

Possibilities for stimulation of *Bifidobacterium* growth by propionibacteria

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Abstract — Twenty-seven propionic acid bacteria (PAB) strains were studied for their ability to produce metabolites, which stimulated the growth of six *Bifidobacterium* strains. In addition, the 27 investigated PAB strains were examined for their influence on the growth of four Gram(–) strains belonging to species *Escherichia coli* and *Yersinia enterocolitica*, their abilities to survive at pH 2.0, 2.5, 3.0 and in the presence of 4% bile salts as well as their sensitivity against 12 selected antibiotics. All PAB strains stimulated growth of from 3 to 6 of the examined *Bifidobacterium* strains and simultaneously produced antibacterial metabolites against Gram(–) rods. Fifteen PAB strains inhibited growth of the particular *Bifidobacterium* strains. Five PAB strains were resistant to acidity surviving for 2 h in pH 2.5 at population level 10^2 – 10^6 cfu·mL⁻¹ and 3 strains were resistant to bile salts. Only one PAB strain survived in both the acidic and the bile salt environments. All PAB strains were sensitive to a majority of the antibiotics used in the investigations.

Bifidobacterium / *Propionibacterium* / Gram(–) rod / stimulation / inhibition

Résumé — Stimulation de la croissance de *Bifidobacterium* par les bactéries propioniques. Vingt sept souches de bactéries propioniques (PAB) ont été examinées pour leur aptitude à produire des métabolites stimulant la croissance de six souches de *Bifidobacterium*. Par ailleurs, ces 27 souches de PAB ont été examinées pour leur influence sur le développement de quatre souches Gram(–) des espèces *Escherichia coli* et *Yersinia enterocolitica*, pour leur capacité de survie dans un environnement à pH 2.0, 2.5, 3.0 et en présence de 4 % de sels biliaires ainsi que pour leur sensibilité à douze antibiotiques sélectionnés. Toutes les souches de PAB ont stimulé le développement de 3 à 6 des souches examinées de *Bifidobacterium* en produisant en même temps des métabolites antibactériens contre les bacilles Gram(–). Quinze souches de PAB ont eu un effet inhibiteur sur le développement des souches individuelles de *Bifidobacterium*. Cinq souches de PAB ont résisté à l'acidité et ont

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survécu pendant 2 h à pH 2.5 à un niveau population de 10^2 – 10^6 cfu·mL⁻¹. Trois souches ont résisté aux sels biliaires. Une seule souche de PAB a survécu à la fois à l'acidité et aux sels biliaires. Toutes les souches de PAB étaient sensibles à la plupart des antibiotiques utilisés dans l'essai.

Bifidobacterium / *Propionibacterium* / bacille Gram(-) / stimulation / inhibition

1. INTRODUCTION

Bifidobacteria are considered a probiotic microflora necessary for proper functioning of the gastrointestinal tract. They colonise and constitute the predominant microflora of mucous membranes in the colon of breast milk-fed infants. In adult people, population of these microorganisms varies depending on age, condition and medical treatment with chemotherapeutics as well as eating habits [2, 3]. Population of these microorganisms can be kept at an appropriate level through regular supplementation of the gastrointestinal tract with these bacteria in a form of freeze-dried pharmaceutical preparations [11, 12] or consumption of fermented milk products (probiotic).

Bifidobacteria belong to fastidious microorganisms of specific nutritional requirements and significant sensitivity to oxygen. For these reasons, their cultivation is difficult and requires appropriate media and methods in particular for determination of their population size. Not all species and strains of *Bifidobacterium* are able to proliferate and adhere in vitro to mucous membranes in human and animal intestines. It is very likely that the ability of an individual species of *Bifidobacterium* to colonise an intestine is connected with the presence of bifidogenic factors in the intestine [2]. Several substances known as bifidogenic factors are introduced to the gastrointestinal tract with food such as carbohydrates raffinose, melibiose, lactose and glycoproteins as well as N-acetyl-glucosamines present in women's milk. These compounds, to different extents, stimulate proliferation of the individual species of *Bifidobacterium* [4, 7]. The propionic acid bacteria (PAB) also

have a beneficial effect on the growth of *Bifidobacterium*. One of the identified active bifidogenic factors is 2-amino-3-carboxy-1,4-naphthoquinone produced by a *P. freudenreichii* strain. Stimulation of growth of *Bifidobacterium* was observed both after the addition of the compound to the growth medium as well as during the mixed cultivation of the producer strain with bifidobacteria [8]. Several features of *Propionibacterium* strains suggest that they can be used as probiotic microflora. The PAB together with lactic acid bacteria were successfully used in feeding animals leading to bigger body weight gains [6, 9, 10]. However, little is known about the presence of PAB in human intestines, their abilities to colonise and proliferate there as well as methods of supplementation in order to keep a proper size of their population in the guts.

