

The Effect of Tartaric Acid-modified Enzyme-resistant Dextrin from Potato Starch on Growth and Metabolism of Intestinal Bacteria

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Abstract

In present study, enzyme-resistant dextrin, prepared by heating of potato starch in the presence of hydrochloric (0.1% dsb) and tartaric (40% dsb) acid at 130°C for 2 h (TA-dextrin), was tested as the source of carbon for probiotic lactobacilli and bifidobacteria cultured with intestinal bacteria isolated from faeces of three healthy 70-year old volunteers. The dynamics of growth of bacterial monocultures in broth containing tartaric acid (TA)-modified dextrin was estimated. It was also investigated whether lactobacilli and bifidobacteria cultured with intestinal bacteria in the presence of resistant dextrin would be able to dominate the intestinal isolates. Prebiotic fermentation of resistant dextrin was analyzed using prebiotic index (PI). Fermentation products were determined by HPLC. It was shown that all of the tested bacteria were able to grow and utilize TA-modified dextrin as a source of carbon, albeit to varying degrees. In co-cultures of intestinal and probiotic bacteria, the environment was found to be dominated by the probiotic strains of *Bifidobacterium* and *Lactobacillus*, which is a beneficial effect.

Keywords: Resistant dextrin; Prebiotic; Intestinal bacteria

Introduction

Nowadays, one's lifestyle is indicative of one's future health. Many factors determine the risk of disease, or conversely, the possibility of remaining healthy. Physical activity and an appropriate diet are examples of daily routines that may influence one's health. A lack of physical activity, particularly if associated with overconsumption, increases the risk of development of nutrition-related chronic diseases, such as obesity, hypertension, cardiovascular diseases, osteoporosis, type II diabetes, and several types of cancer. Over the last decade, dramatic changes have taken place in the perception and understanding of the importance of the daily diet. Foods are no longer judged in terms of taste and immediate nutritional needs, but also in terms of their ability to improve the health and well-being of the consumers. The role of diet in human health has led to the recent development of the so-called functional food concept. A functional food is a dietary ingredient that has cellular or physiological effects above its normal nutritional value. It can also contain prebiotics [1].

"A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" [2]. The definition updated by Gibson specifies that a prebiotic is "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" [3]. The current definition of prebiotics was suggested during the ISAPP experts' meeting in 2008, according to which "a dietary prebiotic is a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" [4]. For a substance to qualify as a prebiotic, it must meet certain criteria: it must be chemically characterized, exhibit health benefits that are measurable and outweigh any adverse effects, and appropriately modulate the composition or activity of the microbiota in the target host [5].

Some carbohydrates, such as fructo oligosaccharides (FOS) [6,7], inulin [8]; Van Loo, [9], transgalacto-oligosaccharides (TOS)

[10,11] and lactulose [12,13] are well-accepted prebiotics, while isomaltooligosaccharides (IMO) [14] and xylooligosaccharides [15] are candidate prebiotics. The fermentation of some oligosaccharides is not as selective as that of FOS, so their prebiotic status remains in doubt. Promising sources of prebiotics are starch products, especially resistant starch (RS) [16-18], and products of partial degradation of starch, that is, dextrans [19,20].

The objective of this study was to determine whether dextrin obtained as a result of heating starch with tartaric acid (patent claim no. 392894) is a substance with prebiotic properties [7]. Thus, it was examined whether the dextrin would be utilized as a source of carbon by probiotic and intestinal bacteria. It was also investigated whether probiotic lactobacilli and bifidobacteria cultured with intestinal bacteria in the presence of resistant dextrin would be able to dominate the intestinal isolates. In the study, the prebiotic index (PI) and the fermentation products of resistant dextrin were determined.

Materials and Methods

Materials

Potato starch and tartaric acid ($\geq 99.5\%$) were purchased from Sigma-Aldrich Corp. (Poznan, Poland). Hydrochloric acid and ethanol (96%) were procured from POCH (Gliwice, Poland). *Lactobacillus*,

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Bifidobacterium, *Clostridium*, *Fusobacterium*, *Escherichia coli* and *Enterococcus* bacteria strains were isolated from feces of three healthy 70-year old men volunteers. The 24-h cultures were frozen at -20°C: (a) *Lactobacillus*, *Bifidobacterium* bacteria in MRS broth with 20% glycerol, (b) *Fusobacterium*, and *Clostridium* in VL broth, and (c) *Escherichia*, *Enterococcus* in a nutrient bouillon with 20% glycerol. Prior to experiments bacteria were activated by twofold inoculation (3%) in: (a) liquid MRS broth (*Lactobacillus*, *Bifidobacterium*), (b) liquid VL broth (*Fusobacterium*, *Clostridium*), and (c) nutrient broth (*Escherichia*, *Enterococcus*).

