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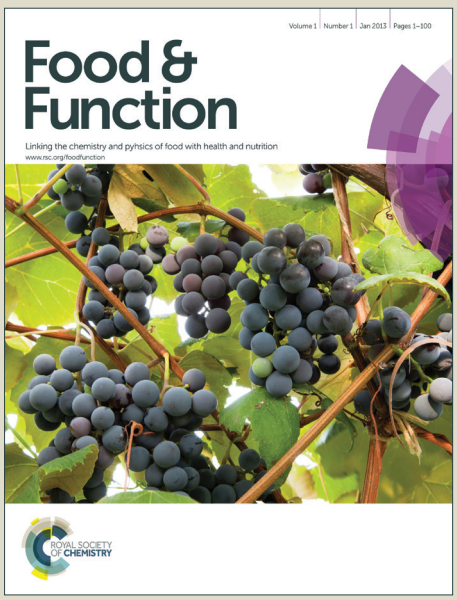

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Binding of bile acids by pastry products containing bioactive substances during *in vitro* digestion.

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Abstract

The modern day consumer tends to choose products with health enhancing properties, enriched in bioactive substances. One such bioactive food component is dietary fibre, which shows a number of physiological properties including the binding of bile acids. Dietary fibre should be contained in everyday, easily accessible food products. Therefore, the aim of this study was to determine sorption capacities of primary bile acid (cholic acid - CA) and secondary bile acids (deoxycholic – DCA and lithocholic acids - LCA) by muffins (BM) and cookies (BC) with bioactive substances and control muffins (CM) and cookies (CC) in two sections of the *in vitro* gastrointestinal tract. Variations in gut flora were also analysed in the process of *in vitro* digestion of pastry products in a bioreactor. Enzymes: pepsin, pancreatin and bile salts: cholic acid, deoxycholic acid and lithocholic acid were added to the culture. Faecal bacteria, isolated from human large intestine, were added in the section of large intestine. The influence of dietary fibre content in cookies and concentration of bile acids in two stages of digestion were analysed. Generally, pastry goods with bioactive substances were characterized by a higher content of total fibre compared with the control samples. These products also differ in the profile of dietary fibre fractions. Principal Component

Analysis (PCA) showed that bile acid profile after two stages of digestion depends on the quality and quantity of fibre. The bile acids profile after digestion of BM and BC forms one cluster, and with the CM and CC forms a separate clusters. High concentration of H (hemicellulose) is positively correlated with LCA (low binding effect) and negatively correlated with CA and DCA contents. The relative content of bile acids in the second stage of digestion was in some cases above the content in control sample, particularly LCA. This means that the bacteria introduced in the 2nd stage of digestion synthesize the LCA.

Key words: pastry goods, bioactive substances, dietary fibre, bile acid.

Abbreviations: CM – control muffins, BM – bioactive muffins, CC – control cookies, BC – bioactive cookies, NDF – neutral detergent fiber, C – cellulose, H – hemicellulose, L – lignin, TDF – total dietary fiber, SDF – soluble dietary fiber, IDF – insoluble dietary fiber, CA – cholic acid, DCA – deoxycholic acid, LCA – lithocholic acid.

1. Introduction

In view of increasingly common diet-related diseases, the modern consumer shows a growing demand for food which, in addition to its basic functions – providing the human body with energy and nutrients – should also exhibit health-promoting properties, and its consumption should support the treatment of diet-related disorders. Food manufacturers and food technologists come up with new product formulae, to meet the requirements for "functional foods" with programmed health-promoting attributes. However, before a food product is labelled as healthy, its composition needs to be tested to see how its biologically active components behave in the digestive process and whether they still preserve their pro-health attributes. To that end, *in vitro* or *in vivo* research is carried out, in order to assess the bioavailability of biologically active substances in the designed food product. Digestive

processes occurring in human gastrointestinal tract are very complex, yet crucial for the preservation of health. They include simultaneous mechanical transformation of food, leading to the reduction in the size of food portions (occurring mainly in the mouth and stomach) and enzymatic transformation by which macromolecules are hydrolysed to small particles and then absorbed into the bloodstream (small and large intestine**).** Conducting research on digestion and food absorption in the complex, multi-level *in vivo* digestive system is technically demanding (especially the issue of accessing the small intestine section), and also very costly and limited by ethical reasons. A growing demand for food component testing and their interactions in the gastrointestinal tract leads to the increased popularity of GSI (gastric and small intestinal model) testing as an alternative to *in vivo* research[1]. Many research studies confirm the importance of dietary fibre in the metabolism of bile acids. Bile acid binding capabilities of dietary fibre depend, among other factors, on its fractional composition. Bile acids, the major metabolites of cholesterol, are a mixture of steroids, and play an important physiological role in the elimination of cholesterol from the body and in facilitating the absorption of dietary lipids and fat-soluble vitamins by formation of micelles[2,3]. The concentration of individual bile acids can be related to various diseases, such as: hepatitis, gallstone and other liver diseases[4]. The effect of dietary fibre on blood lipid profile has been researched to reveal a number of mechanisms responsible[5,6,7]. Dongowski[8] observed that the viscosity of dietary fiber plays an important role in the lowering of cholesterol levels. Viscosity interferes with bile acids absorption from the ileum. As a result, LDL cholesterol is removed from the blood and converted into bile acid with the participation of the liver, to replace the bile acids removed in faeces. The hypocholesterolaemic action of fibre is to a degree mediated by a lower absorption of digestive bile acid, because of the interruption of the enterohepatic bile acid circulation increasing the loss of faecal bile acid, and its recurring synthesis in liver[9]. Trautwein[10] put forward the idea that the physicochemical

properties of soluble fibre result in important variations in volume, bulk and viscosity of the intestinal lumen, which influence the metabolic pathways of hepatic cholesterol and lipoprotein metabolism, also resulting in the lowering of plasma LDL-cholesterol. Others suggested that dietary fibre increases the enzymatic activity of cholesterol-7-α- hydroxylase, which is the major regulatory enzyme responsible for the hepatic conversion of cholesterol to bile acids[11]. Jones[12] observed a reduction in the hepatic lipogenesis stimulated by insulin. It has also been put forward that the fermentation of dietary fibre by the intestinal microflora might modify the short chain fatty acids production, and thus lead to a reduction in acetate and an increase in the propionate synthesis. This, in turn, can reduce the endogenous synthesis of cholesterol, fatty acids and low density lipoproteins[13]. Lignin is the major fraction responsible for bile acid binding; also, pectins and β-glucans show significant binding abilities[14,15]. The affinity of dietary fibre for bile acids is determined by the number and location of hydroxyl groups in the given bile acid, with increasing number of –OH groups in steroid ring leading to lower bile acid binding ability of fibre. As demonstrated by Dongowski[16], bile acid binding process is more efficient in acidic environments as compared to basic environments. Apart from dietary fibre, also Maillard reaction products, protein and carbohydrates[16] have bile acid binding capacity, as well as polysaccharides produced by *Lactobacillus* and *Streptococcus* bacteria[17]. Liver cells are known to be responsible for the synthesis of bile acids. The influence of bacteria active in the transformation of primary bile acids into secondary bile acids has been investigated[2,3]. It has been suggested that some faecal bacteria may contain Cytochrome P450, responsible for the transformation of cholesterol and its derivatives to primary bile acids[18]. Ample research conducted thus far has demonstrated the ability of dietary fibre fractions, starch and proteins to bind bile acids[19]. It needs to be emphasized, however, that these research models emulated the human gastrointestinal environment taking into account only its pH and additive of pepsin, pancreatin and bile

