

EXTENDED ABSTRACT

Propionic acid bacteria as probiotics

LENA I. VOROBYEVA, EVGENIY YU. KHODJAEV & NINA V. VOROBYEVA

Moscow State University, Moscow, Russian Federation

Abstract

Propionic acid bacteria (PAB) possess a set of physiological and biochemical properties that allows their inclusion in probiotic compositions. Their potential resources are underestimated as yet. The list of the described probiotic characteristics of PAB must be enlarged by the addition of antimutagenic, reactivative and protective activities, first discovered by our group. Live and dead cells of PAB and *Luteococcus casei* as well as their cultural liquid (CL) revealed antimutagenic (AM) effect on spontaneous and induced mutagenesis. Protective and reactivative activities of *Propionibacterium freudenreichii* cells are bound up with the intracellular protein, identified as cystein synthase, whose synthesis is induced by some stress factors. Under unfavourable conditions leading to lysis of the majority of cells, the released protein may play a vitally important role in the cell population as a whole, supporting the existence of the species. The active protein reveals cross-reactive properties, both the protective and reactivative effects on cells of *Escherichia coli* and yeasts *Saccharomyces cerevisiae* and *Candida guilliermondii*. Phylogenetically close to PAB, *L. casei* produces and excretes in the medium a proteinaceous metabolite, possessing protective and reactivative effects on cells of the producer, *E. coli*, *S. cerevisiae* and *C. scottii*, treated by heating and UV irradiation. The exometabolite is synthesized by cells in the log phase of growth. The effectiveness of its impact inversely depends on the survival of microbes. CL of *L. casei* is considered as the source of a new prebiotic with reactivative and protective properties.

Key words: *Luteococcus casei*, prebiotics, probiotics, propionic acid bacteria, protective and reactivative properties, exometabolites

Background

The totality of all microbial biocenoses of humans and animals is considered to be a peculiar extra-corporeal organ. Its total cell number exceeds that of all eukaryotic cells from tissues and organs (1). The concentration of the bacterial cells in the stomach, duodenum and small intestine can be up to 10^5 cells/g. Alkalinization leads to an increase of the bacterial number, which mounts to 10^{10} – 10^{11} cells/g (2). The bacterial variety includes over 500 species. The overall weight of the bacteria in the colon of an adult human is up to 1.5–2.0 kg.

The role of intestinal microorganisms consists of biosynthesis of vitamins of groups B and K, amino acids and proteins; metabolism of bile pigments and bile acids; adsorption of nutrients and microelements; decontamination of pathogens; regulation of intestinal motility; protection against xenobiotics

and formation of volatile acids via degradation of products.

Obligatory gut organisms are not isolated but constantly interact with the environment and the central nervous, endocrine and immune systems. Numerous imbalances lead to severe illnesses. In particular, antibiotics usage suppresses faecal microflora. At the same time, facultative microbes may colonize the intestine. Frequently, gastroenteric infections involve colonization by *Helicobacter pylori*. It is considered that these bacteria provoke gastritis and duodenitis and play some role in cancer progress.

Steady changes in ratios of normal alimentary microflora result in disbacteriosis. Its combined treatment includes restoration of the quantity and/or balance of the microbes of the normal microflora by utilization of probiotics. Probiotics

are monocultures or mixed cultures of living microorganisms that positively affect the health of humans and animals by improvement of endogenous microflora properties (3).

Some lactobacilli, bifidobacteria and non-lactobacteria such as *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Propionibacterium freudenreichii*, and yeasts such as *Saccharomyces cerevisiae* are attributed as human probiotics (4). In all, 96–99% of resident microflora of the human intestine are strict anaerobes growing at low redox potentials of a medium. Thus, it is proposed that usage of electrolyzed water, a catholyte with significant reducing properties, may favour the vital activity of normal microflora (5).

Propionic acid bacteria (PAB) are combined into the family Propionibacteriaceae, genus *Propionibacterium*. Their distinctive features are: formation of propionic acid as a consequence of propionic acid fermentation, dependent on coenzyme B₁₂, and high content of G+C (65–67%).

PAB are divided into two groups owing to peculiarities of their habitat: lactic or classical and cutaneous. The former are isolated from milk, fermented dairy products and cheese; they are also found in 24 species of vegetables and fruits. Cutaneous PAB represent a sole anaerobic microflora of healthy human skin and a component of associants in the rumen of ruminants. Lactic PAB have been used in cheese-making from antiquity. In Russia they are included in starters of several dairy products such as PAM and Tonus, and are source of vitamins B₁₂, porphyrins, antioxidative enzymes (SOD and catalase) and propionic acid (6), but their potential as probiotics is generally underestimated.