The present investigations evaluated occurrence of PAB strains able to produce bifidogenic metabolites during cultivation as well as determination of the specificity of their action on *Bifidobacterium* strains.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

Twenty-seven strains of *Propionibacterium* belonging to species: *P. jensenii* (9 strains), *P. freudenreichii* (4 strains), *P. thoenii* (11 strains) and *P. acidipropionici* (3 strains) were examined. The strains were obtained from the Culture Collection of the Institute of Food Biotechnology at the University of Warmia and Mazury in Olsztyn. The strains were stored anaerobically on

APT agar medium (Merck, 64271 Darmstadt, Germany) at 4 °C and resuscitated before use through passages on the same broth medium for 24–48 h, at 30 °C. To test the formation of bifidogenic factors the strains were grown on the following liquid media: reconstituted cheese whey; reconstituted 10% cheese whey supplemented with 1% yeast extract and 0.5% glucose; reconstituted 10% skim milk powder; reconstituted skim milk powder supplemented with 1% yeast extract and 0.5% glucose. The incubation was carried out at 30 °C for 5 to 8 d with manual correction of pH to 6.8, every 24 h.

The strains of bifidobacteria used in the study were isolated from the guts of calves and piglets and were classified as belonging to species of – *B. breve* 2/1, 2/3 – and *B. animalis* 10, 2/4, 2/2. One strain, *Bifidobacterium* sp. B1/1, was obtained from the Culture Collection of the Institute of Food Biotechnology at the University of Warmia and Mazury in Olsztyn. The strains were grown in MRS broth (Merck) for 24 h, at 37 °C or MRS agar under anaerobic conditions using Anaerocult C (Merck).

Two Gram(–) rod test strains, *Escherichia coli* O157: H7 and – 205 –, were obtained from the Culture Collection of the Institute of Food Biotechnology at the University of Warmia and Mazury in Olsztyn and 2 test strains, *Yersinia enterocolitica* 2 and 8, were isolated from the gastrointestinal tract of piglets. The strains were grown on nutrient broth or agar (Merck) for 24 h, at 37 °C.

2.2. Influence of *Propionibacterium* cultures on *Bifidobacterium* strains and Gram(–) rods

The influence of PAB metabolites present in 5 and 8 d-old monocultures on the growth of *Bifidobacterium* strains was examined using the well method. The melted MRS agar inoculated with 10^5 cfu·mL⁻¹ of *Bifidobacterium* strains was poured into

Petri dishes and left to solidify. Wells of 9 mm diameter were cut off and filled with 0.1 mL of the PAB culture. The Petri dishes were incubated at 37 °C for 24 h under anaerobic conditions. After incubation, the zones of intense (stimulation) or reduced (inhibition) growth around the wells caused by the diffusing PAB metabolites were measured.

The influence of PAB on growth of Gram(–) rods was measured using the same diffusion method, however, the medium used for examination was nutrient agar.

2.3. Survival of *Propionibacterium* strains in an acidic environment

Suspensions of 24 h-old PAB strains of concentrations from 10^6 to 10^7 cfu·mL⁻¹ were added to test tubes containing 10 mL physiological solution adjusted with HCl to pH 2.0, 2.5 and 3.0, and kept at temperature at 37 °C for 1 or 2 h. The suspensions of the cells were transferred onto APT agar medium and incubated at 30 °C for 3–4 d, under anaerobic conditions, for determination of the living cells.

2.4. Survival of *Propionibacterium* strains in bile salts

The PAB strains were added into 4% (w/w) solution of bile salts (Merck) in physiological solution of pH 7.0. After 2 h of incubation at 37 °C, the surviving cells were detected using the method presented above in the chapter 2.3.

2.5. Susceptibility of *Propionibacterium* strains to antibiotics

During the experiment, the following 12 antibiotics were examined: β -lactam antibiotics: penicillin (P 10), ampicillin (Am 10), carbenicillin (CB 100); aminoglycoside antibiotics: streptomycin

(S 10), gentamycin (GM 10), neomycin (N 30), kanamycin (K 30); polypeptide antibiotics: colistin (CL 50); riphamycines: riphampicin (RA 30); furan derivatives: nitrofurantoin (F/M 300); naldixic acid (NA 30); chloramphenicol (C 30) (BioMerieux). Four paper discs, each saturated with a particular antibiotic were placed on a Petri dish with Mueller-Hinton agar inoculated with suspension of a PAB strain of concentration 10^5 cfu·mL⁻¹. The incubation was carried out at 30 °C for 4 d, under anaerobic conditions. The susceptibility or resistance of the examined strains against the used antibiotics was assessed by measuring the diameter of the inhibition zone.