acids[20,21,22,23,24,25,26]. The research presented in this paper a model is used which emulates the environment of human gastrointestinal tract with its pH, the presence of enzymes i.e. pepsin and pancreatin, bile acid salts and a mix of faecal bacteria in anaerobic conditions. Therefore the focus of this paper is not only on the ability of digested food to bind bile acids, but also on the influence of faecal bacteria on the changes in bile acid levels in the large intestine.

2. Materials and methods

2.1.Materials

The material for this research consisted of pastry goods containing bioactive substances: muffins, and cookies as well as control samples of muffins and cookies. In products with bioactive substances, wheat (Kupiec mills, Poland) and potato flour (Kupiec mills, Poland) was replaced by buckwheat flour (Niedźwiady mills, Poland). The fat was replaced by inulin (Hortimex, Poland), sugar was replaced by sweeteners: aspartame (Hortimex, Poland) and acesulfame-K (Hortimex, Poland) in 1:1 proportion. Other ingredients such as buckwheat husk (Niedźwiady mills, Poland), mulberry extract and chokeberry (Institute of Agricultural and Food Biotechnology, Poland) were also added. Maltodextrin (Hortimex, Poland) was added as the bulking agent (Table 1).

2.2. *In vitro* digestion

The digestive process was carried out according to Fig. 1. A bioreactor was used as a simple digestive tract, and the samples were collected in two stages of digestion (1 – small intestine and 2 – large intestine). The simulation of the gastrointestinal tract was conducted at the temperature of 37°C and the stirring speed of 200 rpm. The environment of the stomach, small and large intestine was reproduced as closely as possible[27]. In this paper uses a model which emulates the environment of human gastrointestinal tract with its pH, the presence of enzymes i.e. pepsin (5.76 g in 120 mL of 0.1M sterilized hydrochloric acid – Sigma-Aldrich, Germany), pancreatin (1.2 g – Sigma-Aldrich, Germany) and bile acid salts (CA, DCA, LCA

– each 3.6 g, Sigma-Aldrich, Germany) mixed together in 300 mL of 0.1M sterilized sodium bicarbonate. In order to further enhance the resemblance of this environment to the human digestive tract (large intestine in particular), a mix of faecal bacteria, previously isolated from a healthy 24 year old male, was added at stage 2, in the amount of 10^6 cfu/mL.

2.3. Dietary fibre assay

2.3.1. Total Dietary fibre (TDF)

The contents of TDF, consisting of soluble dietary fibre (SDF) and insoluble dietary fibre (IDF) were estimated in accordance with AOAC[28]. The assumption in this method is to determine the content of dietary fibre under conditions similar to those found in the human alimentary tract using the following enzymes: thermostable α-amylase (pH 6.0, 90°C Termamyl 120 L, Novozymes, Denmark), pepsin (pH 1.5, 40°C) and pancreatin (pH 6.8, 40°C). Following enzymatic extraction samples were washed with 3 x 20 mL of hot water, 3 x 10 mL of 96% ethanol (Poch, Poland, pure p.a.) and 3 x 20 mL of acetone (Poch, Poland, pure p.a.). Filters with the residue (IDF) were dried at 135°C for 2 h and then incinerated for 5 h in an oven at 525°C. In order to determine SDF the filtrate was made up with 96% ethanol (400 mL, 60°C) and left for 2 h. The precipitated dietary fibre was washed with 3 x 20 mL of hot water, 3 x 10 mL of 96% ethanol and 3 x 20 mL of acetone, and then filters with the residue (SDF) were dried at 135°C for 2 h and incinerated in an oven at 525°C for 5 h. Analyses were performed using a Fibertec System 1023 apparatus (Foss, Sweden).

$$\%\, IDF\ or\ \%SDF = \frac{\big((Weight\ of\ residue - protein - ash) - blank\big) * 100}{weight\ of\ sample}$$

2.3.2. Detergent fibre determination

The content of neutral dietary fibre (NDF), consisting of acid detergent fibre (ADF) and acid detergent lignin (ADL), was determined using the detergent method according to Van

Soest[29], as modified by McQueen & Nicholson[30]. Thermostable α-amylase was used to digest starch. Reagents to estimate the content of neutral detergent fiber (NDF): neutral disodium versenate dehydrate (Poch, Poland, pure p.a.), disodium tetraborate decahydrate (Poch, Poland, pure p.a.), disodium hydrogen phosphate (Poch, Poland, pure p.a.), ethylene glycol (Poch, Poland, pure p.a.) and destylate water. Reagents to estimate the content of ADF: sulfuric acid (1N, Poch, Poland, pure p.a.), N-cetyl-N,N,N- trimethylammoniumbromid (Merck, Germany, GR for analysis). Reagent to estimate the content of ADL: sulfuric acid (72%, Poch, Poland, pure p.a.). Hemicellulose (H) content was calculated from the difference between NDF and ADF. Cellulose (C) content was calculated from the difference between ADF and ADL. Analyses were conducted using a Fibertec System M 1020 apparatus by Tecator (Foss, Sweden).

