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NATIVE AND PHYSICALLY-MODIFIED STARCHES – UTILIZATION OF RESISTANT STARCH BY BIFIDOBACTERIA (*IN VITRO*)

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The capability of selected *Bifidobacterium* strains to utilize the resistant starch fraction from native starches of the following origin: wheat, potato, pea, and their preparations, obtained experimentally by physical modification (iterated syneresis), was studied. *Bifidobacterium* strains were preselected according to their ability to ferment starch. The following strains: *B. pseudolongum* KSI9, *B. animalis* KS20a1 and *B. breve* KN14, were chosen for the next step of the investigation. Native starches (wheat, potato, pea) and their preparations were characterized by different contents of the resistant starch fraction, which was metabolized during *in vitro* fermentation as a source of carbon and energy for *Bifidobacterium* growth. A significant decrease in resistant starch content was noted after 24-h fermentation by *Bifidobacterium* strains for pea and potato starches and their preparations. It indicates that these starches and experimental preparations may be good substrates for *Bifidobacterium* fermentation in the large intestine. The gelatinization process had a negligible influence on resistant starch metabolism by the strains selected for the experiment.

INTRODUCTION

The major role of carbohydrates in a diet is to produce energy. Starch is primarily digested in the small intestine by enzymatic degradation, but its part can escape digestion and be fermented in the large bowel [Bednar *et al.*, 2001]. The major substrates available for microflora fermentation are oligosaccharides (DP 3–9), non-starch polysaccharides of the plant cell wall (DP>9 *i.e.* hemicelluloses, pectins) as dietary fiber components, and the starch fraction resistant to enzymatic hydrolysis in the upper part of the gastrointestinal tract. Starch digestibility is affected by intrinsic factors, such as starch structure and composition, and by associations between starch granules and proteins and cell wall structures within the diet. Furthermore, extrinsic factors, such as starch processing and conditions in the gastrointestinal tract, also affect starch digestibility [Soral-Śmietana, 2000; Weurding *et al.*, 2001]. It is known that the amount of digested and absorbed starch is different, and that its part is transferred to the colon. It is estimated that approximately 8–10% of starch consumed daily is not digested and reaches the large bowel [Cummings & Macfarlane, 1997]. Amylase-resistant starch is acknowledged as an efficient substrate undergoing fermentation with the microflora colonizing the large intestine. This fermentation is of significant importance to the colon environment and functioning of this part of the gastrointestinal tract. Amylase-resistant starch is often referred to as "colonic food". Resistant starch affects an increase in the fecal bulk and a decrease in the colonic pH, and is the major substrate for colonic butyrate production. Butyric acid is a short-chain fatty acid with the strongest protective effect against colorectal cancer [Ahmed *et al.*, 2000].

The human large intestine contains an excess of 200 g of material, of which approximately half is microbial biomass. The pattern of microbial metabolism is unique because the vast majority of colonic bacteria do not use oxygen as a terminal electron acceptor in respiratory processes, energy generation occurs primarily through substrate-level phosphorylation reactions or fermentation [Cummings & Macfarlane, 1997]. From culture-based data, it is thought that at least 500 different microbial species exist in the human gastrointestinal tract, but ten to twenty probably predominate. While some of them can be pathogenic in nature (*e.g.* proteolytic, clostridia), lactobacilli and bifidobacteria are generally considered beneficial to human health [Gibson, 2002]. Bifidobacterial numbers in the human gut tend to decrease with age, but they can be increased either by continuous ingestion of bifidobacteria-containing preparations, or food supplementation with substrates (bifidogenic factors or prebiotics) that promote the growth of endogenous bifidobacteria in the gut [Alander *et al.*, 2001]. Among all bacteria colonizing the intestine, particular attention should be paid to *Bifidobacterium*, being one of the largest groups of saccharolytic bacteria constituting *ca.* 25% of the total population of bacteria present in the intestines of adults and *ca.* 95% of these in the intestines of infants [Gibson & Roberfroid, 1995]. The major probiotic property of these bacteria

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is their activity against pathogens. Bacteria such as certain species of *Bifidobacterium* metabolize undigested polysaccharides such as resistant starch, using this component as a source of carbon and energy necessary for the growth of bacteria beneficial to the host's health. In this way pathogenic bacteria can be reduced or eliminated, and the survival/proliferation of some probiotic bacteria in the large bowel can be enhanced [Fooks & Gibson, 2002].

