobacteria (genera: *Escherichia*, *Shigella*, *Citrobacter*, *Desulfovibrio*). Thus, the cultures grown in the NBGT medium were described as Proteobacteria-*Bacteroides*. The highest diversity was observed in the non-selective Wilkins-Chalgren medium, where representatives typical for the intestinal microbiota were detected. Those bacteria belonged to the families of Clostridiaceae, Lachnospiraceae, Eubacteriaceae, Enterobacteriaceae (Gammaproteobacteria), Sutterellaceae (Betaproteobacteria), Veillonellaceae and Bacteroidaceae (Table 1).

Phytate Degradation Ability in Selected Groups of Intestinal Microbiota *In Vitro*

The HPLC method was used to determine the level of InsP_6, its intermediate derivatives (InsP_3, InsP_4, InsP_5; INT-Ins*P*s) and its lower derivatives (InsP_1, InsP_2 and *myo*-inositol; LD). The ability to degrade phytate by particular populations of the intestinal microbiota was analysed independently from the diets. The cultures of the Gam-positive anaerobes and lactic acid bacteria appeared to be the least effective in the hydrolysis of phytic acid (Fig. 3). No more than 20% of the phytic acid was degraded to lower *myo*-inositol phosphates (INT-Ins*P*s and lower derivatives) by those bacteria. In the case of

271 the Proteobacteria-*Bacteroides* cultures the amount of INT-Ins*P*s and lower derivatives
272 in the culture medium reached up to 65% and 12%, respectively. Bacteria belonging to
273 *E. coli* group (MacConkey medium) appeared to be more efficient in the decomposition
274 of phytic acid and generated mainly InsP_3 and InsP_4 (23 % and 65%, respectively) so
275 that together they constituted the majority of all the *myo*-inositol phosphates. The lower
276 derivatives made the remaining amount of the *myo*-inositol phosphates. The highest
277 degree of phytate degradation was, however, obtained in the non-selective Wilkins-
278 Chalgren medium, where all phytate was hydrolysed either to the lower inositol
279 phosphates (65%) or to INT Ins*P*s (35%) (Fig. 3).
280
281 Phytate Degradation – Influence of the Diet on the Bacterial Phytase Activity
282 When comparing the phytate degradation by Gram-positive anaerobes, the only
283 significant difference observed was the higher level of INT-Ins*Ps* generated by the
284 infant-originating bacteria than the vegetarians' one ($P<0.05$; Fig.4). The analysis of
285 single intermediate derivatives InsP_3, InsP_4 and InsP_5 revealed a non-significantly
286 higher concentration of InsP_3 and InsP_4 ($P=0.059$ and $P=0.051$, respectively) in the
287 GPA cultures from infants (Supporting Information).
288 In the LAB cultures (MRS medium), a higher proportion of INT-Ins*P*s in the
289 conventional diet group when compared to the vegetarian group was observed ($P<0.01$;
290 Fig.4). This resulted from a significantly higher concentration of InsP_5 ($P< 0.01$; see
291 Supporting Information). No significant differences in *myo*-inositol phosphates
292 concentrations were observed for the *E. coli* cultures (MacConkey medium). In this
293 case, the bacteria from all the volunteer groups showed a high phytase activity
294 generating mainly INT-Ins*P*s (Fig.4). In the Proteobacteria-*Bacteroides* cultures, the
295 concentration of the lower *myo*-inositol phosphate derivatives generated by the bacteria