$$\%Hemicellulose = \%NDF - \%ADF$$
$$\%Cellulose = \%ADF - \%ADL$$

2.4 Bile acid assay

Reversed-phase (C18 column) ultra high-performance liquid chromatography electrospray ionization mass spectrometry (RP-UHPLC-ESI-MS) analysis was performed using a Dionex UltiMate 3000 UHPLC (Thermo Fisher Scientific, Sunnyvale, CA, USA) coupled to a Bruker maXis impact ultrahigh resolution orthogonal quadrupole-time-of-flight accelerator (qTOF) equipped with an ESI source and operated in positive-ion mode (Bruker Daltonik, Bremen, Germany). The RP chromatographic separation was achieved with a Kinetex™ 1.7 µm C18 100 Å, LC column 100 × 2.1 mm (phenomenex, Torrance, CA, USA). The ESI-MS settings were as follows: capillary voltage 4500 V, nebulizing gas 1.8 bar, and dry gas 9 L/min at 200° C. The scan range was from mass-to-charge ratio (*m*/*z*) 80 to 1200. The mobile phase was composed of water containing 1% formic acid (A) and acetonitrile containing 5% water and 1% formic acid (B). The flow rate was 0.2 mL/min with a gradient elution of 5%–95% B over 35 min, and standing at 95% B for 20 min. The sample injection volume was 2 µL. The

column temperature was set to 40 °C. The ESI-MS system was calibrated using sodium formate clusters introduced by loop-injection at the beginning of the LC-MS run. The LC-MS data were processed using Data Analysis 4.1 software (Bruker Daltonik, Bremen, Germany). Molecular ions: $[M-2H_2O+H]^+$ for CA, DCA and $[M-H_2O+H]^+$ for LCA, dehydrocholic acid were extracted from full scan chromatograms and peak areas were integrated. The extraction window of individual ion chromatograms was ± 0.05 *m/z* units. The compounds present in each sample were identified by comparing their retention times with those of standards, and based on molecular mass and structural information from the MS detector. The dehydrocholic acid was used as an internal standard. It was added in a constant amount to samples, the blank and calibration standards. This substance was used for calibration by plotting the ratio of the analyte signal to the internal standard signal (Relative response – y) as a function of the analyte concentration of the standards (Relative concentration – x). The concentration of bile acids were calculated according to formulas – CA: $y = 2.71889x - 0.061$, $R^2 = 0.9999$, DCA: $y = 4.8769x - 0.09$, $R^2 = 0.9998$, LCA: $y = 3.3214x - 0.005$, $R^2 = 0.9999$.

2.5 Content of faecal bacteria

Microbiological research was carried out according to international standards[31]. The numbers of *Enterococcus*, *Bifidobacterium*, *E. coli* and *Lactobacillus* in the experimental samples were determined using the pour plate with overlay method[32] on**:** Kanamycine Esculine Azide Agar (Merck, Germany) for *Enterococcus,* TOS agar with MUP Selective Supplement (Merck, Germany) for *Bifidobacterium,* Endo agar (Merck, Germany) for *E. coli,* MRS agar (Merck, Germany) for *Lactobacillus.*

2.6. Statistical analysis

Experiments were conducted in triplicate. Each value was the mean of three independent trials. Hierarchical cluster analysis was performed using Ward amalgamation rule with the Euclidean distance *(d)* measure. The tree plots were scaled to a standardized scale

(*dlink/dmax**100). Non-hierarchical cluster analysis (*k*-means clustering) was performed to form a grouping of pastry goods due to the different contents of dietary fiber. V-fold cross-validation algorithm was used to determine the best number of clusters. Principal component analysis (PCA) technique was used to reduce the dimensionality reduction of data and to present the samples in new coordinate system. The statistical analysis was performed using Statistica software, Version 10, StatSoft Inc. (OK-US).

3. Results

The variation in the content of dietary fibre in cookies was considered (Table 2). Similarities in the dietary profiles of cookies were noted. The analysis of hierarchical tree showed that BC and CC profiles are similar, the most different were the samples coded as CM (Figure 2). CM sample contained the least fibre. However, the profile of dietary fiber fraction is different for bioactive and control samples. Control samples (CM and CC) contain larger proportion of hemicellulose as compared to cellulose or lignin. Indeed, the samples of CC have the highest content of H. Then this proportion changes for bioactive samples (BM and BC), which contain a larger proportion of lignin as compared to H and C. Analysis of bile acid content conducted in two stages of digestive system model showed that the concentration of bile acids in the first stage of digestion is lower in comparison to control sample (Table 3). The relative content of bile acids in the second stage of digestion was in some cases above the content in the control sample. The influence of dietary fibre content in cookies on the concentration of bile acids in two stages of digestion was observed. The k-Means algorithm was used to detect clusters in observations and to assign those observations to the clusters. The ANOVA (Analysis of variance) results pertaining to the differences in the means for the continuous variables across the clusters were used to evaluate which variables have discriminant power ($p<0.05$) – Table 4. It was noted that H from variables set in the first stage of digestion and C, IDF, H, LCA from variables set in the second stage of digestion did not

have sufficient discrimination power. In general, the k-means method will produce k different clusters of greatest possible distinction. The highest number of clusters k leading to the greatest separation is not known a *priori* and must be computed from the data. V-fold cross-validation algorithm was used to determine the best number of clusters. This type of clustering procedure is useful when no test sample is available and the learning sample is too small to have the test sample taken from it. The classification generated using only the variables with discriminant power ($p<0.05$) were presented in Table 5, the final classification was the same for both stages of digestion. The cluster analysis showed that the data have clear structure and both fibre and bile acids have impact on the effect of clustering, BM, BC form one cluster. The PCA was used to explain and interpret interdependences between variables and their impact on the classification of data. The variables with discriminant power (computed in k-Means procedure) in PCA analysis were used. In this study, PCA was used to reduce number of variables of the dataset (fibre profile of cookies and bile acid content at two stages of digestion). The plot of principal component (PC) coordinates of variables for the first two factors (PCs) makes the interpretation of principal components much easier. The principal component coordinates are commonly referred to as loadings plot. This graph shows a unit circle (*x*-loadings on PCs) - Figure 3A, 4A. Because the current analysis is based on correlations, the largest principal component coordinate (variable-PC correlation) that can occur is equal to 1.0. The variables, when projected onto this circle, help analyse the relationship between the original variables, the way they correlate to each other and their influence in determining the new coordinate system. The quantity at the centre of such analyses is the *x*-loadings plot. The *x*-loadings of a principal component with respect to a variable is the cosine of the angle between the directions of that component and the axis of the respective variable. For instance, LCA is positively correlated with H - low angle between vectors, additionally these variables are more correlated to principal component 2; CA and H