The aim of this work was to investigate the possibility of using the resistant starch fraction from native starches of three origins and their experimental preparations with a different resistant starch content, as substrates for selected *Bifidobacterium* strains.

MATERIALS AND METHODS

MATERIAL

Polish wheat and potato starches and pea starch produced by Cosucra S.A. (Belgium) were reference materials and raw materials for physical modification process. Physically-modified starch samples were obtained by solubilization of native starch samples in water at a concentration of 3%, and by isolation from a solution without any non-solvents or complexing agents, acc. to the procedure described in the Polish patent P. 325981 [Lewandowicz *et al.*, 1998]. Cold syneresis of starch gels is the main technological process that enables obtaining physically-modified starch preparations. It was described in detail in a previous publication [Lewandowicz & Soral-Śmietana, 2004]. Three strains of *Bifidobacterium*: *B. pseudolongum* KSI9, *B. animalis* KS20a1, and *B. breve* KN14 used were from the own collection of the Department of Food Microbiology IAR&FR PAS Olsztyn, Poland.

METHODS

The study was aimed at identifying the bifidogenic properties of 75 different *Bifidobacterium* strains subjected to a preliminary analysis which examined its reference to native starches and their preparations according to the fermentation profile. Of these (the first step of the experiment), 16 *Bifidobacterium* strains were selected for further investigation. Native starches and their preparations were sterilized in a thin layer with UV (125 μW/m^2) for 15 min and added to a modified liquid Garche's medium [Rasic, 1990] without sugar. The medium was warmed to 58–60°C and mixed at a ratio of 1:1 with a Garche's double-agar medium without sugar, melted and cooled to 58–60°C. The final medium, containing 1% of native starch of a different origin, was poured onto Petri-dishes and left overnight at room temperature. The control sample of modified Garche's medium contained 1% glucose. The media were inoculated on the surface with active strains of bifidobacteria using a bacterial loop. The inoculated plates were incubated at 37°C/24 h under anaerobic conditions in jars equipped with Atmosphere Generation System AnaeroGenTM, Oxoid, UK. The results of bacterial growth on the medium containing different starches were determined on the basis of growth intensity described on a scale from "no growth" to "good growth". Three strains of *Bifidobacterium*: *B. pseudolongum* KSI9, *B. animalis* KS20a1, and *B. breve* KN14 were selected for liquid culturing.

The second step of the experiment included the assessment of the influence of hydrothermal processing (gelatinization) on bacterial utilization of resistant starch in comparison to the non-treated samples. Native starches and their preparations were sterilized in a thin layer with UV (125 μW/m^2) for 15 min to inactivate microflora. The gelatinisation temperatures were determined in a Brabender apparatus. The hydrothermal process provoking gelatinization was run at individual temperatures as follows: (a) native starches: 65°C for wheat, 60°C for potato, and 68°C for pea; and (b) starch preparations: 39°C for wheat, 52°C for potato or pea. The liquid modified Garche's medium containing 1% of native starches or their preparations (non-gelatinized or gelatinized) was inoculated with ~10^5 of selected strains of bifidobacteria and incubated at 37°C/24 h under anaerobic conditions. After 24-h microbiological fermentation, the samples were freeze-dried and moisture content [AOAC, 1990] and content of resistant starch was determined.

Resistant starch analysis was carried out using the method by Champ *et al.* [1999]. Resistant starch is the starch not hydrolyzed by pancreatic α-amylase. The products of hydrolysis, solubilized in 80% ethanol, were discarded. Resistant starch present in the pellet was solubilized in 2 mol/L KOH, then hydrolyzed into glucose with amyloglucosidase. Glucose was then quantified with a glucose oxidase/peroxidase analysis kit (Cormay, Poland).