Preparation of dextrin

Enzyme-resistant tartaric acid-modified dextrin (TA-dextrin) was prepared following the method of Jochym et al. [21]. Thus potato starch was sprayed with hydrochloric acid solution (0.5% w/w) to obtain a final HCl concentration of 0.1% on a dry starch basis (dsb). The tartaric acid solution (20% w/v) was then added to obtain a final organic acid concentration of 40% dsb. Thoroughly mixed sample was dried at 110°C to obtain final moisture content below 5%. Dried sample (10 g) was placed in an anti-pressure bottle (SIMAX), capped and heated at 130°C for 2 h in an ELF 11/6 EUROTHERM CARBOLITE oven (Hope, England). Product was cooled in a desiccator and milled into powder with a particle size of <1 mm. Dextrin was then washed with 80% EtOH to remove excess of tartaric acid, and low molecular weight material formed during dextrinization, dried overnight at 50°C, and then at 110°C for 1 h, and finally milled in a cyclone lab sample mill (UDY Corp., Fort Collins, CO, USA) fitted with a 0.50 mm screen.

The dynamics of growth of mixtures of bacteria

The intestinal bacteria *Lactobacillus*, *Bifidobacterium*, *Escherichia coli*, *Enterococcus*, *Clostridium* and *Fusobacterium* were co-cultured in the presence of resistant dextrin to determine whether the beneficial bacteria *Lactobacillus* and *Bifidobacterium* can dominate their environment in the presence of a mixture of isolated intestinal bacteria. Inoculants of bacterial monocultures were prepared in such a way that after 24 h of growth the number of particular bacteria ranged from 3.50×10^7 to 4.50×10^7 CFU/mL, corresponding to the number of these bacteria in the terminal section of the ileum [22]. The monocultures of bacteria isolated from three 70-year-old persons were incubated in liquid MRS (*Lactobacillus* and *Bifidobacterium*), in liquid VL medium (*Clostridium* and *Fusobacterium*) and in liquid broth (*Escherichia coli* and *Enterococcus*). All monocultures were incubated in sterile 15 mL test tubes (Marfour) – *Lactobacillus*, *Escherichia coli*, *Enterococcus* under aerobic conditions and *Bifidobacterium*, *Fusobacterium* and *Clostridium* under anaerobic conditions. After incubation, the cultures were centrifuged in a MPW-350R centrifuge (Med. Instruments, Poland) at 9.000 rpm for 10 min at 22°C, the supernatant was decanted and the biomass was transferred to 100 mL of medium of Wynne et al. [23] with the addition of resistant dextrin (TA-dextrin). The cultures were incubated for 168 h under anaerobic conditions (in similar conditions as in the intestine). Following dilution in physiological salt, the cultures were plated (Koch's plate method) in duplicate immediately after inoculation (0 h) and after 24, 48, 72 and 168 h on selective media: *Lactobacillus* on Rogosa agar, *Bifidobacterium* on RCA agar with the addition of the antibiotic dicloxacillin, *Escherichia coli* on ENDO agar, *Enterococcus* on bile-aesculin agar, *Clostridium* on DRCM agar and *Fusobacterium* on Schaedler agar with an antibiotic. The plates were incubated for 48 h at 37°C; *Lactobacillus*, *Escherichia coli*, and *Enterococcus* under aerobic conditions and *Bifidobacterium*, *Fusobacterium* and *Clostridium* under anaerobic conditions in a Concept 400 anaerobic chamber (Ruskinn Biotrace, USA). The control

trial was determined by the trial without the addition of the source carbon.

Determination of prebiotic index (PI)

Prebiotic fermentation of resistant dextrans were analyzed using quantitative equation (prebiotic index – PI). The PI equation is based on the changes in key bacterial groups during fermentation. The bacterial groups incorporated into this PI equation were bifidobacteria, lactobacilli, clostridia and *Fusobacterium*. The equation assumes that an increase in the populations of bifidobacteria and/or lactobacilli is a positive effect while an increase in *Fusobacterium* and clostridia are negative [8].