Some bacteria were isolated from cheese at the early phase of ripening. They conduct fermentation typical of the genus *Propionibacterium* and have high similarity in content of G+C (63.4%). They were attributed to the genus *Luteococcus* on the basis of analysis of linear polymorphism of restricted DNA fragments and MALDI-TOF mass-spectral analysis of cytoplasmic proteins (data not published). The strain was identified as *L. japonicus* subsp. *casei*. They synthesize exometabolites possessing both protective and reactivative properties and are of undoubted interest in connection with the problems discussed in the present paper.

Probiotic properties of PAB

Probiotic properties of PAB are bound with: i) formation of helpful metabolites and antimicrobial compounds; ii) synthesis of β -galactosidase, the enzyme-splitting lactose, thus preventing lactose intolerance in some humans; iii) bifidogenous activity; iv) massive synthesis of trehalose, low-calorie

carbohydrate; v) content of some microelements (Mn–267, Fe–535, Cu–102; in $\mu\text{g}/\text{kg}$) in PAB biomass (7) exceeds their concentration in lactic acid and bifidobacteria; vi) decrease of activities of carcinogenic enzymes, β -glucuronidase, nitroreductase and azoreductase, which convert faecal procarcinogens into active forms of carcinogens (8) (data were obtained in animals but PAB may cause similar effects in humans); vii) NO formation and accumulation during reduction of nitrates and nitrites (9) (nitrogen monoxide controls many essential functions such as neurotransmission, vasodilatation, intestine peristalsis and mucous tunic protection; chronic intestinal diseases are sometimes caused by lack of NO formation in the organism); viii) synthesis of the compound with helpful physiological and anticarcinogenic properties via linoleic acid isomerization in *P. freudenreichii* ssp. *shermanii*; ix) apoptosis of colorectal carcinoma cells *in vitro* by the same strain (10).

Antimicrobial products

The final products of energy metabolism in PAB are propionic and acetic acids and CO₂. The acids do not support growth of putrefactive bacteria, clostridia, yeasts and fungi. CO₂ participates in the redox potential decrease and is toxic to a number of aerobic putrefactive bacteria. Some PAB strains generate bacteriocins, which inhibit the growth and survival of *Listeria monocytogenes* and *Yersinia enterocolitica* cells and also, unlike lactic bacteria, the growth of Gram-negative bacteria, yeasts and moulds (11,12).

Antimutagenic activity

At present the natural protective mechanisms do not always cope with a drastic rise of mutation pressure, therefore a search for and availability of antimutagens must be regarded as an element of a compensatory approach to the genofond protection. Thus, an opportunity of lowering of mutagen quantity, using bacteria, primarily inhabiting the intestinal tract, as an antimutagenic (AM) factor is of great importance in the improvement of human health. For the first time we demonstrated AM activity of PAB against the mutations induced by 4-nitro-quinoline and N-nitro-N-nitrosoguanidine (transition mutations), as well as 9-aminoacridine and 2-nitrofluorene (frame-shift mutations) (13). Live and dead cells as well as the cultural liquid (CL) possess the AM effect. It was shown that AM activity of CL is caused by the proteinaceous compound. The AM properties of PAB cannot be overemphasized, taking into

consideration the presence of different amounts of mutagens in food.

Stimulation of growth of bifidobacteria

PAB generate and excrete bifidogenic metabolites, contributing to growth of some strains of bifidobacteria (11). 2-Amino-3-carboxy-1,4-naphthoquinone, synthesized by *P. freudenreichii*, is an active bifidogenic stimulator.

Sources of nutraceuticals

Nutraceuticals possess both nutritious and medicinal properties. The unique metabolism of PAB provides their cells with a set of cofactors, mainly participating in transfer and rearrangement of C-1-compounds. It is noteworthy that the key reaction of propionic fermentation (methylmalonyl-CoA transformation to propionyl-CoA) presents in human cells but is oppositely directed, resulting in the formation of succinate necessary for the synthesis of cytochromes, catalase and other compounds of porphyrin origin. PAB produce other nutraceuticals such as B₁₁ and B₁₂ vitamins and the nucleotide derivatives. PAB are record-holders in the biosynthesis of vitamin B₁₂. It is produced as a result of the industrial cultivation of these bacteria. Vitamin B₁₂ takes part in hematosis and is applied in the therapy of pernicious anaemia. Some strains also generate folic acid (vitamin B₁₁). Folic acid is a cofactor in numerous metabolic reactions including biosynthesis of nucleotides, the structural blocks of RNA and DNA. The low content of folates correlates with increased amounts of homocysteine in the blood and, consequently, heart diseases (14). Some evidence was reported for a role of folic acid in preventing certain tumours. PAB may be used as cell factories in the production of folic acid.