The degree of RS utilization was determined as a difference in the contents of resistant starch fraction of samples before and after fermentation by individual *Bifidobacterium* strains. The utilization was expressed in per cents.

Scanning electron microscope (JSM 5200, Japan) micrographs were obtained after spraying starch preparations with gold, and visualized at an acceleration of 10 KeV.

RESULTS AND DISCUSSION

Preliminary analyses of the fermentation profiles of 75 *Bifidobacterium* strains freshly isolated from infants (16), adults (17), laboratory rats (16), bioyogurts (15), and reference strains purchased from ATCC and DSMZ collections (11) were used for the initial selection of strains able to hydrolyse the starches examined in the study. Starches were fermented only by certain strains belonging to the species: *B. animalis*, *B. breve*, and *B. pseudolongum*. The preliminary analyses indicated not only a differentiated ability of bifidobacteria to utilize starch, but also a differentiated susceptibility of native starches of various origins and their preparations to hydrolysis with bacterial enzymes [Wronkowska *et al.*, 2002b]. Therefore screening analyses of 16 *Bifidobacterium* strains belonging to *B. breve*, B. infantis, *B. pseudolongum*, *B. animalis*, and B. longum species were performed. They were aimed at determining their ability to grow in a minimum medium used for the analysis of native starches and their preparations, being the only source of carbon and energy (Table 1). On the basis of the results obtained, three *Bifidobacterium* strains: *B. pseudolongum* KSI-9, *B. animalis* KS20a1, and *B. breve* KN14 were used for further analyses.

The materials, which were tested *in vitro* as substrates for *Bifidobacterium* strains, demonstrated different contents of resistant starch (Table 2). The content of resistant starch in

TABLE 1. Screening of *Bifidobacterium* strains described as: - no growth, +/- slightly visible growth, + weak growth, ++ medium growth, +++ good growth.

No.	*Bifidobacterium* strains	Glucose control	Starches					
			Wheat		Potato		Pea	
			native	preparation	native	preparation	native	preparation
1	*B. breve* ATTC 15700	+++	++	++	+	++	-	+
2	*B. breve* KN10	++	++	++	+	+++	-	+
3	*B. breve* KN11	++	++	++	+	++	-	+
4	*B. breve* KN14	++	++	++	+	++	-	+
5	*B. infantis* ATCC 15697	+++	+	+/-	+/-	-	-	-
6	*B. pseudolongum* DSMZ 20099	+++	+++	+++	++	+++	+	++
7	*B. pseudolongum* KSI-9	+++	+++	+++	++	+++	+++	++
8	*B. pseudolongum* PS36	+++	+++	++	+++	++	+++	++
9	*B. longum* KNA1	+++	+	+/-	+	-	-	+/-
10	*B. longum* KN29.1	+++	+	+/-	+/-	-	-	-
11	*B. animalis* ATCC 25527	+	+	+/-	+/-	+/-	-	-
12	*B. animalis* KS29a3	+++	++	++	++	++	+	+
13	*B. animalis* KS1b2	+++	++	++	++	++	+	+
14	*B. animalis* KS20a1	+++	+++	++	+++	++	+++	++
15	*B. animalis* 30	++	+	+/-	+/-	-	-	-
16	*B. animalis* 45	++	+	+/-	+/-	-	-	+/-

the native starches analyzed ranged from low in the case of native wheat starch, through medium in that of pea starch, to high in potato starch. After applying cold syneresis of gel of these starches of different botanical origin [Lewandowicz *et al.*, 1998], the resistant starch content of these preparations was higher in the wheat starch preparation, and lower in pea starch and potato starch preparations (Table 2). Previous investigations of native starch granules of wheat, potato and pea origin, performed on SEM-microphotographs, showed a significant disproportion between the shape and size of starch granules of a different botanical origin. Also the kinetics of hydrolysis and the way of enzymatic attack by pancreatic α-amylase were various [Soral-Śmietana, 2000]. After physical modification of starches, their structure in the preparations was quite different from the granular structure of native starches (Figures 1ab, 2ab, 3ab). The SEM-microstructure of wheat and potato starch preparations (Figures 1b, 2b) indicated an integrated gel composition of two-fractions, amylose and amylopectin, and seemed to be more dense in the potato starch preparation. The amylose/amylopectin complex in the pea starch preparation was different than in the other preparation – (no block-like particles), but showed granular cluster structure (Figure 3b). Its resistant starch content was close to that found in the potato starch