296 in the conventional diet group was significantly higher than that in the vegetarians
297 ($P<0.05$) and infants ($P<0.05$; Fig.4). The InsP_3 content in Proteobacteria-*Bacteroides*
298 cultures of the conventional-diet adults was lower when compared to the infants'
299 microbiota ($P<0.05$; see Supporting Information), which manifested as a significantly
300 lower total content of INT-Ins*P*s ($P<0.05$; Fig.4).
301 In the cultures of the anaerobes (non-selective Wilkins-Chalgren medium), some
302 significant differences were observed between the vegetarians' and infants' microbiota
303 (Fig.4). In the vegetarians' bacterial cultures, lower concentrations of INT-Ins*P*s
304 ($P<0.05$) and NDPA ($P<0.01$) as well as a higher content of lower *myo*-inositol
305 phosphates ($P<0.01$) were determined. The low content of INT-Ins*P*s resulted from the
306 decreased level of InsP_4 ($P<0.01$) and InsP_5 ($P<0.05$) (Supporting Information**)**.
307 The differences in the content of NDPA, INT-Ins*P*s and LD in cultures' supernatant is
308 presented in Table 2 as relative proportions of those. The ability of bacterial cultures to
309 degrade phytate (Table 2) clearly showed that GPA and LAB were characterized by low
310 F_{total} (maximal outcome 21) comparing to other examined bacterial cultures (most of the
311 outcomes over 60) and consequently low $F_{INT\text{-}InsPs}$ and F_{LD}. Interestingly, some cultures
312 of similar F_{total} (P-*B* cultures) differed as to $F_{INT\text{-}InsPs}$ (two times higher in BF group
313 comparing to C) and F_{LD} (four times higher in C comparing to BF group). On the other
314 hand, in anaerobes' cultures of different F_{total} (over twice higher in C than in BF group)
315 comparable outcome for $F_{INT\text{-}InsPs}$ was obtained (Table 2). The analysis of *F* factors
316 provided an overview of the activity of bacterial populations towards phytate
317 degradation. Moreover, in case of high F_{total} value, the $F_{INT\text{-}InsPs}$ and F_{LD} factors allow to
318 describe more precisely whether the cultures are consisted in majority of bacteria with
319 specific phytase activity ($F_{INT\text{-}InsPs} > F_{LD}$) or there are also bacteria expressing non-
320 specific phosphatase activity (or phytases of broader substrate specificity). The latter

case would lead to accumulation of lower derivatives of phytate degradation (F_{LD} > $F_{INT\text{-}InsPs}$).

Discussion

Although *in vitro* methods are burdened with some drawbacks and do not fully reflect *in vivo* conditions, they enable to investigate the specific metabolic activity of intestinal bacteria. In this study, microbiological media were used to examine populations of Gram-positive anaerobes, lactic acid bacteria, Enterobacteriaceae, Proteobacteria-*Bacteroides* and total anaerobes in three human groups that differed in age (adults-infants) or followed a different diet (conventional, vegetarian and breastfeeding). The media composition and the experimental design limited the changes observed during sub-culturing of complex bacterial populations. The PCR-DGGE approach with sequencing allowed verifying specificities of the applied microbiological media. The selectivity of the MRS and MacConkey media for both lactic acid bacteria and coliforms was confirmed. In the case of Garche's and NBGT media, the identification of predominating DGGE bands resulted in re-naming of the cultures to Proteobacteria-*Bacteroides* and Gram-positive anaerobes (GPA), respectively. In the non-selective medium (Wilkins-Chalgren medium), representatives of the physiological intestinal microbiota were detected (Table 1), including Firmicutes (*Lachnospiraceae, Clostridiaceae, Eubacteriaceae, Veillonellaceae*), Bacteroidetes (*Bacteroides vulgatus*), Betaproteobacteria (*Sutterellaceae*), and Gammaproteobacteria (*Enterobacteriaceae*).

In this study, the coliforms proved to be the most efficient phytate degraders. Such high activity in phytate degradation might result from the sensitivity of that bacterial group to a deficiency of iron ions which, when bound with phytate, cannot be utilised by the bacteria. *E. coli* is equipped with large iron uptake systems and its metabolism is

346 strongly regulated by iron depletion (McHugh et al. 2003). This may explain why *E.*
347 *coli* degraded phytic acid mainly to InsP_4 and InsP_3 (phytate derivatives with no ability
348 of binding minerals) and thus making the iron ions (as well as other minerals too)
349 available to bacteria. Moreover, as it has been reported for *Pseudomonas* (member of
350 the Gammaproteobactria class), InsP_3 mediates in transporting the iron into bacterial
351 cells (Hirst et al. 1999).
352 Proteobacteria-*Bacteroides* were following coliforms as the second bacterial culture
353 characterised by a high efficiency in phytate degradation. When compared to *E. coli*
354 cultures, the differences observed in the profiles of phytate derivatives indicated that
355 changes in the environment (different medium composition and anaerobic conditions)
356 and differences in the bacterial composition may trigger considerable changes in the
357 metabolic activity of bacterial consortia.
358 The results obtained in this study coincide with results of an experiment of Steer et al.
359 (2004), where a medium enriched with phytate was inoculated with human adult
360 microbiota and phytate degrading strains were isolated. Among 63 identified strains as
361 many as 27 belonged to Proteobacteria, 11 to *Bacteroides* and 2 to *Fusobacterium*,
362 giving in total 64,5 % of Gram-negative bacteria among phytate degraders. Such a high
363 proportion of Proteobacteria in the total number of phytate degraders (Steer et al. 2004)
364 correlated with a high effectiveness in phytate degradation in the Proteobacteria-
365 *Bacteroides* cultures showed in this study.
366 The cultures of GPA and lactobacilli were characterised by their lowest ability to
367 degrade phytic acid, however, among the examined GPA cultures those of infants'
368 origin showed the highest phytase activity (although still low when compared to the
369 other bacterial groups). In the studies by Steer et al. (2004), Gram-positive aerobic
370 *Enterococcus* and *Staphylococcus*, and anaerobic clostridia formed about 35% of the