The nucleotide derivatives are applied in therapy for prevention and cure of thrombolytic illnesses.

Trehalose accumulation

Under different stresses PAB cells generate significant amounts of trehalose, a low-caloric diet sugar. Trehalose serves as a protein stabilizer, it keeps the natural colours and taste of food and its freshness.

Adhesive properties and ability to survive in intestinal tract

Effective probiotic bacteria ought to possess high adhesion and capacity for preservation of viability despite a variety of unfavourable factors such as stomach acids and enzymes, salts of bile acids and enzymes of small intestine, as well as the antagonistic

influence of other bacteria. It was shown in model experiments *in vitro* that the level of adhesion of PAB numbers up to 0.2–0.6% of all bacteria added (15). The level of adhesion of lactobacillus and bifidobacteria was significantly higher: from 1.3 to 24.3%. It has been established that the adhesion of PAB may be enhanced by their preliminary coaggregation with other probiotic bacteria. The stability of PAB towards acidity and salts of bile acids was enhanced by preliminary adaptation to the above-mentioned stress factors (16).

PAB amount to a minor part of the total microflora in the intestinal tract of healthy humans (0.001%); however, daily peroral administration for a week resulted in a significant rise of their content (up to 10⁶ cells/g). This amount is quite enough for manifestation of favourable effects on the intestine ecology and physiology of the host.

The PAB effect as a growth stimulator of other helpful bacteria is achieved without colonization and adhesion in the intestinal tract.

Protective and reactivative activities – new criteria for usage of PAB as probiotics

Under natural conditions, in human and animal organisms, during food processing and administration of medicines, bacteria are periodically exposed to different stress factors. Sublethal and lethal doses of stressors do not always block the cell division. The survival ability of bacteria under stress situations depends on the functioning of inducible adaptation mechanisms including autoinducers, components of a proteinaceous and non-proteinaceous nature (17).

The protective exometabolites are found in *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas fluorescens* and *Lb. casei* cells. They can directly interact with cellular structures, neutralize active molecules (18) or function as signal molecules, inducing the activation of a stress response (17).

A protein of molecular mass 35 kDa was isolated from *P. freudenreichii* cells (19). It possessed both protective and reactivative effects on the producer and *E. coli* cells subjected to UV irradiation, heating and impact of bile acids. By sequencing the N-terminal and internal regions of the protein it was identified as cystein synthase. The protein is produced constitutively and its synthesis is enhanced by heating, UV light and treatment with detergent. The active protein is not excreted in the environment. Seemingly it displays protective properties under stress situations. When cell mortality is high, being exposed to lysis, they excrete their contents. At the same time, part of a population, potentially capable of replication but shocked, gets a signal, activating natural reparation systems within cells. In other words, the intracellular

reactivative protein we discovered may play a vitally important role in the cell population as a whole, supporting the existence of the species under unfavourable conditions.

The proteinaceous exometabolite with reactivative and protective effects was isolated by filtration of CL from *L. casei* through mixed acetate membranes. Its biological activity is bound up with a component (m.m. of nearly 8 kDa), obtained by HPLC gel filtration. Mass-spectral analysis demonstrated that this peptide has m.m. 7.6 kDa and makes up to 80% of the total protein of the fraction eluted from the membrane filter. The exometabolite is produced during the log phase of *L. casei* growth and is accumulated in the medium in negligible amounts. On the contrary, its amount is enough to produce a 4–6-fold rise in the survival of the bacteria in the stressed cells in comparison with control cells. For evaluation of the protective or reactivative effects the cells were incubated for 10 min before or after the impact of stress factor before they were harvested on solid medium. The control cells were incubated in 3% solution of NaCl (the eluent). The reactivative effect of exometabolite was inversely dependent on the survival of bacteria. The active metabolite does not possess both the mitogenic and bactericidal effects on the producer and different strains of PAB. Proteinaceous exometabolite from *L. casei* possesses a unique breadth of microbial spectrum of its anti-stress effect. The cross-reaction of the active factor on stressed cells of *E. coli* and primitive eukaryotes, yeasts *S. cerevisiae* and *Candida scottii* was demonstrated (20,21). The broad width of anti-stress action and simplicity of isolation of the exometabolite from CL allow its consideration as a new promising prebiotic.

Conclusion

The study of PAB, their relationship with immune functions and metabolism of the human organism must be continued in view of the obvious positive effects of these bacteria. The increase in PAB content in the intestinal tract of humans serves as a significant task. It demands the creation of selective bacterial strains capable of settling down and competing with components of intestinal microflora and being compatible with other probiotics.

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