TABLE 2. The content of the resistant starch fraction in native starches and their preparations.

Starches	Moisture content (%)	Resistant starch (% d.m.)
Wheat starch:		
native	10.38±0.09	3.06±0.21
preparation	14.75±0.03	7.67±0.50
Potato starch:		
native	8.76±0.08	60.82±2.85
preparation	5.23 ±0.11	18.57±0.56
Pea starch:		
native	9.52±0.13	31.53±1.60
preparation	10.72±0.60	16.45±1.59

A) B)

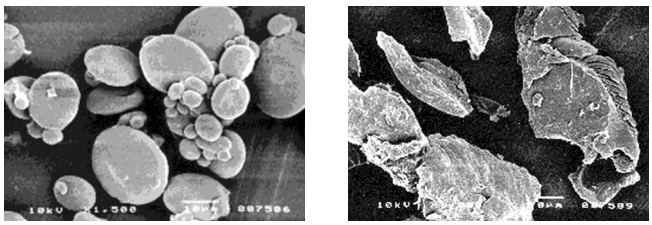

FIGURE 1. SEM-microstructure of native wheat starch (A) and its preparation (B).

A) B)

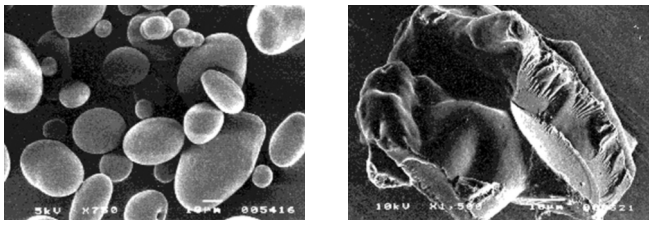

FIGURE 2. SEM-microstructure of native potato starch (A) and its preparation (B).

A) B)

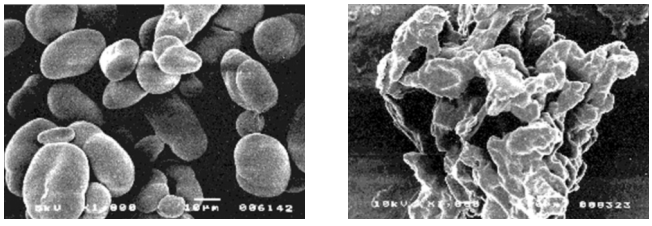

FIGURE 3. SEM-microstructure of native pea starch (A) and its preparation (B).

preparation (Table 2). The microstructure of the wheat starch preparation was similar to the microstructure of the potato preparation, but its resistant starch content was over twice as low as that determined in the potato starch preparation. Upon such a technological process, apart from pota-

to starch, the pea starch is also a good material for obtaining this preparation with a high or medium content of resistant starch, compared to starches of different botanical origins [Soral-Śmietana & Wronkowska, 2000; Wronkowska & Soral-Śmietana, 2000; Lewandowicz & Soral-Śmietana, 2004]. It should be pointed out that the content of resistant starch in native wheat starch was very low and substantially different from that recorded in native potato or pea starches (Table 2). The results of this study indicated a very low level of resistant starch in native wheat starch. This is interesting, but unexpected and quite different from the results of our previous research [Wronkowska *et al.*, 2002a,b]. The low level of resistant starch in native wheat starch suggests that the content of this fraction of starch can vary not only in starches of a different origin but also in those of the same origin. However, this phenomenon would be difficult to explain at the moment.