371 total identified phytate degraders, whereas bifidobacteria and lactobacilli were not
372 recovered from the chemostat effluent. In the present study, *Staphylococcus* and
373 *Enterococcus* were not detected in the bacterial cultures, and most of the identified
374 DGGE bands from the GPA profiles represented clostridia, which is partially consistent
375 with the results obtained by Seer et al. (2004). Moreover, in the GPA cultures,
376 bifidobacteria were also detected. According to Steer et al. (2004), bifidobacteria and
377 lactobacilli are not involved in the process of phytate degradation in gastrointestinal
378 tract. However, findings of Haros et al. (2005, 2007) showed that single
379 *Bifidobacterium* strains manifest phytase activity to different extends, with *B.*
380 *pseudocatenulatum* ATCC 27919 and *B. longum* subsp. *infantis* ATCC 15697 isolated
381 from infant faeces being the most active strains. The species composition of
382 *Bifidobacterium* populations is probably crucial for that activity since the genes coding
383 phytase have been found in the genomes of *B. longum* subsp. *infantis* and *B.*
384 *pseudocatenulatum* whereas they are absent in most of the remaining bifidobacteria
385 species (Tamayo-Ramos et al. 2012). The physiological role of phytase activity in
386 microbiota of breastfed infants is not clear since we did not detect inositol phosphates
387 (InsP_3-InsP_6) in mothers' milk (at micromolar level per gram of dry matter; data not
388 shown). The substrates for phytases are introduced in infant's diet after weaning, and
389 this can be the time when phytase activity gives an advantage for phytate degrading
390 bacteria. Another issue, that needs investigations, is whether the effects of phytase
391 activity are of importance solely for bacteria or triggers consequences to the infant's
392 health like increased bioavailability of minerals and amino acids or impact of phytate
393 degradation products on physiology (development, maturation) of infant's intestinal
394 epithelium. In the case of lactic acid bacteria, the phytase activity is detected mostly in
395 the strains isolated from sourdough and other naturally fermented plant material (De

396 Angelis et al. 2003), which accounts for an adaptation to the conditions of an extremely
397 high-phytate content.
398 The results obtained in the study show that Gammaproteobacteria are the main phytate
399 degraders in the human intestine and those are probably them that initiate phytate
400 degradation in the small intestine and continue it in the large intestine. Other groups of
401 bacteria are also involved in this process and they may both degrade phytates starting
402 from InsP_6 (*B. longum* subsp. *infantis*, *B. pseudocatenulatum*) and hydrolysed the
403 intermediate derivatives of phytate degradation (InsP_3, InsP_4, InsP_5) to the lower
404 inositol phosphates and inorganic phosphorus. The effectiveness of the phytate
405 degradation seems to be higher when the microbiota is already adapted to a higher-
406 phytate concentration, as it is in the case of the vegetarians' microbiota. The results
407 obtained in these examinations allow for the conclusion that more diverse microbiota
408 (adults' *vs.* infants' microbiota), shaped additionally by the diet (omnivores' *vs.*
409 vegetarians') ensures a higher degree of phytate degradation. The substrate specificity
410 for phytases of the intestinal anaerobes has not been investigated in detail yet.
411 Therefore, the impact of particular bacterial groups or species on the phytate hydrolysis
412 is difficult to evaluate. Whether the degradation of *myo*-inositol phosphates is a result of
413 specific phytase activity or a result of the activity of non-specific phosphatases acting
414 on phytate, as those described for *Lactobacillus pentosus* (Palacios et al., 2005), is still
415 to be elucidated.
416 Degradation of phytate in the stomach and small intestine is an activity effect of dietary
417 phytases of plant or microbial (fungi) origin. In the intestine, the solubility of phytates
418 plays a crucial role in terms of accessibility for bacterial phytases since phytates that
419 reach the large intestine are mostly in an insoluble form (Schlemmer et al. 2009), which
420 decreases their susceptibility for degradation. Moreover, the higher concentration of