The PI equation is described below:

$$PI = (\text{Bif}/\text{Total}) - (\text{Fus}/\text{Total}) + (\text{Lac}/\text{Total}) - (\text{Clos}/\text{Total})$$

where PI is prebiotic index; Bif, bifidobacterial numbers at sample time/numbers at inoculation; Fus, *Fusobacterium* numbers at sample time/numbers at inoculation; Lac, lactobacilli numbers at sample time/numbers at inoculation; Clos, clostridia numbers at sample time/numbers at inoculation; Total, total bacteria numbers at sample time/numbers at inoculation.

pH changes

Changes in pH were monitored with an Elmetron CP-401 pH-meter (Elmetron, Zabrze, Poland).

Determination of fermentation products by High Performance Liquid Chromatography (HPLC)

Organic acids, aldehydes and ethanol concentrations were determined by HPLC in supernatant liquid. The chromatographic analysis was performed by Finnigan Surveyor HPLC system (Thermo Scientific, Riviera Beach FL, USA) with refractive index (RI Plus) and photodiode (PDA Plus) detectors. The column used was an Aminex HPX 87H, 300 × 7.8 mm (HPLC Organic Acid Analysis Column, Bio-Rad, Hercules CA, USA). The mobile phase was 0.005 M H₂SO₄. The separation was carried out by isocratic elution with a flow rate of 0.6 ml/min, and the column temperature was maintained at a constant 60°C.

Quantification of fermentation products was carried out using the external standard method. Lactic acid, formic acid, acetic acid, propionic acid, butyric acid, succinic acid, ethanol, and acetaldehyde of known retention times were used as external standards. For each standard, solutions were prepared, filtered through 0.22 μm syringe filters (Milipore, Belford, USA), and injected into the HPLC system to provide standard curves (concentration versus peak area), and for calculating the quantities of organic acids, aldehydes and ethanol. Linear regression curves based on peak areas were calculated for the individual standards covering a broad range of concentrations (Table 1).

Results and Discussion

It seems likely that prebiotic activity will be exhibited by dextrin obtained by simultaneous thermolysis and chemical modification of potato starch in the presence of a volatile inorganic acid (hydrochloric acid) as a catalyst of the dextrinization process and an excess amount of an organic acid (tartaric acid) as a modifying factor (patent claim no. 392894 "Preparation with prebiotic qualities"). In previous research, Kapusniak et al. [24] analyzed this dextrin in terms of the solubility and pH of its 1% aqueous solution, the content of reducing sugars,

Substance	Calibration regression equation	Retention times (min)
Lactic acid	$C=2.36 \times 10^{-7} \times p$	12.7
Acetic acid	$C=4.08 \times 10^{-7} \times p$	15.7
Formic acid	$C=2 \times 10^{-6} \times p+0.0576$	14.4
Propionic acid	$C=2 \times 10^{-6} \times p+0.0218$	18.5
Butyric acid	$C=2 \times 10^{-6} \times p+0.0182$	16.7
Succinic acid	$C=1.97 \times 10^{-7} \times p$	12.2
Acetaldehyde	$C=4.27 \times 10^{-7} \times p$	17.5
Ethanol	$C=3.85 \times 10^{-7} \times p$	22.6

C: The concentration of the analyzed substances (mg/100 ml).
P: Peak area.

Table 1: Regression equation and retention time for HPLC standards.

molecular mass distribution, weight average molecular mass using high performance size-exclusion chromatography (HPSEC), average chain length using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), and the content of the resistant fraction using the enzymatic-gravimetric method AOAC 991.43 [25], the enzymatic-gravimetric-chromatographic method AOAC 2001.03 [26], the enzymatic-spectrophotometric method [27], and the pancreatin-gravimetric method [28]. It has been shown that the use of tartaric acid in the process of starch thermolysis yields acidic dextrin characterized by high water solubility (about 68%) and a high content of reducing sugars (about 29%). It has also been found that dextrin modified with tartaric acid does not contain any traces of unreacted starch, and the percentage share of the main fraction (having a weight average molecular mass of about 1.800 g/mol) was 80%. The average length of the carbohydrate chain in dextrin obtained with tartaric acid was 8.2 as determined by means of HPAEC. A study by Kapusniak et al. [24] revealed that the content of the resistant fraction in dextrin modified with tartaric acid, determined by means of AOAC 991.43, amounted to 44.5%. However, the results obtained by the Englyst enzymatic-spectrophotometric method showed that the actual content of the resistant fraction was above 68% [27]. Kapusniak et al. [24] used the official AOAC 2001.03 method to determine the content of the resistant fraction in dextrin modified with tartaric acid. This is the latest approved method for determining the total content of dietary fiber in foods containing resistant maltodextrins. Apart from measuring the content of insoluble dietary fiber and the high molecular weight fractions