are negatively correlated. The PC1 and PC2 were computed using only the active variables. The supplementary variables was projected onto the vector subspace generated by the factors. Supplementary variables were extracted because some of them do not have discriminant power as ANOVA showed (Table 4). Two new variables (PCs) were introduced: component 1, which has the highest explanatory power, and component 2, which together explains about 90 and 97% of the data variance, respectively for the 1st and 2nd stage of digestion. As observed in Figure 3B and 4B, it is possible to distinguish three groups. The first cluster represents BC and BM, the second one including samples CC and the third is related to CM. It was indicated that parameters for this group are definitely different. The simultaneous comparison loadings plot (Figure 3A, 4A) with appropriate score plot (Figure 3B and 4B) allows to identify the relationships between samples and variables. The high concentration of H is positively correlated with LCA content and negatively correlated with CA and DCA - Figure 3A, 4A. It means that the H variable has the highest impact on concentration of bile acids in two stages of digestion and additionally least bind the LCA to the lowest extent. It should be noted that samples CC have the highest value of H - Table 2 - and the 50% higher level of LCA compared to blank sample (Table 3). Based on the results of the study it was concluded that all tested pastry goods exhibited good prebiotic properties. The most abundant growth of *Enterococcus* bacteria was observed after 18 h for the CM (by 13%), while BC cookies did not constitute a favourable environment for the growth of this type of bacteria (Table 6). The process of *in vitro* digestion of BM affected the count of *Lactobacillus* bacteria – an increase by 22%, while after the digestion of BC the number of bacteria was observed at the level of 8.36 - 8.41 $\log_{10}$ cfu mL^{-1}. The highest growth of *E. coli* bacteria was observed as a result of digestion of BM and CC (41%). In the case of *Bifidobacterium*, the richest growth (34%) was observed after 18 h using CC. For all other types of cookies no *Bifidobacterium* bacteria were identified.

4. Discussion

The high content of TDF in pastry goods containing bioactive substances (Table 2) is attributed to the addition in their recipes of buckwheat hulls and buckwheat flour. It has been shown by research[2] that buckwheat hulls and buckwheat flour contain 91.2%, and 8.2% of TDF, respectively. Therefore bioactive samples showed a much higher dietary fiber content. A similarly high content of dietary fibre in buckwheat flour was found by Górecka[33] and Przybylski[34]. High content of dietary fibre can also be related to the presence of resistant starch. Kim[35] observed an increase in the content of resistant starch from 1.3% to 7% in bakery products made from wheat flour, after 7-day storage. They also showed that the content of resistant starch and humidity in the tested pastry goods increases during the production process. Leeman[36] showed that potato starch contains approx. 5.2% of resistant starch. Similar observations were made by Dongowski[16] in his research on wheat flour. The samples were characterised by a varying content of ingredients containing starch (Table 1). In order to minimize the impact of retrograded starch, only samples immediately after baking were used in this research. The composition of dietary fibre fractions in the tested bakery products varied, which was also attributed to the addition of buckwheat hulls and flour. According to Dziedzic[24] hulls contain 32% of lignin and 36% of cellulose. Escarnot[37] demonstrated that wheat flour, also used in sweet bakery products, contains 7.3% of hemicelluloses.

Basing the conclusion on the results of *in vitro* research, it was found that the ability of bakery products to bind bile acids depended both on the type of product and the type of analysed bile acid (Figure 3 and 4). Individual bile acids exhibit varied physicochemical and biological properties, of which hydrophilicity is the most important. Of the primary bile acids, CA is the most hydrophilic, while LCA — the least[38]. Also, the amphiphilicity of bile salts is strongly influenced by subtle variations in molecular structure as stated by Carey[39]. The

author report that the molecular hydrophobicity of bile salts depends on the cholate conjugation group. Between deoxycholate (DCA), cholate (CA), chenodeoxycholate (CDC) and ursodeoxycholate (UDC) it decreases in the order DCA >CDC >CA >UDC with free bile salts >glycine-conjugates > tauro-conjugates. Deoxycholic acid is a more active surfactant than taurocholic acid, which can be attributed to stronger hydrophobic properties of DCA as compared to taurocholic acid[40]. This data is consistent with the observations made at the first stage of digestion, i.e. DCA as the most reactive of the three investigated bile acids was bound at the first stage of digestion to the highest degree (Table 3).

Bile acid binding capacity depends, among other things, on the fractional composition of dietary fibre, chemical structure of the acid in question, the osmotic concentration and the pH of the environment. Lignin is an excellent adsorbent of bile acids. Bioactive samples eg. BM and BC showed higher percentage content of lignin fraction than control samples. However, higher concentrations of CA and DCA were demonstrated in case of BC and BM samples during the first stage of digestion (Table 3). Thos maybe attributed to a different ratio of different fractions of dietary fiber. According to Dziedzic[24] sorption of bile acid binding depends on the proportion of dietary fiber fractions. Cellulose shows the lowest bile acids binding ability. Additionally ability to bind bile acids depends on the molecular weight of the individual molecule and the presence of methoxyl and β-carbonyl groups. Eastwood and Hamilton[41] demonstrated that methylation of lignin increased bile acid adsorption. It follows, then, that a simple interdependence between the concentration of individual bile acids and the concentration of dietary fiber fractions cannot be determined. The obtained results showed a limited ability of LCA binding by H (Figures 3 and 4).

Kahlon and Woodruff[20] showed that the ability to bind bile acids is greater for products with higher TDF levels. Many researchers have demonstrated that the degree of comminution (atomization) of foods rich in fibre has a direct impact on their ability to bind

bile acids: the less comminuted the food, the higher the bile acid binding capacity. When *in vitro* digestion of pastry products was conducted, food comminution which normally occurs in the mouth was emulated using a mechanical mill, therefore each investigated sample featured the same degree of comminution. For the above reason, the degree of comminution was disregarded as a variable in statistical analysis[42, 43].

Chezem[35] found that potato starch had a higher capacity to bind cholic acid (0.06 mg/g faeces) as compared to cellulose (0.01 mg/100 g). Moreover, LCA and DCA were bound by potato starch in the amounts of 0.81 and 1.45 (mg/g faeces) respectively, as compared to cellulose (0.06 and 0.31 mg/g faeces). The influence of starch was not considered, because, as has been mentioned, only freshly baked samples were used for this research. The binding effect in the large intestine can only be exhibited by starch resistant to endogenic amylolytic enzymes, therefore a spectacular effect demonstrated by Chezem[44] did not have any serious influence in the investigated system. Bile acid salts are also bound by proteins[45, 46], in an emulated gastrointestinal environment, however, peptidases deactivate them. In the large intestine an increase in the content of bile acids was observed for bioactive and control samples, as compared to the small intestine (Table 3). Hepatocytes are responsible for biotransformation of cholesterol and phytosterols to cholic acid[47]. To emulate digestive environment under this research, faecal bacteria e.g. *E. coli* were used in the bioreactor. These bacteria have cytochrome P450 enzymes, similar in their composition to the enzymes produced by hepatocytes[48,49]. For the above reason the supposition that *E. coli* can take part in biotransformation of cholesterol or phytosterols to primary bile acids seems justified. Cornfine[43] demonstrated that in an *in vitro* environment, longer digestion times lead to increased bile acid binding capabilities. This is partly because enzymes, such as amylase, peptidase and cellulase, which are produced by faecal bacteria in the digestive tract, have a longer time to act. The research presented in this paper demonstrated an increase in DCA