3. RESULTS AND DISCUSSION

3.1. Influence of *Propionibacterium* metabolites on *Bifidobacterium* spp.

Preliminary results indicated that a substantial percentage of the PAB strains from the Culture Collection of the Institute of Food Biotechnology at the University of Warmia and Mazury in Olsztyn growing on whey produced bifidogenic metabolites,

however, not all *Bifidobacterium* strains were stimulated by them. Among the 27 examined 5 d-old PAB cultures, between 44 and 100% of the strains produced metabolites stimulating growth from 3 to 6 of the individual *Bifidobacterium* strains (Tab. I). After 8 d, the stimulating activities of PAB disappeared in the majority of the cultures and the active bifidogenic factors were detected in only 15–55% of the PAB cultures (Tab. I). In 15 PAB cultures, 5 and/or 8 d-old, there were metabolites that inhibited the growth of 1 or 2 *Bifidobacterium* strains. The same cultures, however, did not influence the growth of the remaining *Bifidobacterium* strains. This may suggest specific requirements of the individual *Bifidobacterium* strains for bifidogenic factors as well as differences in their sensitivity towards antibacterial metabolites produced in the PAB cultures. Of the examined 27 PAB strains, 12 strains produced bifidogenic metabolites in whey stimulating all or at least 3 of the *Bifidobacterium* strains used in the experiment and did not inhibit any of the *Bifidobacterium* strains. The extent of stimulation varied, which was seen from different diameters of the growth stimulation zones of the individual test strains.

Table I. Number of 5 and 8 d-old PAB cultures on whey medium influencing the growth of *Bifidobacterium* strains.

<i>Bifidobacterium</i> strains	PAB cultivation time (days)							
	5		8		5		8	
	Number of stimulating strains		Number of inhibiting strains		No influence			
B 1/1	22	15	4	0	1	12		
B 10	15	12	4	4	8	11		
B 2/4	13	10	3	1	11	6		
B 2/2	27	9	0	2	0	6		
B 2/1	22	6	3	1	2	20		
B 2/3	17	4	1	4	9	9		

Strains: *Bifidobacterium* sp. B1/1, *B. animalis* B10, B2/4, B2/2, *B. breve* B2/1, 2/3.

3.2. Influence of *Propionibacterium* metabolites on Gram(–) bacteria

The same PAB cultures also produced antibacterial metabolites against Gram(–) bacterial strains (Tab. II). The antibacterial activity was dependent on the age of the cultures as well as on the sensitivity of the Gram(–) strains. The metabolites inhibiting the growth of the Gram(–) strains were mainly present in the 8 d-old cultures (Tab. II). Among the 27 8 d-old PAB cultures examined, between 63% and 81% of the strains exhibited strong antibacterial action. Of the 5 d-old cultures, only some individual PAB strains produced metabolites inhibiting the growth of the Gram(–) strains the majority of the cultures did not have any influence on their growth. This was confirmed during our previous research with *P. acidipropionici* [14].

Among the tested Gram(–) strains, the most sensitive one was *Yersinia enterocolitica* 2, which was inhibited strongly by 81–89% of both 5 and 8 d-old PAB cultures (Tab. II). The results of the first experiments allowed us to exclude 15 PAB strains from the further research due to lack of influence on growth of the tested strains. Among the selected 12 PAB strains, 9 strains stimulated all or the majority of the

tested *Bifidobacterium* strains (Tab. IIIa) and simultaneously exhibited antibacterial activity against Gram(–) rods (Tab. IIIb). In addition, 3 strains, *P. acidipropionici* T122, *P. thoenii* 115 and 124, were selected that inhibited growth of one *Bifidobacterium* strain but simultaneously stimulated growth of the remaining *Bifidobacterium* strains (Tab. IIIa).

Abilities of the selected PAB strains to produce bifidogenic factors were investigated through their cultivation on reconstituted skim milk or reconstituted whey supplemented with additional carbon sources. The results demonstrated that production of bifidogenic metabolites by the investigated PAB strains resulted from the characteristics of the producing strain rather than composition of the cultivation medium. Almost all selected PAB strains produced bifidogenic factors stimulating growth of *B. animalis* 10, 2/2 and *B. breve* 2/1, in the 3 cultivation media used. The growth of the remaining *Bifidobacterium* strains was stimulated by a limited number of the PAB strains only. In our previous research concerning antibacterial activity of the *P. acidipropionici* strain, the influence of the milk or whey medium on production of antimicrobial metabolites was not stated; however, such metabolites were not produced in synthetic media, either [14].