of soluble fiber, this method makes it possible to determine resistant oligosaccharides (by using high-performance liquid chromatography, HPLC). Currently, many authors define resistant starch as the starch fraction that remains undigested by amylolytic enzymes after 16 h. In the Englyst method, fractions undigested after 120 min are considered resistant. In the pancreatin-gravimetric method, similarly as in the Englyst method, samples are digested with pancreatin, but resistant fractions are determined gravimetrically only after 16 h. In the case of dextrin modified with tartaric acid, the results of determination by the pancreatin-gravimetric method (67%) were similar to those obtained in previous studies using the Englyst method (68%), but much higher than those obtained using the AOAC 2001.03 method (50%) [27,24]. The observed differences among the various methods in terms of the measured content of the resistant fraction in dextrin modified with tartaric acid was caused by the fact that, according to the latest reports, enzymatic-gravimetric methods (including AOAC 2001.03) using thermostable α -amylase can determine only part of resistant starch type 4 [29]. And based on enzymatic tests, it can be argued that dextrin obtained using an excessive amount of tartaric acid may be classified as resistant starch type 4.

In the present study, enzyme-resistant dextrin, prepared by heating of potato starch in the presence of hydrochloric (0.1% dsb) and tartaric (40% dsb) acid at 130°C for 2 h (TA-dextrin), was tested as the source of carbon for probiotic lactobacilli and bifidobacteria cultured with the intestinal bacteria isolated from the feces of three healthy 70-year-old volunteers.

In media where TA-dextrin was the source of carbon, all *Lactobacillus* and *Bifidobacterium* strains reached the stationary phase at 24 h of incubation. The number of bacteria of the genus *Lactobacillus* and *Bifidobacterium* in the stationary phase was similar and amounted to: 8.70 log cfu/mL and 8.41 cfu/mL, respectively. At 168 h of culture in a medium with dextrin modified with tartaric acid, the number of lactobacilli and bifidobacteria remained high and ranged from 7.75 to 7.91 log cfu/mL, which shows their substantial viability (Figure 1).

The control strains were cultured in media with glucose. At 24 h of incubation, in cultures with glucose the number of lactobacilli and bifidobacteria amounted to from 9.35 to 8.83 log cfu/mL. The bacteria entered the stationary phase, similarly as in media containing dextrin,

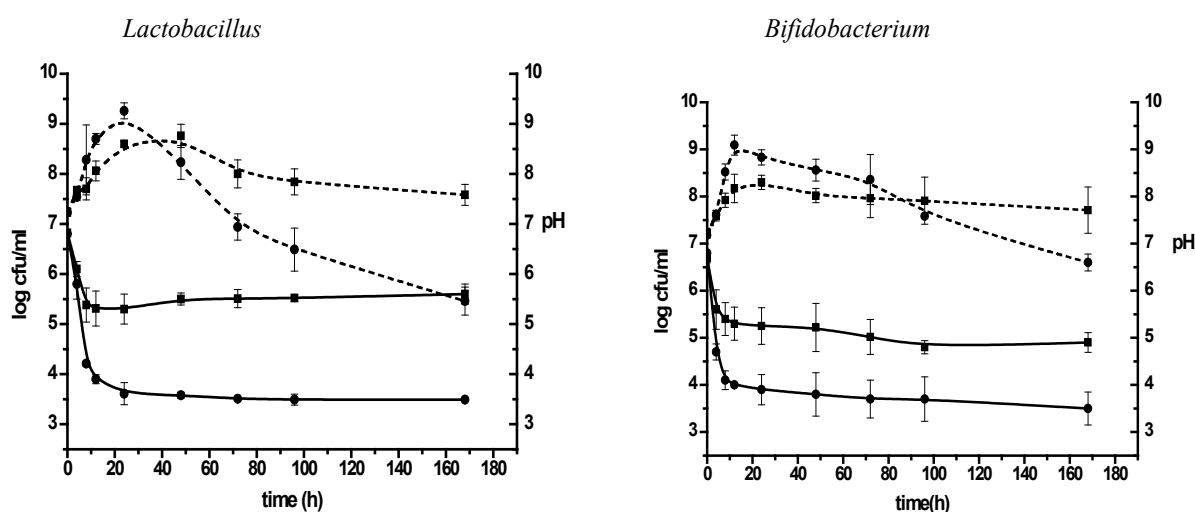


Figure 1: The growth curves (---) and changes in pH (-) for *Lactobacillus* and *Bifidobacterium* bacteria grown in the medium containing TA-dextrin (■) or glucose (control) (●). Results show means and standard deviations of n=3 replicates.