421 InsP_6 present in the small intestinal chyme, the stronger the InsP_6 hydrolysis in the large
422 intestine (Schlemmer et al. 2009). The degree of phytate solubilisation may be the
423 reason why approximately 50% of phytates remain non-degraded *in vivo* (Schlemmer et
424 al. 2009), whereas in the *in vitro* tests, such as the one conducted in this study, the
425 soluble phytate could be completely hydrolysed by the most diverse bacterial cultures
426 (*i.e.* cultures in the Wilkins-Chalgren non-selective medium).
427 Studies on intact InsP_6 and hydrolysates of InsP_6 reported that both may strongly affect
428 the physiology of human and animal cells displaying antitumor activity (Vucenik and
429 Shamsuddin, 2003; Kumar et al. 2010). Although the mode of action of InsP_6 and its
430 hydrolysates is different (Suzuki and Hara, 2010; Ishizuka et al. 2011), a suppression of
431 cell proliferation and an induction of cell specialization, especially in conditions of
432 colon carcinogenesis, is undoubtedly a desired effect triggered by these compounds.
433 From this point of view, the complete degradation of phytate does not seem to be a
434 desirable phenomenon. However, the microbiota investigated in this study represented
435 bacteria living in the lumen of the intestine, and this part of microbiota differs from
436 bacterial populations adhering to the mucus layer (Zoetendal et al. 2002). Moreover,
437 bacterial isolates obtained from faeces express different abilities to adhere to epithelial
438 cells and the mucus (Wasilewska, Markiewicz and Bielecka, 2008). Thus, the ability to
439 degrade phytate to different lower *myo*-inositol phosphates by bacterial populations
440 adhered to the epithelium deserves further investigation.
441 Recapitulating, out of all the bacterial groups examined, the GPA and LAB showed the
442 lowest potential for phytate degradation. The vegetarians' GPA and LAB were
443 characterised by the lowest phytase activity when compared to both the omnivorous
444 adults and the breastfed infants. Out of the bacterial populations examined, the highest
445 phytase activity was determined for microbiota in the Wilkins-Chalgren non-selective

446 medium and in the *E. coli* cultures. The results obtained from the non-selective medium
447 showed that the intestinal bacteria cooperate in the gradual decompositions of phytate,
448 which may reflect both the taxonomical and metabolic diversity (substrate specificity of
449 phytases) of bacteria. Moreover, it has been shown that the microbiota from the
450 environment of a high phytate content (vegetarians' intestine) was the most effective at
451 degrading phytate, which suggests that microbiota adapt to such an environment and
452 that the diet modulates metabolic activities of intestinal bacteria what was reported in
453 early enrichment studies.
454

455 **Acknowledgements**

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460

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569

570 **Supporting Information**

571 Composition of microbiological media

572 **Figure S1** DGGE profiles of faecal bacterial cultures grown in the modified media

573 **Figure S2** Degradation of InsP_6 (NDPA) to intermediate derivative inositol phosphates
574 (InsP_3, InsP_4 and InsP_5) in the modified media.

575

576

577 **Table 1** Identification of DGGE bands taken from the DGGE profiles of bacterial
578 cultures cultivated in the non-selective modified Wilkins-Chalgren medium (anaerobes,
579 A), modified MRS medium (MRS), modified MacConkey medium (MC), modified
580 NBGT (N) and modified Garche's medium (G).