content in the large intestine, for CM, BC and BM, by 13%, 15% and 28% respectively, and also an increase in LCA content by 29% for BM and 55% for CC cookies (Table 3). Research conducted by Kailasapathy and Chin[50], Gomes and Malcata[51], shows that faecal bacteria take active part in the biotransformation of primary bile acids (cholic acid) to secondary bile acids i.e. deoxycholic and lithocholic. During *in vitro* digestion in the large intestine a growth of *Enterococcus*, *Lactobacillus* and *Esherichia coli* bacteria was noted (6.83-9.68 $\log_{10}$ cfu mL^{-1}) –bacteria, which in all probability are responsible for the transformation of primary bile acids into secondary acids. In BC sample there was not an appreciable growth of *Enterococcus*, *Lactobacillus* and *E. coli* bacteria. Licht[52] showed that glucose and fructose are responsible for growing of faecal bacteria. The control samples contained sucrose as compared to bioactive samples (which contained a replacement in the form of aspartame and acesulfam K). Moreover, BM contained more SDS fraction in comparison to other samples. The authors [48,49] showed that soluble dietary fiber fractions affect the growth of faecal bacteria. In the conducted research, at the 2nd stage of digestion bacteria synthesize the LCA. This fact was possible to observe because the sample CC has the highest H content which does not bind LCA (as was mentioned above) and as a result it was possible to note that relative content of LCA exceeded significantly the value of 1. It is possible that other variables stimulate or inhibit this synthesis but they were not recognised (sample BM have the lowest content of H but the concentration of LCA and DCA exceeded by 30% the initial value). The highest concentration of other bile acids was observed also after the second stage of fermentation in comparison to the first stage (Table 3). However, it cannot be ruled out that this effect results from the release of binding bile acids at the 1st stage. It is highly probable that desorption of bile acids can occur with a change of pH in the gastrointestinal tract[16], however, the obtained results led to the conclusion that the concentration of lithocholic acid increased at stage 2 of the digestive process to a level above the concentration introduced into the growth vessel. It

can therefore be suggested that biotransformation of lithocholic acid (LCA) from cholic or deoxycholic acid occurs after the second stage of digestion; this should be confirmed with further research.

The results obtained within this research can lead to new dietary recommendations being used in the future for people at risk of lipid metabolism disorders such as hypercholesterolemia and atherosclerosis, or colorectal cancer – as preventive measures. They can also be used in developing new treatment methods for lipid disorders.

Conclusions

Cookies and muffins containing bioactive ingredients were characterised by a higher content of NDF and TDF dietary fibre, as compared with the control samples. In addition, a difference in the fractional composition of dietary fibre was observed between muffins and cookies made with bioactive substances on one hand and products made to original recipe on the other. It was found that the ability to bind bile acids depended both on the type of tested product and the type of bile acid. Despite the well documented ability of dietary fibre to bind bile acids, a higher concentration of DCA and LCA was observed in the large intestine. It can be assumed that faecal bacteria used for *in vitro* digestion of food play an active role in the biotransformation of primary bile acids into secondary bile acids. This supposition, however, needs further laboratory research to confirm its accuracy.

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References

1. A. Guerra, L. Etienne-Mesmin, V Livrelli, S. Denis, S. Blanquet-Diot and M. Alric, *Trends Biotechnol.*, 2012, **30**, 591-600.
2. Burkard, A.V. Eckardstein and K.M. Rentsch, *J. Chromatogr*. 2005, **826**, 147–159.

3. Y. Wang, J. Zhang, X.X. Zhu and A. Yu, *Polymer*, 2007, **48**, 5565–5571.
4. A. Gültekin, A. Ersöz, A. Denizli and R. Say, *Sens. Actuators B*, 2012, **162**, 153-158.
5. N. Babio, R. Balanza, J. Basulto, M. Bulló and J. Salas-Salvadó. *Nutr Hosp.* 2010, **25**, 327-340.
6. J.L. Slavin. *Nutrition.* 2005, **21**, 411-418.
7. M.M.A.K. Khattak. *Pak. J. Nutr.*, 2002, **1**, 161-168.
8. G. Dongowski, M Huth and E. Gebhardt. *Br. J. Nutr.,* 2003, **90**, 895–906.
9. L.M. Morgan, J.A. Tredger, Y. Shavila, J.S. Travis and J. Wright. *Br. J. Nutr.* 1993, **70**, 491-501.
10. E.A. Trautwein, A. Kunath-Rau and H.F. Erbersdobler. *J. Nutr.* 1999, **129**, 896-902.
11. S. Roy, H.C. Freake and M.L. Fernández. *Atherosclerosis.* 2002, **163**, 29-37.
12. P.J. Jones, C.A. Leitch and R.A. Pederson. *Am. J. Clin. Nutr.* 1993, **57**, 868-74.
13. H.H. Cheng and M.H. Lai. *J. Nutr*. 2000, **130**, 1991-95.
14. J. Boatright, J. Nickerson and A. Moring, *J. Ocul. Biol. Dis. Infor*., 2009, **5**, 149-159.
15. M. Norlin and K. Wikvall, *Cur. Molek. Med*., 2007, **7**, 199-218.
16. G. Dongowski, *Food Chem*., 2007, **104**, 390-397.
17. R. M. Pigeon, E. P. Cuesta and S. E. Gillilandt, *J. Dairy Sci*.,2002, **85**, 2705–2710.
18. W. Karam and J. Y. Chiang, *J. Lipid Res*., 1994; **35**, 1222–1231.
19. J. U. Kongo-Dia-Moukala, H. Zhang, P. C. Irakoze, *Int. J. Mol. Sci.,*2011, **12**, 1066-1080.
20. T. S. Kahlon and C. L. Woodruff , *Cereal Chem.* 2003, **80**, 260-263.
21. T. S. Kahlon, M. H. Chapman and G. E. Smith, *Food Chem*., 2007, **103**, 676-680.
22. Ch. Barbana, A. C. Boucher and J. I. Boye, *Food Res. Int*., 2011, **44**, 174-180.
23. D. Górecka, K. Dziedzic and M. Hęś, *Italian J. of Food Sci*., 2014, **21**, 103-108.

24. K. Dziedzic, D. Górecka, M Kucharska and B. Przybylska, *Food Res. Int*., 2012, **47**, 279-283.

25. D. Lairon, H. Lafont, J. L. Vigne, G. Nalbonne, J. Leonardi and J. C. Hauton. *Am. J. Clin. Nutr.*, 1985, **42**, 629-638.