From the nutritional point of view, according to the classification proposed by Englyst and co-workers [Englyst *et al.*, 1992; Englyst & Hudson, 1996] or Eerlingen & Delcour [1995], the resistant starch fraction determined within the native starch granules is of the RS II type. Retrograded or crystalline non-granular starch represents another type of resistant starch, RS III. The physical modification process used in this study changed the RS II type in native starches to the RS III type in their preparations.

The RS content after 24-h hydrolysis with selected *Bifidobacterium* strains decreased significantly in the case of native potato and pea starches. Generally, the resistant starch fraction included in native pea starch was preferentially used in *ca.* 70% or more by *Bifidobacterium* strains (Table 3, Figures 4–6), whereas RS present in native potato starch (Table 2) was utilized in *ca.* 42–63%. On the other hand, the following *Bifidobacterium* strains: *B. pseudolongum* KSI9, *B. animalis* KS20a1, and *B. breve* KN14, utilized 1–2.6% of resistant starch from native wheat starch, which accounts for 30 to almost 85% of the material.

The gelatinization of native starches had an insignificant effect on the metabolization of the resistant starch fraction (Figures 4–6, Table 3). It was found that gelatinized native potato starch was a better substrate in terms of its utilization. It was used by *Bifidobacterium breve* in *ca.* 69% and by *Bifidobacterium pseudolongum* in *ca.* 59%, compared with the values recorded before the treatment. No effect of gelatinization of native potato starch on resistant starch utilization by *Bifidobacterium animalis* was, however, observed (Figure 5).

The starch preparations obtained after the physical modification of native starches [Lewandowicz *et al.*, 1998], were subjected to fermentation with the same *Bifidobacterium* strains as those used for native starches. Taking into account the RS content of the preparation and native

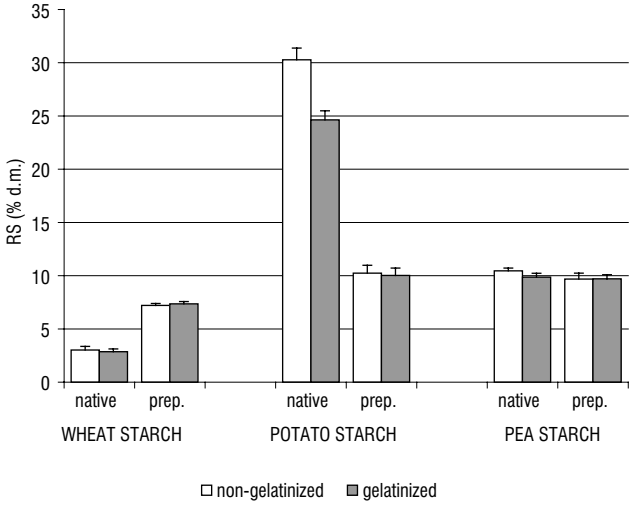

FIGURE 4. Resistant starch content in medium after fermentation with *B. pseudolongum* KSI9.

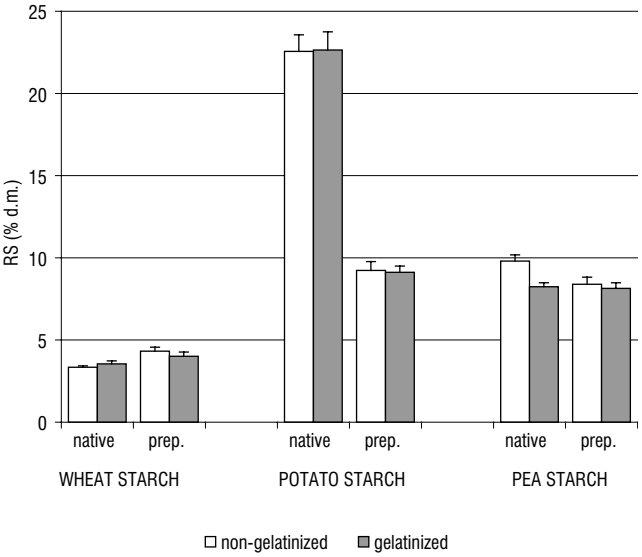

FIGURE 5. Resistant starch content in medium after fermentation with *B. animalis* KS20a1.