Band number*	**Closest relative**	**similarity %**	**Class, Family**
A2, A6, A9, A13, A14	*Escherichia coli*	98-99	Proteobacteria, Enterobacteriaceae
A3	*Enterobacter* sp.	99	Proteobacteria, Enterobacteriaceae
A5	*Dorea longicatena*	97	Clostridia, Lachnospiraceae
A7	*Sutterella wadsworthensis*	99	Betaproteobacteria/Burkholderiales
A8	*Clostridium lactatifermentans*	96	Clostridia, Clostridiaceae
A10-A11	*Dialister invisus*	98	Negativicutes, Veillonellaceae
A12	*Eubacterium eligens*	96	Clostridia, Eubacteriaceae
A15	*Allisonella histaminiformans*	99	Negativicutes, Veillonellaceae
A16	*Sutterella stercoricanis*	98	Proteobacteria, Sutterellaceae
A17	*Citrobacter freundii*	100	Proteobacteria, Enterobacteriaceae
A18,A20	*Clostridium perfringens*	98-100	Clostridia, Clostridiaceae
A19	*Bacteroides vulgatus*	99	Bacteroidia, Bacteroidaceae
MC1-MC4, MC13, MC18	*Escherichia coli*	99-100	Proteobacteria, Enterobacteriaceae
MC5, MC10, MC11	*Enterobacter cloacae*	99	Proteobacteria, Enterobacteriaceae
MC7-MC9	*Enterobacter* sp.	100	Proteobacteria, Enterobacteriaceae
MC10, MC12	*Enterobacter cloacae*	100	Proteobacteria, Enterobacteriaceae
MC11, MC14-MC16	*Citrobacter freundii / Klebsiella oxytoca*	100	Proteobacteria, Enterobacteriaceae
MC6, MC15, MC17, MC20, MC21	*Escherichia coli / Enterobacter cloacae*	99-100	Proteobacteria, Enterobacteriaceae
MC19	*Klebsiella pneumoniae*	99	Proteobacteria, Enterobacteriaceae
MRS1, MRS7, MRS16	*Lactobacillus salivarius*	99-100	Lactobacillales, Lactobacillaceae
MRS2, MRS10, MRS11, MRS15	*Streptococcus infantarius*	99-100	Lactobacillales, Streptococcaceae
MRS3, MRS14	*Escherichia coli*	99-100	Proteobacteria, Enterobacteriaceae
MRS5	*Lactococcus garvieae*	100	Lactobacillales, Streptococcaceae
MRS6	*Streptococcus salivarius*	100	Lactobacillales, Streptococcaceae
MRS8	*Lactobacillus mucosae*	100	Lactobacillales, Lactobacillaceae
MRS9, MRS17	*Lactobacillus gasseri*	99	Lactobacillales, Lactobacillaceae
MRS12, MRS18	*Lactobacillus fermentum*	100	Lactobacillales, Lactobacillaceae
MRS13	*Lactococcus lactis* subsp. *lactis*	99	Lactobacillales, Streptococcaceae
N1, N2, N5-N7, N10, N12	*Escherichia coli*	98-100	Proteobacteria, Enterobacteriaceae
N4	*Citrobacter freundii*	100	Proteobacteria, Enterobacteriaceae
N8	*Bacteroides vulgatus*	97	Bacteroidia, Bacteroidaceae
N9	*Escherichia coli* *Enterobacter cloacae subsp. cloacae*	100	Proteobacteria, Enterobacteriaceae
N11	*Desulfovibrio piger*	98	Deltaproteobacteria
N13-N15	*Shigella flexneri* *Escherichia coli*	100	Proteobacteria, Enterobacteriaceae

N16	*Klebsiella oxytoca*	100	Proteobacteria, Enterobacteriaceae
G1, G9	*Mitsuokella jaluladini*	98-99	Clostridia, Veillonellaceae
G2	*Clostridium sp.*	99	Clostridia, Clostridiaceae
G3	*Ruminococcus torques*	100	Clostridia, Lachnospiraceae
G4, G16	*Eubacterium hadrum*	99	Clostridia, Eubacteriaceae
G5	*Bifidobacterium pseudocatenulatum*	99	Actinobacteria, Bifidobacteriaceae
G6	*Clostridium ramosum*	100	Erysipelotrichi, Erysipelotrichaceae
G7	*Streptococcus infantarius*	100	Lactobacillales, Streptococcaceae
G8,G11	*Dorea longicatena*	99-100	Clostridia, Lachnospiraceae
G10	*Ruminococcus lactaris*	98	Clostridia, Ruminococcaceae
G12, G13	*Bifidobacterium adolescentis*	99-100	Actinobacteria, Bifidobacteriaceae
G14, G15	*Clostridium perfringens*	100	Clostridia, Clostridiaceae

581 * To see the band position in DGGE gels see Supporting Information.
582

583

Table 2 Ability of bacterial cultures to degrade phytate.

Bacterial group*	Diet†	Ratio of inositol phosphates in bacterial cultures‡ (LD : INT-Ins*P*s : NDPA)	Ability of bacterial cultures to degrade phytate§		
			F_{LD}	$F_{INT\text{-}InsPs}$	F_{total}
GPA	C	1 : 1 : 7	3	5	8
	V	2 : 1 : 7	8	3	12
	BF	1 : 4 : 5	4	17	21
LAB	C	2 : 1 : 7	4	3	7
	V	2 : 0 : 8	5	1	6
	BF	2 : 1 : 8	4	2	5
E. coli	C	3 : 5 : 2	27	42	70
	V	0 : 9 : 1	4	81	84
	BF	3 : 7 : 1	23	60	83
P-*B*	C	4 : 4 : 2	33	30	62
	V	2 : 6 : 2	13	50	64
	BF	1 : 7 : 2	8	60	68
Anaerobes	C	6 : 2 : 2	47	18	64
	V	9 : 1 : 0	88	7	95
	BF	1 : 4 : 5	7	22	28

* GPA – Gram-positive anaerobes, LAB – lactic acid bacteria, P-*B* – Proteobacteria-*Bacteroides*.