26. T. S. Kahlon and G. E. Smith. *Food Chem.*, 2007, **100**, 1531-1536.

27. J. S. Hur, B. O. Lim, E. A. Decker and D. J. McClements, *Food Chem*. 2011, **125**, 1-12.

28. M. N.-G. Asp, C.-G. Johansson, H. Hallmer and M. Siljestrom, *J. Agric. Food Chem.*, 1983, **31**, 476-482.

29. P. J. Van Soest, *AOAC*, 1967, **50**, 50-55.

30. R. E. McQueen and J. W. G. Nicolson, *AOAC*, 1979, **62**, 676-680.

31. F. Pędziwilk, *Acta. Alim. Pol.,*1975, **25**, 127-130.

32. C. Vanderzant and D. F. Splittstoesser. C. *American Public Health Assoc*., Washington, DC 1992.

33. D. Górecka, M. Hęś, K. Szymandera-Buszka and K. Dziedzic, Acta *Sci. Pol., Technol. Aliment*., 2009, **8**, 75-83.

34. R. Przybylski and E. Gruczyńska, *Eur. J. Plant Sci. and Biotech*., 2009, **3**, 10-22.

35. J. H Kim, E. J. Tanhehco and P. K. W. Ng, *Food Chem*., 2006, **99**, 718-723.

36. A. M. Leeman, M. E. Karlsson, A.-Ch. Eliasson and I. M. E. Bjӧrck, *Carbohyd. Polym*., 2006, **65**, 306-313.

37. E. Escarnot, R. Agneessens, B. Wathelet and M. Paquot, Food Chem., 2010, **122**, 857-863.

38. F. Meijide, J. V. Trillo, S. de Frutos, L. Galantini, N. V. Pavel,V. H. Soto, A. Jover and J. V. Tato, *Steroids*, 2013, **78**, 247-254.

39. M. C. Carey, *Hepatology*, 1984, **5**, 66-71.

40. J. Maldonado-Valderrama, J. L. Muros-Cobosb, J. A. Holgado-Terrizab and M. A. Cabrerizo-Vílchez, *Coloids and Surfaces B*, 2014, **120**, 176-183.

41. M. A. Eastwood, and D. Hamilton, *Biochim. Biophys. Acta*,1968, **162** 165-173.

42. Ch.-M. Huang and N. H. Dural, *J. Food Proc. Ing*. 1995, **18**, 243-266.

43. C. Cornfine, K. Hasenkopf, P. Eisner and U. Schweiggert, *Food Chem*., 2010, **122**, 638-644.

44. J. C. Chezem, E. Furumoto and J. Story, *Nutr. Res*., 1997, **17**, 1671-1682.

45. L. Yang, J.-H. Chen, H. Zhang, W. Qiu, Q.-H. Liu, X. Peng, Y.-N. Li and H.-K. Yang, *Food Chem*., 2012, **132**, 925-930.

46. Ch. Barbana, A. C. Boucher and J. I. Boye, *Food Res. Int*., 2011, **44**, 174-180.

47. S. Steckelbroeck, M. Watzka, D. Lutjohann, P. Makiola, A. Nassen, V. H. Hans, H. Clusmann, A. Reissinger, M. Ludwig, L. Siekmann and D. Klingmuller, *J. Neurochem.*, 2002, **83**, 713–726.

48. R. B. Vail, M. J. Homann, I. Hanna and A. Zaks, *J. Ind. Microbiol. Biotechnol*., 2005; **32**, 67–74.

49. H. Yamazaki, M. Nakamura, T. Komatsu, K. Ohyama, N. Hatanaka, S. Asahi, N. Shimada, F. P. Guengerich, T. Shimada, M. Nakajima and T. Yokoi, *Protein. Expr. Purif.*, 2002; **24**, 329–337.

50. K. Kailasapathy and J. Chin, *Immunol. Cell Biol*., 2000, **78**, 80-88.

51. A. M. P. Gomes and F. X. Malcata, *Trends Food Sci. Technol*., 1999, **10**, 139-157.

52. T. R. Licht, M. Hansen, M. Poulsen, L. O. Dragsted, *BMC Microbiol.*, 2006, doi: 10.1186/1471-2180-6-98.

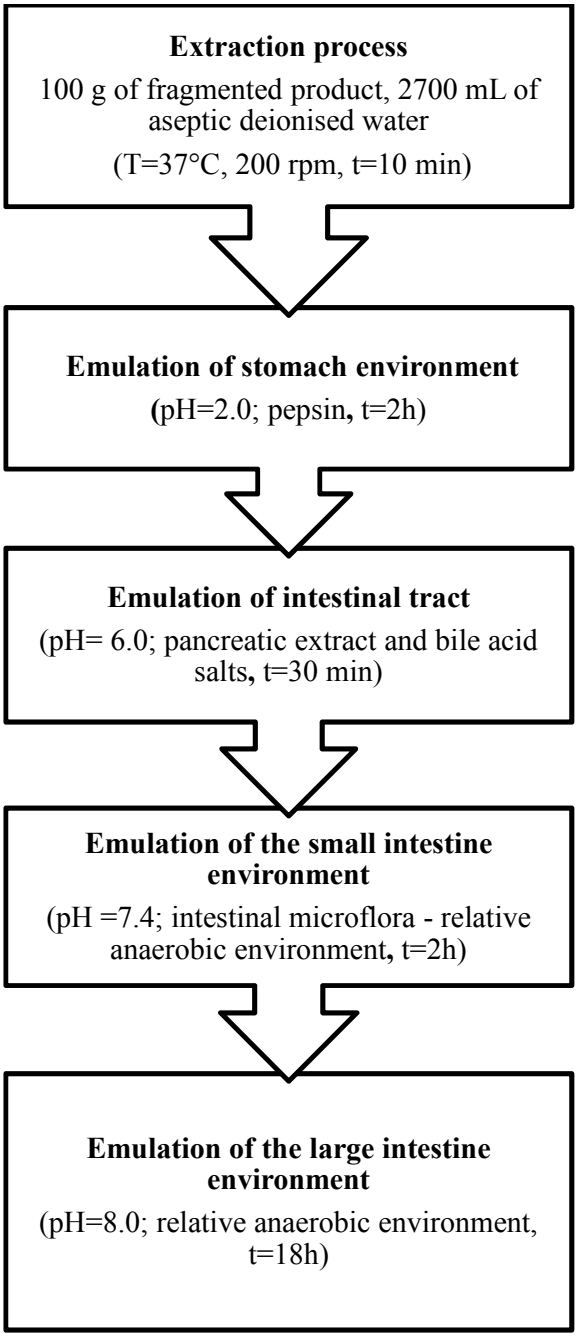

Figure 1

In vitro digestion model.