TABLE 3. Degree of utilization of the resistant fraction from native and modified starches by selected *Bifidobacterium* strains (%).

Starches	*B. pseudolongum* KSI9		*B. animalis* KS20a1		*B. breve* KN14	
	non-gelatinised	gelatinised	non-gelatinised	gelatinised	non-gelatinised	gelatinised
Wheat starch:						
native	1.3	6.5	0	0	2.6	0
preparation	6.0	4.0	43.7	47.7	31.8	33.0
Potato starch:						
native	50.2	59.5	62.9	62.8	42.4	68.5
preparation	44.9	46.0	50.3	50.9	39.5	41.5
Pea starch:						
native	66.8	68.7	68.9	73.9	72.2	77.0
preparation	41.2	41.0	49.0	50.5	43.1	45.4

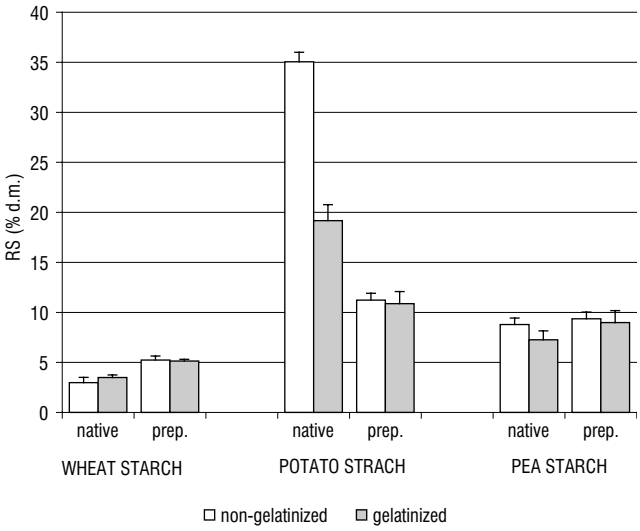

FIGURE 6. Resistant starch content in medium after fermentation with *B. breve* KN14.

starch before fermentation (Table 2), they differed in terms of the utilization of native starches and their preparations by selected *Bifidobacterium* strains (Figures 4–6). After 24-h fermentation, the resistant starch content of pea starch preparation was similar to that determined in native starch (Figures 4,6), but the average utilization of the non-digested starch fraction by *B. animalis* or *B. breve* was higher (Table 3). The preparation obtained from potato starch appeared to be as good a fermentation substrate for *Bifidobacterium* strains as the pea starch preparation. As regards the wheat starch preparation, only *B. animalis* and *B. breve* strains utilized the resistant starch fraction from this preparation, at an average level of 44% and 32%, respectively, which was substantially different from that indicated for potato or pea preparations.

Many factors influence starch digestion and absorption in the gastrointestinal tract. One of the factors affecting starch availability is the effect of hydrothermal processes occurring during starch gelatinisation. This factor was also used in this study to modify the structure of starches and their preparations gelatinization. Generally, the gelatinization process had a negligible influence on resistant starch metabolism by *Bifidobacterium*. Its slight effects were visible in the case of native potato and pea starches only. The hydrothermal processing of native potato starch increased the utilization of resistant starch by *B. breve* and *B. pseudolongum* (*ca.* 26% and *ca.* 9% of RS, respectively) in comparison to non-treated starch (Table 3, Figures 4, 6). A slight positive influence of the gelatinization process on native pea starch, being a source of carbon and energy, was noted during its fermentation by *B. animalis* and *B. breve* strains (Figures 5, 6).