after 24 h of incubation. However, the stationary phase lasted much shorter than in media containing dextrin. At 168 h of culture, the number of viable *Lactobacillus* and *Bifidobacterium* cells cultured with glucose was much lower than that of cells cultured with resistant dextrin, and amounted to from 5.56 to 6.70 log cfu/mL (Figure 1).

In the medium containing dextrin, the acidifying activity of bifidobacteria was higher than that of lactobacilli. After incubation, a test of culture pH revealed that *Bifidobacterium* had the highest acidifying activity (pH 4.9), while *Lactobacillus* – the lowest (pH 5.6). In the control medium containing glucose, the pH of *Lactobacillus* and *Bifidobacterium* cultures decreased much more than that in the medium containing dextrin; at 168 h the pH was 3.50 (Figure 1).

In media containing TA-dextrin, the other bacteria isolated from human feces were able to grow, but the degree of dextrin utilization depended on the strain. In media with resistant dextrin, bacteria entered the stationary phase between 12 and 48 h of incubation and it lasted for 20-30 consecutive hours. In this phase, the highest cell count was found for *Fusobacterium* strains (8.50 log cfu/mL) (Figure 2). Also *Enterococcus* strains grew successfully, with the number of cells in the stationary phase reaching 8.49 log cfu/mL. Lower growth was found for *E. coli* and *Clostridium* strains (8.31 and 8.26 log cfu/mL, respectively) (Figure 2). In media containing dextrin, bacteria isolated from human

feces preserved high viability, and at 168 h the number of viable cells was by 1-2.5 log cycles larger than that of the control cells cultured with glucose.

Out of the bacteria isolated from human feces, the most acidifying ones were *Clostridium* strains, which decreased pH to about 3.50. In cultures containing dextrin, no significant differences were found between the remaining strains, as the pH ranged from 4.51 to 5.50. The strains were most active by 24 h of incubation; later on the pH values did not change significantly (Figure 2).

It was shown that all the bacteria isolated from human feces were able to grow and utilize TA-dextrin as a source of carbon, albeit to varying degrees. The highest growth was recorded for *Lactobacillus* and *Bifidobacterium*. *Bifidobacterium* strains were also characterized by the highest acidifying activity (lowering pH to 4.9), which remains consistent with the results reported by other authors [15,30,31]. The weakest growth was observed for *Clostridium* and *E. coli*. It was found that the stationary phase for *Lactobacillus* and *Bifidobacterium* strains was much longer than for other intestinal bacteria. After prolonging culture time to 72-168 h, which corresponds to retarded or pathological passage of digesta through the large intestine, the viability of intestinal bacteria in a medium with resistant dextrin was found to be lower by 1-1.5 log cycles than that of *Lactobacillus* and *Bifidobacterium*. The