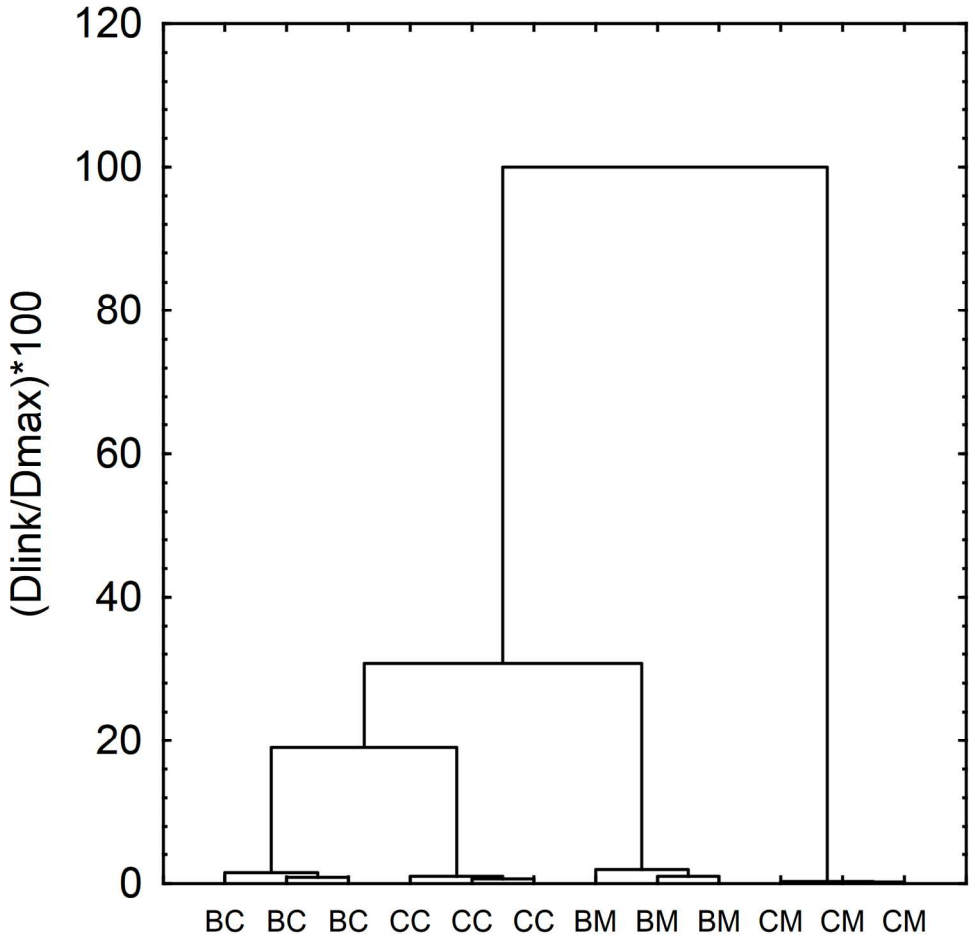

Fig. 2
The cluster analysis results, the variation in the content of dietary fiber in cookies was considered The normalisation of scale tree to dlink/dmax*100 was performed (d – distance, l – linkage, max – maximum of linkage Euclidean distance). Amalgamation rule: Ward's method, distance metric: Euclidean distances.

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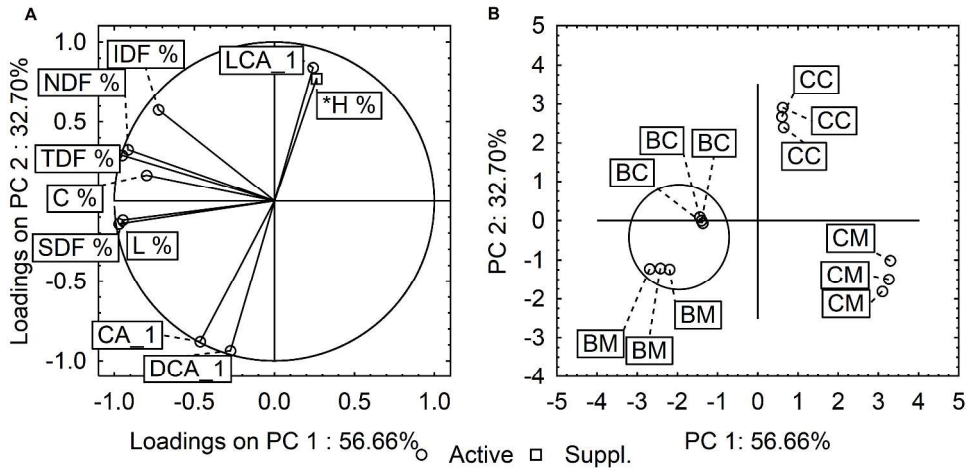

Figure 3
Two-dimensional plot representing the PCA of loadings plot (A) and score plot (B) for tested cookies at the 1st stage of digestion, the supplementary variables were indicated by superscript (*), the principal components (PC1 and PC2) was computed using only the active variables, bile acids were introduced as relative concentration (to blank sample), variables were standardised.

1068x499mm (96 x 96 DPI)

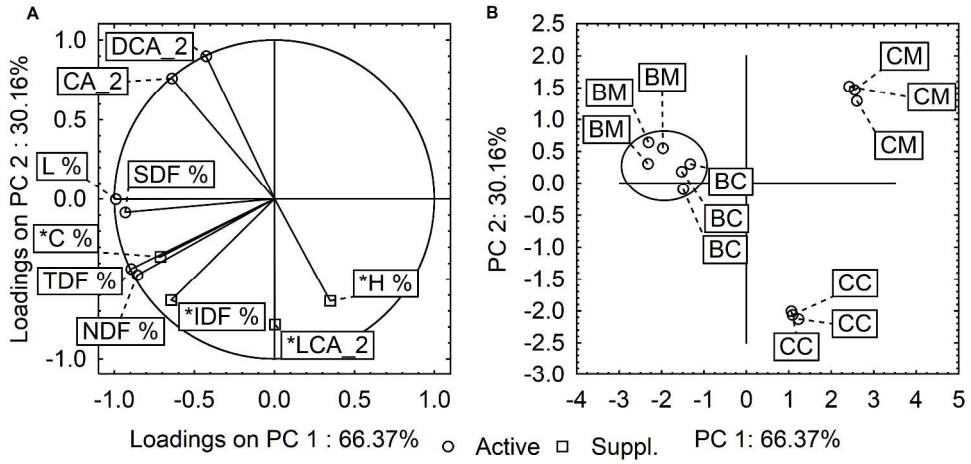

Figure 4
Two-dimensional plot representing the PCA of loadings plot (A) and score plot (B) for tested cookies at the 2nd stage (large intestine) of digestion, the supplementary variables were indicated by superscript (*), the principal components (PC1 and PC2) was computed using only the active variables, bile acid were introduced as relative concentration (to blank sample), variables were standardized.