Table II. Number of 5 and 8 d-old PAB cultures influencing the growth of Gram(–) rods.

Gram(–) rods	PAB cultivation time (days)					
	5		8		8	
	Number of inhibiting strains		No influence		Number of inhibiting strains	
O157 : H7	2	18	25	9	0	0
205	1	20	26	7	0	0
2	24	23	3	4	0	0
8	7	17	13	10	7	0

Strains: *Escherichia coli* O157 : H7, 205, *Yersinia enterocolitica* 2, 8.

Table III a. Influence of 5 and 8 d-old PAB cultures on growth of *Bifidobacterium* strains.

PAB strains	Diameter of <i>Bifidobacterium</i> growth stimulation zone (mm)											
	B1/1		B10		B 2/4		B 2/2		B 2/1		B 2/3	
	Age of culture (days)											
	5	8	5	8	5	8	5	8	5	8	5	8
T 123	19	17	20	12	0	11	19	12	21	0	14	14
T 128	15	12	19	12	14	13	20	11	20	0	14	0
P 2/5	20	12	20	0	16	0	21	0	20	0	12	0
T 83	16	12	19	11	16	15	20	11	0	11	15	14
T 109	12	0	19	11	13	0	20	12	20	14	14	0
T 110	15	16	21	11	15	17	19	12	20	14	13	13
T 112	20	15	20	0	16	0	21	0	21	0	12	0
T 115	I	0	18	0	0	12	20	0	21	13	14	0
T 120	14	12	0	12	0	12	19	0	19	0	15	0
T 124	20	17	19	0	15	0	20	I	12	0	14	0
T 126	22	18	21	0	0	0	21	12	22	0	0	0
T 122	14	19	0	12	0	0	19	0	I	0	14	0

I-inhibition zone, 0-no influence.

Strains: *P. jensenii*: T123, T128, P2/5, *P. freudenreichii*: T83, T 109, *P. thoenii*: T 110, T112, T115, T120, T124, T126, *P. acidipropionici* T122.

Table III b. Influence of 5 and 8 d-old PAB cultures on growth of Gram(-) rods.

PAB strains	Diameter of growth inhibition zone for Gram(-) rods (mm)											
	<i>E. coli</i> O157 : H7			<i>E. coli</i> 205			<i>Y. enterocolitica</i> 2			<i>Y. enterocolitica</i> 8		
	Age of culture (days)											
	5	8	5	8	5	8	5	8	5	8	5	8
T 123	0	22	0	19	19	24	0	14				
T 128	14	0	0	20	17	24	0	15				
P 2/5	0	16	0	14	19	20	S	13				
T 83	14	21	0	19	20	20	0	15				
T 109	0	20	0	21	21	25	0	0				
T 110	0	19	0	20	25	18	0	0				
T 112	0	22	0	23	17	21	S	14				
T 115	0	19	0	16	19	16	14	14				
T 120	0	0	12	0	19	0	0	14				
T 124	0	0	0	0	21	21	12	15				
T 126	0	20	0	21	20	20	0	0				
T 122	0	24	0	25	23	24	17	19				

S-stimulation zone, 0-no influence.

3.3. Survival of *Propionibacterium* strains in a low pH or in the presence of bile salts

The selected PAB strains also varied in their abilities to survive in an adverse environment similar to that in the gastrointestinal tract (Tab. IV). It has been stated that 9 of the 12 examined PAB strains survived for 1 h at pH 2. Only one strain, *P. thoenii* T124, survived for 2 h in the same conditions, but its population density was reduced by 3 logs. At pH 2.5, all 12 PAB strains survived for 1 h but only 5 strains survived for 2 h. The initial population density, about 10^6 cfu·mL⁻¹, was reduced by 1 to 4 logs. All the PAB strains survived at pH 3.0 for 2 h and their population density was virtually not affected. The most resistant to the action of HCl were *P. thoenii* 124, surviving for 2 h at pH 2.0 and 2.5 as well as *P. jensenii* 123, 128 and P2/5 and *P. freudenreichii* 83 surviving at pH 2.5 for 2 h. The final population densities varied from 10^2 to 10^6 cfu·mL⁻¹ (Tab. IV). This suggests the possibility of survival of these strains in a gastric juice environment for 1 to 2 h. Fewer strains were resistant to bile salts. Of the 12 examined PAB strains only *P. jensenii* P2/5, *P. thoenii* 124 and *P. acidipropionici* 122 survived at 4% concentration of bile salts for 2 h. Of these 3 strains, *P. thoenii* 124 was resistant to both the bile salts and pH 2.0 and 2.5.