† Diets: C – conventional, V – wegetarian, BF – breastfeeding.

‡ LD – lower derivatives of phytate degradation (InsP_1, InsP_2 and *myo*-inositol), INT-Ins*P*s – intermediate derivatives of phytate degradation (the sum of InsP_3, InsP_4 and InsP_5), NDPA – non-degraded phytic acid.

§ ability to hydrolyse phytate to the lower inositol phosphates (F_{LD}) or to generate intermediate inositol phosphates ($F_{INT\text{-}InsPs}$); F_{total} – a general ability to degrade phytate.

FIGURE CAPTIONS

Figure 1 Schema of the study design. Modified media used in the study: Garche's (G), de Man Rogosa medium (MRS), the neomycin brilliant green taurocholic acid medium with blood (NBGT), MacConkey (MC) and Wilkins-Chalgren medium with horse's blood (W-Ch). For the detailed description see Materials and Methods.

Figure 1 Counts ($\log_{10}$ CFU ml^{-1}) of faecal bacterial cultures in modified media: Garche's (G), de Man Rogosa medium (MRS), MacConkey (MC), the neomycin brilliant green taurocholic acid medium with blood (NBGT) and Wilkins-Chalgren medium with horse's blood (W-Ch). The media were inoculated with diluted faecal bacteria of the adults on conventional or vegetarian diet, as well as the breastfed infants.

Figure 2 Dendrogram of the composite data set consisting of DGGE patterns of faecal bacteria cultures in the modified media: NBGT, MacConkey, MRS and Garche's. The media were inoculated with the faecal bacteria (10^7 cells) retrieved from the adults on the conventional diet (C1-C8), the adult vegetarians (V1-V8) and the breastfed infants (I1-I6).

Figure 3 Ability of selected intestinal groups of bacteria to degrade phytate *in vitro*. GPA – Gram-positive bacteria, LAB – lactic acid bacteria, P-B – Proteobacteria-*Bacteroides* culture. The graph shows means (medians) for the percentage distribution for the determined inositol phosphates (InsP_3, InsP_4, InsP_5), non-degraded phytic acid (NDPA) and lower derivatives of phytate degradation (LD) in relation to the initial content of phytic acid (1 mM).

Figure 4 Degradation of InsP_6 (NDPA) to intermediate derivative inositol phosphates (INT-Ins*P*s, the sum of InsP_3, InsP_4 and InsP_5;) and the lower inositol phosphates (InsP_1 and InsP_2; LD) in modified media: a) Garche's medium; b) MRS medium; c) MacConkey medium; d) NBGT medium, and e) non-selective Wilkins-Chalgren medium inoculated with faecal bacteria of the adults on conventional diet, the adult vegetarians and the breastfed infants. The brackets show values different at $p<0.05$ or $p<0.01$.