1068x499mm (96 x 96 DPI)

Table 1 The recipe of pastry goods (g/100g)

	CM	BM	CC	BC
Wheat flour	20.00	16.00	20.00	15.50
Potato flour	15.00	5.00	14.00	11.50
Buckwheat flour	-	7.5	-	5.00
Buckwheat husk	-	2.5	-	2.00
Baking powder	0.70	0.70	-	-
Baking soda	0.35	0.35	-	-
Sugar	15.25	-	20.00	-
Maltodextrin	-	14.40	-	15.00
Aspartame	-	0.05	-	0.05
Acesulfame K	-	0.05	-	0.05
Oil	16.50	6.50	4.00	3.5
Inulin	-	2.00	-	1.70
Water	-	8.00	-	3.00
Buttermilk	16.50	16.50	7.00	7.00
Eggs	7.00	7.00	4.00	4.00
Chokeberry	-	4.50	-	4.50
Dark chocolate	9.7	8.00	21.00	21.00
Vegetable butter	-	-	8.00	3.80
Vanilla sugar	-	-	1.00	1.00
Cinnamon	-	-	1.00	1.00
Mulberry leaf extract	-	0.40	-	0.40

Table 2 Content of dietary fiber and its fractions in pastry products (% d.m.)

cookies	NDF	C	H	L	TDF	SDF	IDF
CM	5.24 ±0.03	0.48 ±0.04	3.87 ±0.07	0.91 ±0.10	1.10 ±0.10	0.40 ±0.05	0.68 ±0.06
BM	9.08 ±0.73	2.47 ±0.20	2.97 ±0.04	3.64 ±0.04	20.50 ±0.46	12.14 ±0.21	8.44 ±0.26
CC	8.36 ±0.00	1.68 ±0.02	4.93 ±0.00	1.75 ±0.05	15.66 ±0.41	4.54 ±0.20	11.16 ±0.26
BC	9.36 ±0.54	1.19 ±0.34	4.66 ±0.23	3.51 ±0.11	20.47 ±0.25	6.60 ±0.02	13.77 ±0.21

Table 3 Relative content of bile acids estimated in two stages of digestive system model

cookies	CA_1	DCA_1	LCA_1	CA_2	DCA_2	LCA_2
CM	0.45 ±0.02	0.04 ±0.01	0.18 ±0.04	0.93 ±0.04	1.13 ±0.01	1.00 ±0.06
BM	0.66 ±0.01	0.05 ±0.01	0.18 ±0.02	1.09 ±0.04	1.28 ±0.01	1.29 ±0.07
CC	0.10 ±0.01	0.00 ±0.00	0.32 ±0.04	0.65 ±0.02	0.19 ±0.01	1.55 ±0.07
BC	0.50 ±0.01	0.03 ±0.00	0.15 ±0.02	1.02 ±0.02	1.15 ±0.02	0.98 ±0.07

The stages of digestion 1 – small intestine, 2 – large intestine. The values lower than 1 were considered with process of binding of bile acid and the values higher then 1 - the concentration of bile acid in sample is above the content in adequate control sample.

Table 4 ANOVA results, evaluation of discriminant power of variables ($p<0.05$) at 1st and 2nd stage of digestion, generated in k-Mean clustering procedure of cookies

Stage 1

Variable	Between - SS	df	Within - SS	df	F	p value
NDF	10.429	2	0.571	9	82.203	0
C	6.395	2	4.605	9	6.248	0.0199
H	4.197	2	6.803	9	2.776	0.115
L	10.946	2	0.054	9	914.977	0
TDF	10.987	2	0.013	9	3745.968	0
SDF	8.628	2	2.372	9	16.37	0.001
IDF	9.358	2	1.642	9	25.648	0.0002
CA_1	10.148	2	0.852	9	53.611	0
DCA_1	8.288	2	2.712	9	13.752	0.0018
LCA_1	9.576	2	1.424	9	30.252	0.0001

Stage 2

Variable	Between - SS	df	Within - SS	df	F	p value
NDF	5.694	1	5.306	10	10.731	0.0083
C	2.782	1	8.218	10	3.385	0.0956
L	10.234	1	0.766	10	133.674	0
TDF	6.374	1	4.626	10	13.781	0.004
SDF	7.31	1	3.69	10	19.807	0.0012
IDF	3.076	1	7.924	10	3.881	0.0771
H	1.612	1	9.388	10	1.717	0.2194
CA_2	7.021	1	3.979	10	17.643	0.0018
DCA_2	4.498	1	6.502	10	6.919	0.0251
LCA_2	0.91	1	10.09	10	0.902	0.3645

Table 5 k- Means clustering, final classification of pastry goods

cookies (case)	Final classification	Distance to centroid (1st stage)	Distance to centroid (2nd stage)
CM	1	0.051	0.046
CM	1	0.188	0.094
CM	1	0.172	0.070
CC	2	0.023	0.026
CC	2	0.167	0.054
CC	2	0.161	0.039
BM	3	0.495	0.293
BM	3	0.572	0.254
BM	3	0.533	0.317
BC	3	0.531	0.241
BC	3	0.632	0.313
BC	3	0.433	0.256

The variables with discriminant power ($p<0.05$) in two stages of digestion were considered

Table 6 Changes in the concentration of *Enterococcus*, *Lactobacillus, E. coli* and *Bifidobacterium* bacteria during „*in vitro*"digestion ($\log_{10}$ cfu mL^{-1})

	Enterococcus					***Lactobacillus***				
Time (h)	control sample	CM	BM	CC	BC	control sample	CM	BM	CC	BC
2	8.30	8.34	7.99	8.18	8.42	8.34	8.36	8.15	8.32	8.41
4.30	9.04	8.78	9.04	8.28	8.32	8.62	9.08	9.18	8.23	8.28
18	9.36	9.26	8.65	8.70	8.41	8.43	9.30	9.95	9.70	8.36
	Esherichia coli					***Bifidobacterium***				
2	8.08	7.26	6.83	6.86	7.20	n.d.	6.56	6.32	5.72	6.30
4.30	8.23	7.46	8.09	7.41	7.88	n.d.	6.04	5.53	6.00	4.74
18	8.41	9.20	9.64	9.68	8.23	n.d.	n.d.	n.d.	7.67	n.d.

n.d. – not detected