The results obtained suggest that the saccharides (starches and their preparations) analyzed can be good substrates for fermentation with selected *Bifidobacterium* strains. The metabolism of the resistant starch fraction by caecal and faecal microflora of rats, leading to the production of short-chain fatty acids, has already been shown by Kleessen *et al.* [1997]. They proved that growth stimulation occurs not only in the case of *Bifidobacterium*, but also in that of other genera: *Bacteroides*, *Fusobacterium*, *Clostridium*, *Lactobacillus*, *Streptococcus*, and *Enterobacterium*.

Because of its presence in everyday diet, starch is an important component which may influence the colon microecosystem [O'Keefe *et al.*, 1999] due to the bifidogenic properties of the resistant starch fraction. The decrease in the autochtonous *Bifidobacterium* population in the colon, observed with age, makes it necessary to search for substrates preventing the development of *Bifidobacterium* strains and species. Wang *et al.* [1999a, b] ascertained the specific affinity of bifidobacteria to high amylose corn starch, amylopectin and soluble starch, however they did not note this affinity in *Lactobacillus*. The study based on an *in vivo* rat analysis showed that corn RS-preparation stimulated bifidobacteria growth, increasing at the same time the ammonia content and β-glucuronidase activity [Bielecka *et al.*, 2002]. The analysis of native starches (wheat, potato, pea) and their modified preparations (RS content about 10%) indicated their positive influence on the blood index and caecal microflora enzyme activity in the gastrointestinal tract of rats, in comparison to cellulose [Wronkowska *et al.*, 2002a].

If resistant starch is preferred to fiber by the intestinal microflora, the content of this starch fraction should be determined to provide such a substrate to human and animal organisms. At the same time, attention should be paid to the different botanical origins of resistant starch as a fermentation substrate for the production of SCFA which are formed in different proportions as a result of *in vitro* fermentation by faecal medium [Soral-Śmietana *et al.*, 2001] and may contribute to maintaining a good health state in both humans and animals.

CONCLUSIONS

The starches and their preparations examined in the study can be good substrates for fermentation with selected *Bifidobacterium* strains. *B. breve* KN14, *B. pseudolongum* KSI9, *B. animalis* KS20a1 utilized, as a source of carbon and energy, the resistant starch fraction from native starches or starch preparations to a different degree (0–70%). A significant decrease in resistant starch content was observed in native potato and pea starches and their preparations after 24-h fermentation with the strains of bifidobacteria examined in the experiment. The gelatinization process of native starches and their preparations had a negligible influence on resistant starch metabolism by selected *Bifidobacterium* strains.

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SKROBIE NATYWNE I MODYFIKOWANE FIZYCZNIE – WYKORZYSTANIE SKROBI OPORNEJ PRZEZ BIFIDOBAKTERIE (*IN VITRO*)

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Podjęto badania oszacowania zdolności wybranych szczepów *Bifidobacterium* do wykorzystywania frakcji amylazoopornej naturalnych skrobi z następujących źródeł botanicznych: pszenna, ziemniaczana, grochowa oraz preparatów otrzymanych z nich w wyniku fizycznej modyfikacji (postępująca synereza). Szczepy *Bifidobacterium* zostały wyselekcjonowane na podstawie ich zdolności do fermentowania badanych skrobi. Do dalszych badań wybrano następujące szczepy: *B. pseudolongum* KSI9, *B. animalis* KS20a1 and *B. breve* KN14. Naturalne skrobie (pszenna, ziemniaczana, grochowa) i uzyskane z nich preparaty charakteryzowały się różną zawartością frakcji amylazoopornej, która była metabolizowana podczas fermentacji *in vitro* stanowiąc źródło węgla i energii do wzrostu *Bifidobacterium*. Znaczące zmniejszenie zawartości skrobi amylazoopornej w skrobi ziemniaczanej i grochowej oraz ich preparatach stwierdzono w następstwie 24-h fermentacji przez szczepy *Bifidobacterium*. Powyższy materiał może być zatem dobrym substratem do fermentacji przez bifidobakterie w jelicie grubym. Proces kleikowania/żelatynizacji miał niewielki wpływ na wykorzystanie frakcji skrobi amylazoopornej przez wybrane w tych badaniach szczepy.