3.4. Sensitivity of *Propionibacterium* strains to selected antibiotics

In addition, the examined PAB strains were tested for their sensitivity to antibiotics because this feature is taken into account during evaluation of probiotic strains. The examined PAB strains were sensitive to the majority of the antibiotics used and were resistant to a single antibiotic only. The strain *P. thoenii* 112 was sensitive to all antibiotics used and the remaining strains were resistant to nalidixic acid (NA 30) –

10 strains, to colistin (CL 50) – 3 strains as well as 2 medium sensitive strains, to chloramphenicol (C 30) – 1 strain, to nitrofurantoin (F/M 300) – 1 strain and 2 medium sensitive strains, to neomycin (N 30) – 1 strain and rifampycin (RA 30) – 1 strain. Significant sensitivity of the examined PAB strains does not diminish their suitability for practical use in food production. It must only be considered that they will be rapidly eliminated from an environment where chemotherapeutics are present. The abilities of the PAB strains to produce bifidogenic metabolites indicates the possibility of using these bacteria together with *Bifidobacterium* strains in dietetic products. As indicated in mice studies, continuous supplementation of the diet with PAB strains influenced reduction of coliform bacteria as well as elimination of enzymes detrimental to the body [3]. There are only limited possibilities to introduce these beneficial bacteria into the human diet to supplement the population of these bacteria in human intestines. Promising results were obtained using selected PAB strains together with lactic acid bacteria in fermented vegetables and vegetable juices [5]. This allowed the production of dietetic products with a bacterial population in the range 10^5 – 10^7 cfu·g⁻¹ [1, 11, 13]. Such populations, as indicated in the presented research, have a chance of surviving in the drastic environmental conditions prevailing in the stomach and intestines. Products containing these bacteria, consumed regularly, can maintain the population size of probiotic bacteria as well as accompanying PAB in intestines, at a constant level. It is possible that selected PAB strains resistant to gastric juices and bile salts would also be able to support growth of *Bifidobacterium* strains in the colon.

4. CONCLUSIONS

The majority of PAB strains grown in whey media synthesised bifidogenic metabolites; however, some of the PAB strains

Table IV. Survivability of selected PAB strains in low pH or bile salt environments.

PAB strain	Time [h]	Population density (cfu·mL ⁻¹)			
		pH 2.0	pH 2.5	pH 3.0	bile salts [4% solution]
<i>P. jensenii</i> T123	0	5.6×10^6	5.4×10^6	4.9×10^6	8.9×10^5
	1	1.2×10^2	1.1×10^4	2.8×10^5	
	2	<10	2.2×10^3	2.9×10^4	<10
<i>P. jensenii</i> T128	0	1.2×10^6	2.0×10^6	1.6×10^6	3.1×10^6
	1	1.4×10^5	2.1×10^5	8.3×10^5	
	2	<10	7.3×10^2	4.2×10^5	<10
<i>P. jensenii</i> P2/5	0	4.4×10^6	4.7×10^6	3.9×10^6	3.6×10^6
	1	<10	1.2×10^4	2.6×10^6	
	2	<10	1.6×10^3	1.3×10^5	6.3×10^3
<i>P. freudenreichii</i> T83	0	1.2×10^7	9.8×10^6	2.7×10^7	3.2×10^6
	1	<10	4.9×10^6	6.7×10^6	
	2	<10	1.5×10^6	4.8×10^6	<10
<i>P. thoenii</i> T124	0	3.1×10^6	1.3×10^6	3.9×10^6	5.3×10^6
	1	1.7×10^3	3.8×10^3	1.2×10^6	
	2	1.1×10^3	2.6×10^3	1.5×10^6	1.4×10^3
<i>P. acidipropionici</i> T122	0	1.5×10^7	2.1×10^7	2.2×10^7	3.2×10^6
	1	5.7×10^4	1.4×10^5	9.6×10^6	
	2	<10	<10	4.6×10^6	2.1×10^4

produced also inhibited specific individual *Bifidobacterium* strains. The PAB strains grown in natural media, besides bifidogenic substances, can also simultaneously synthesize antibacterial metabolites against strains of *Escherichia coli* and *Yersinia enterocolitica*. Production of the bifidogenic metabolites is a characteristic of a PAB strain only slightly influenced by a growth medium.

A very limited number of PAB strains were able to survive at a low pH or in the presence of 4% of bile salts for 1 or 2 h. The PAB strains were also very sensitive to the examined antibiotics.

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