649 **FIGURES**

650

651 **Figure 1**

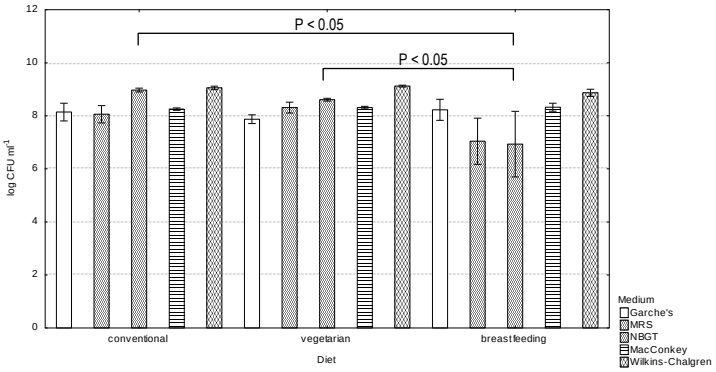

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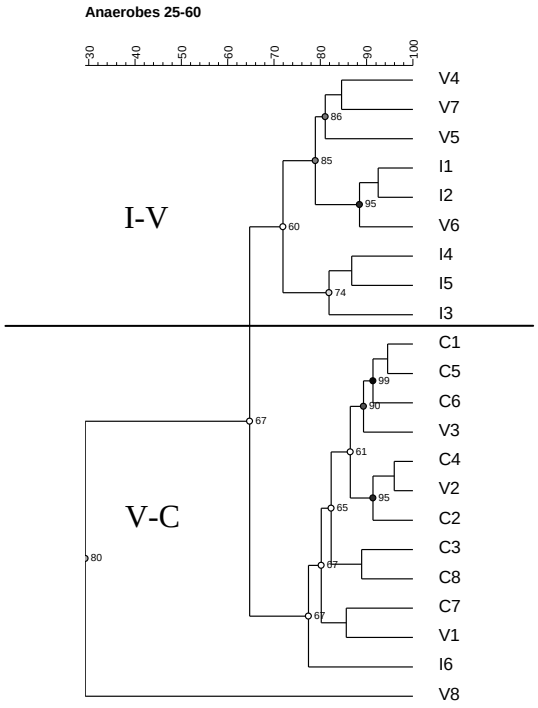
Figure 2


Anaerobes 25-60
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V4
V7
V5
I1
I2
V6
I4
I5
I3
C1
C5
C6
V3
C4
V2
C2
C3
C8
C7
V1
I6
V8
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I-V
V-C

660 **Figure 3**

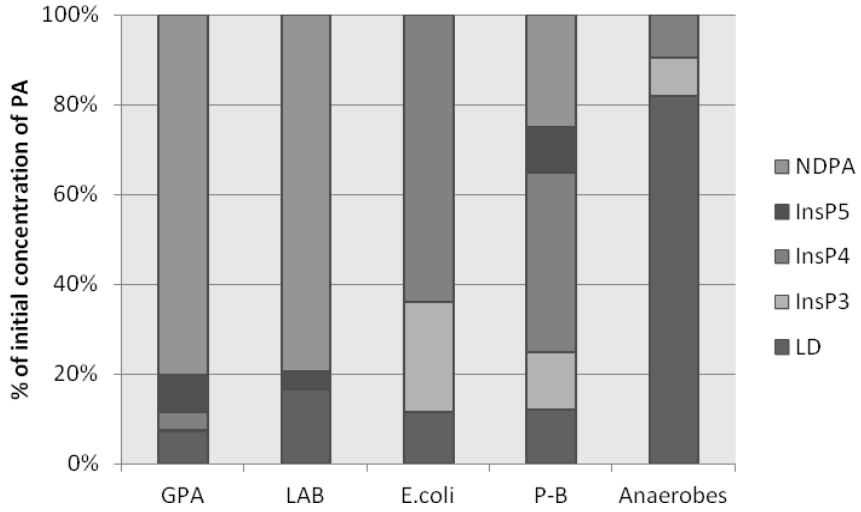

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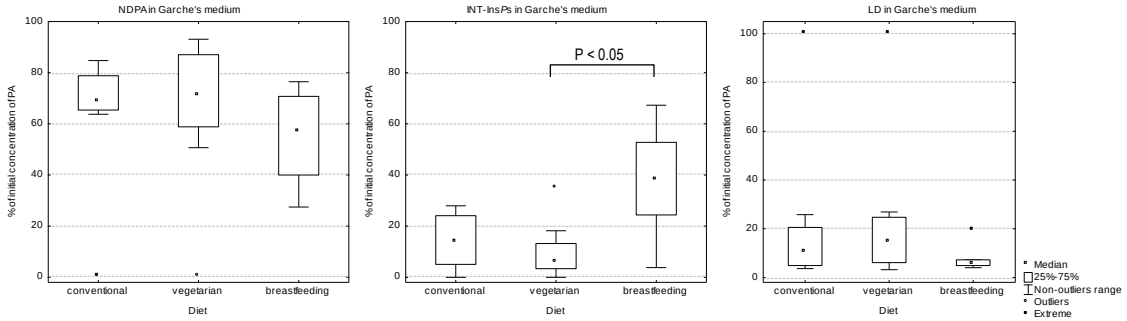
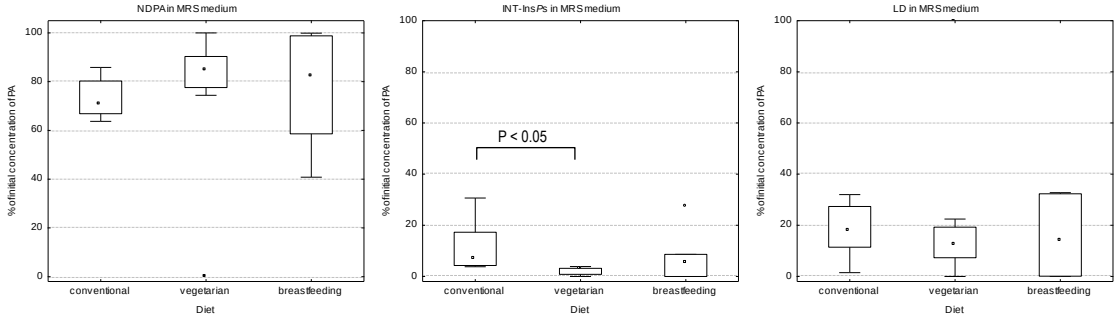
Figure 4