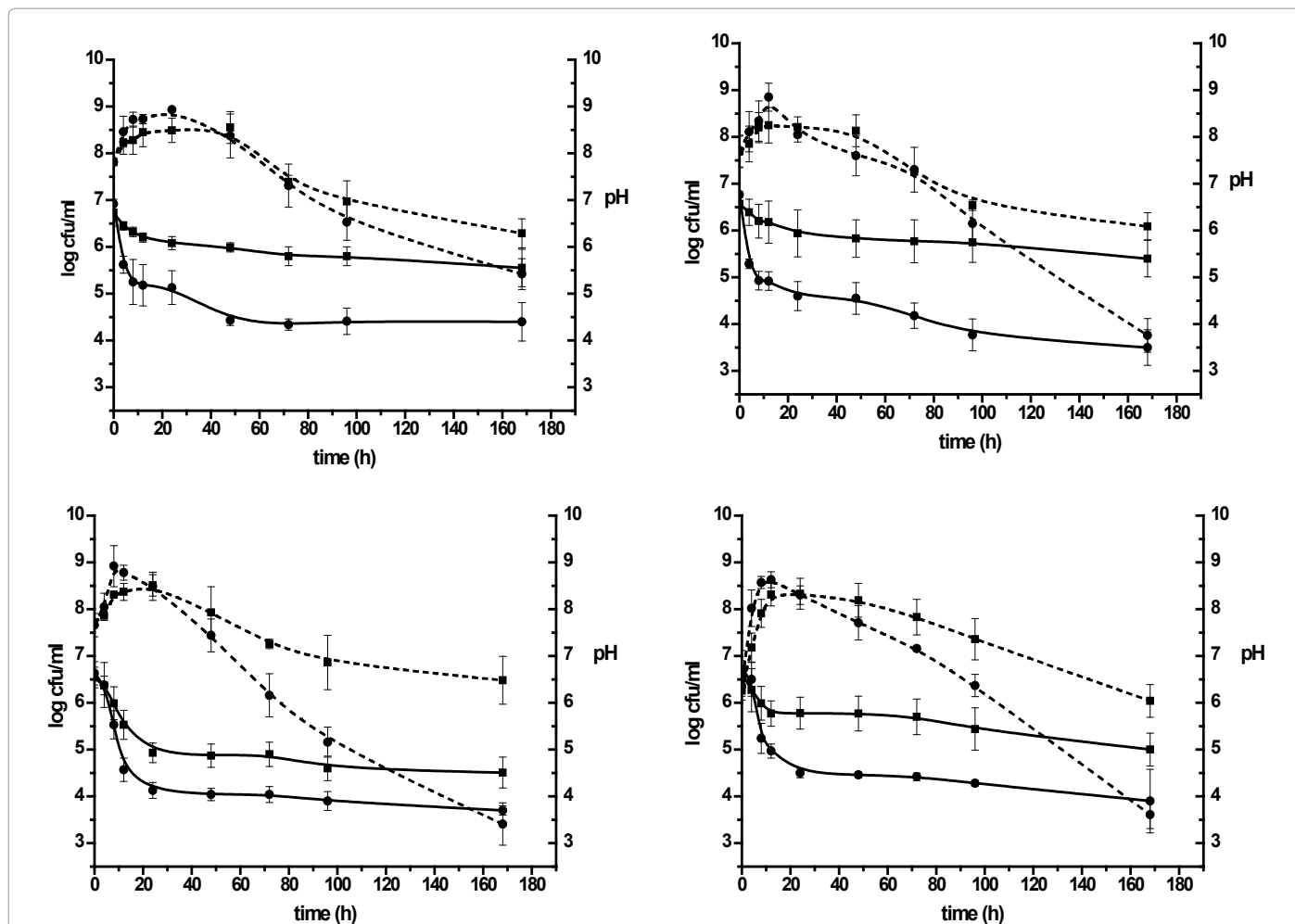


Figure 2: The growth curves (---) and changes in pH (—) for *Fusobacterium*, *Clostridium*, *Enterococcus* and *Escherichia coli* bacteria grown in the medium containing TA-dextrin (■) or glucose (●). Results show means and standard deviations of n=3 replicates.

Fermentation products	<i>Lactobacillus</i>	<i>Bifidobacterium</i>	<i>Escherichia coli</i>	<i>Enterococcus</i>	<i>Clostridium</i>	<i>Fusobacterium</i>
Lactic acid	112.7 ± 0.81	109.5 ± 0.42	85.0 ± 1.00	90.2 ± 1.50	55.2 ± 0.90	53.5 ± 0.21
Acetic acid	1.5 ± 0.15	18.2 ± 0.50	19.5 ± 0.15	6.5 ± 0.30	71.5 ± 0.05	14.0 ± 0.15
Formic acid	0	49.2 ± 0.25	84.5 ± 0.26	60.0 ± 0.15	0	0
Propionic acid	4.5 ± 0.01	4.6 ± 0.45	0	0	0	390.0 ± 1.85
Butyric acid	20.0 ± 0.12	11.5 ± 0.25	0	0	80.5 ± 0.51	0
Succinic acid	0	0	6.0 ± 0.10	1.7 ± 0.10	9.2 ± 0.11	28.1 ± 1.00
Acetaldehyde	2.2 ± 0.15	0.3 ± 0.15	5.5 ± 0.01	5.0 ± 0.15	0.3 ± 0.02	5.1 ± 0.1
Ethanol	0.20 ± 0.01	0.15 ± 0.02	0.09 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0

SD: Standard Deviation; n=3 replicates.