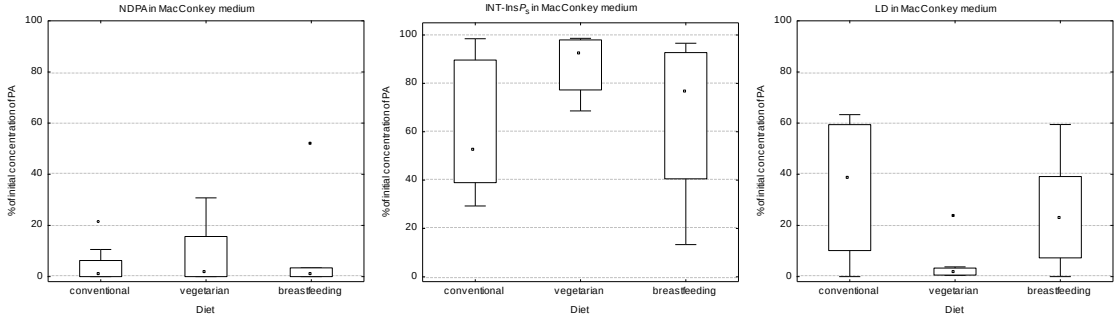
a) Gram-positive anaerobes



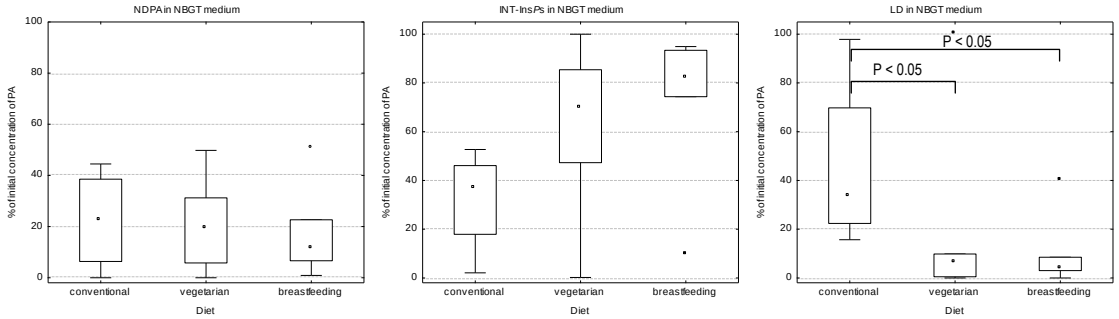
b) Lactic acid bacteria



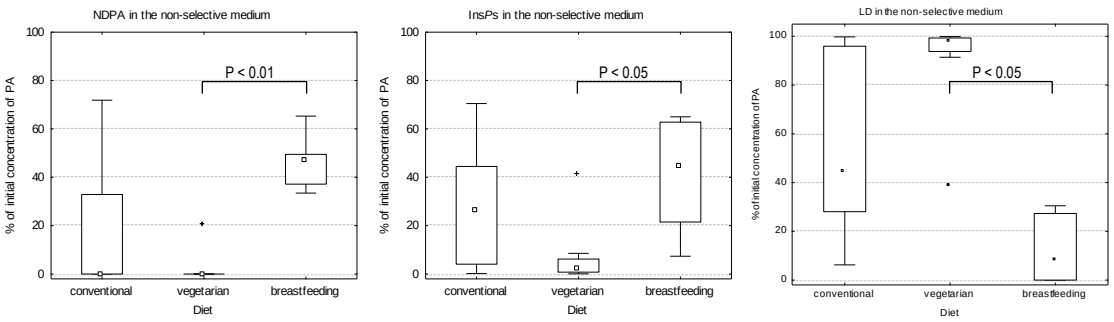
c) *E. coli*



d) Proteobacteria-*Bacteroides* cultures



e) Anaerobes


NDPA in the non-selective medium
% of initial concentration of PA
P < 0.01
conventional
vegetarian
breastfeeding
Diet
InsPs in the non-selective medium
% of initial concentration of PA
P < 0.05
conventional
vegetarian
breastfeeding
Diet
LD in the non-selective medium
% of initial concentration of PA
P < 0.05
conventional
vegetarian
breastfeeding
Diet

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