Table 2: Concentration (mg/100 ml) of fermentation products after 24-h incubation of bacteria isolated from gastrointestinal tract of man in the broth containing TA-dextrin as the only one source of carbon.

number of *Lactobacillus*, *Bifidobacterium*, and other bacteria isolated from fecal samples grown in media containing 1% glucose was lower by 1-2.5 log cycles than that of corresponding bacteria grown in a medium containing dextrin. This may have been caused by the lower pH values of the controls, under which the culture environment became unfavorable to preserving high viability by the studied bacteria. This may have also been caused by the protective effects of dextrin on the bacteria.

It was shown that the PI values in media with TA-dextrin were positive; furthermore, the prebiotic index increased with the time of culture (from 0.24 at 24 h of incubation to 0.31 at 168 h), which proves that beneficial bacteria (*Bifidobacterium* and *Lactobacillus*) can dominate their environment in the presence of a mixture of intestinal bacteria cultured with the addition of resistant dextrin.

The calculated PI values for TA-dextrin were higher than those reported by Olano-Martin et al. [32] or by Kordyl [33] inulin and oligosaccharides under the same incubation conditions (anaerobiosis; 1% prebiotic addition; pH 6.8; incubation temperature of 37°C), which shows that TA-dextrin may act as a prebiotic substance.

The HPLC results indicate that the main metabolite produced by *Lactobacillus* and *Bifidobacterium* was lactic acid (Table 2). Depending on the bacterial strain, its concentration after 24 h of incubation ranged from 109.5 mg/100 mL for *Bifidobacterium* to 112.7 mg/100 mL for *Lactobacillus*.

Another fermentation product was acetic acid (1.5 mg/100 mL for *Lactobacillus* and 18.2 mg/100 mL for *Bifidobacterium*), propionic acid (4.5-4.6 mg/100 mL for *Lactobacillus* and *Bifidobacterium*), and butyric acid (20.0 mg/100 mL for *Lactobacillus* and 11.5 mg/100 mL for *Bifidobacterium*). *Bifidobacteria* generated up to 50 mg/100 mL of formic acid. Furthermore, *Lactobacillus* and *Bifidobacterium* strains fermenting TA-dextrin produced acetaldehyde (0.3 to 2.2 mg/100 mL) and ethanol (0.1-0.2 mg/100 mL).

It was found that the main metabolite produced during the fermentation of TA-dextrin by *Fusobacterium* was propionic acid, with a concentration reaching 390 mg/100 mL. *Fusobacterium* also produced lactic acid (53.5 mg/100 mL), succinic acid (28.1 mg/100 mL), acetic acid (14.0 mg/100 mL), and small amounts of acetaldehyde (5.1 mg/100 mL) (Table 1).

The fermentation of TA-dextrin by *Enterococcus* led mostly to lactic and formic acids. The concentration of those acids, determined by means of HPLC, amounted to 90.2 and 60.0 mg/100 mL, respectively [34-36]. The average concentration of acetic acid and acetaldehyde was comparable and ranged from 6.5 to 5.0 mg/100 mL. Furthermore, *Enterococcus* produced traces of ethanol (0.08 mg/100 mL) (Table 2).

According to the results, the major products of the fermentation of TA-dextrin by *Clostridium* were butyric, acetic and lactic acids. The concentration of these acids was 80.5, 71.5, and 55.2 mg/100 mL, respectively. The concentration of other metabolites, namely succinic acid and acetaldehyde, was considerably lower. Moreover, traces of ethanol were also present (Table 1).

The HPLC results showed that *E. coli* also metabolized TA-dextrin, producing a considerable amount of lactic and formic acids, at concentrations reaching 85 mg/100 mL. The concentration of another fermentation product, acetic acid, was 19.5 mg/100 mL. Succinic acid and acetaldehyde were produced at a similar level (5.5-6.0 mg/100 mL). *E. coli* produced traces of ethanol as well (Table 2).

Conclusions

The experiments showed that dextrin obtained as a result of heating potato starch in the presence of hydrochloric acid (0.1% of starch dry mass) and tartaric acid (40% of starch dry mass) at 130°C for 2 h may have prebiotic properties. The presented results are promising, but, according to the recommendations of FAO experts concerning the applications of prebiotics, it is still necessary to continue with in vivo experiments, which are now being conducted.

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