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The kinetics of bacterial growth in selected pro- and synbiotics – recommendations for family practitioners

Kinetyka wzrostu bakterii w wybranych pro- i synbiotykach – wskazówki dla lekarzy rodzinnych

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A – Study Design, **B** – Data Collection, **C** – Statistical Analysis, **D** – Data Interpretation, **E** – Manuscript Preparation, **F** – Literature Search, **G** – Funds Collection

Summary **Background.** In recent years one may observe increasingly widespread use of products containing probiotic bacterial strains. Development of most optimal content of the products is the purpose of many studies searching for combination of probiotic bacterial strains with high bacterial viability, and for the substances that increase the rate of bacterial growth. Family practitioners when prescribing probiotics must rely on research indicating the comprehensive and comparative value of commercially offered products.
Objectives. The aim of this study was to investigate the kinetics of bacterial growth in commercially available products containing from 1 to 9 probiotic bacterial strains with or without prebiotic substances.
Material and methods. Bacteria from five tested products were cultivated under anaerobic conditions over 48 hours and the number of bacterial cells was calculated according to the method of Koch. Statistical analysis of data was performed using the STATISTICA statistical package program, version 9. Differences were considered statistically significant when $p < 0.05$.
Results. The shortest lag phase of microbial growth and the greatest value of CFU was observed for the product containing the most numerous and diverse bacterial strains with oligofructose added as a prebiotic. The longest lag phase was noted for the product containing one bacterial strain without prebiotic.
Conclusions. Our results suggest that: (1) under anaerobic conditions the most rapid bacterial growth occurs in products containing several bacterial strains; (2) prebiotic substance is very effective in increasing the rate of bacterial growth and maintaining high bacterial viability *in vitro*.
Key words: probiotics, prebiotics, synbiotics, bacterial growth, CFU.

Streszczenie **Wstęp.** Rosnące w ostatnich latach zainteresowanie stosowaniem preparatów zawierających bakterie probiotyczne stało się podstawą do badań nad poszukiwaniem optymalnego ich składu uwzględniającego żywotność kultur bakteryjnych, jak i zastosowania substancji nasilających wzrost bakterii. Przepisując preparaty probiotyczne lekarze rodzinni powinni polegać na obszernych doniesieniach z literatury porównujących właściwości oferowanych przez rynek preparatów.
Cel badań. Zbadanie kinetyki wzrostu bakterii w wybranych preparatach zawierających 1–9 szczepów bakterii z dodatkiem lub bez substancji prebiotycznych.
Materiał i metody. Szczepy bakteryjne z badanych preparatów hodowano przez 48 godzin w warunkach beztlenowych. Ilość komórek bakteryjnych liczono metodą Kocha. Do obliczeń statystycznych użyto programu STATISTICA, wersja 9. Za różnice znamienne statystycznie uznawano wartości, dla których $p < 0,05$.
Wyniki. Faza przygotowania była najkrótsza w przypadku preparatu zawierającego 9 szczepów bakteryjnych z dodatkiem prebiotyku, a najdłuższa w przypadku preparatu zwierającego tylko 1 szczep bakterii bez dodatku prebiotyku.
Wnioski. 1. Najszybciej namnażają się mikroorganizmy znajdujące się w preparatach zawierających największą różnorodność szczepów. 2. Na szybkość namnażania się szczepów bakteryjnych oraz ich żywotność korzystny wpływ wywiera dodatek substancji prebiotycznych umieszczonych w preparatach handlowych.
Słowa kluczowe: probiotyki, prebiotyki, synbiotyki, wzrastanie bakterii, CFU.

Background

More than 500 different species of bacteria exist in human bodies, making up more than 100 trillion cells. Intestinal microflora protects the organism from many environmental pathogens and is necessary for the proper development and functioning of the immune system [1, 2]. The finding that intestinal bacteria beneficially affect human health was the stimulus for development and production of commercially available products named probiotics, containing "good bacteria" and improving human microbial balance. Probiotics are widely used for patients on/after antibiotic therapy, with lactose intolerance, or with different types of diarrhea [3]. The optimal and expected effect of probiotics depends on a dose and time of use [4], and the minimal daily therapeutic dose is described as 10^6–10^9 CFU [5]. Increased effectiveness of probiotics is achieved when a specific substances, prebiotics, are added to the market products. Prebiotics, the substances that modulate microbial growth, are defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" [6]. Prebiotics such as fructo-oligosaccharides (FOS) are found in a diet and affect alimentary tract enhancing, for example, bacterial short-chain fatty acids production, which in turn leads to lowering of pH and improvement of mineral substances absorption by intestinal membrane. This is of particular importance for the patients during/after antibiotic therapy to recover and rebuild the balance of beneficial microorganisms. The most popular prebiotics from FOS group are oligofructose and inulin, they are characterized as most effective, with low calorific value and neutral taste [7].

Functional combination of alive probiotic bacteria with prebiotic substance that works as a kind of medium for selected microbes is termed synbiotic. According to available literature and to our study this combination appears to be the most effective in increasing the number of "good" bacteria capable of colonizing gastrointestinal tract [8].

In recent years numerous studies on individual probiotic bacterial strains have been carried out to evaluate functional benefits of their activity in gastrointestinal tract [9–12]. So far, however, there are limited publications describing pro- and synbiotic products as a whole, without preparation of particular components.

It should be emphasized that probiotics and synbiotics demonstrate variations in properties which are due to differences in quantity and quality of probiotic species (different number and/or different bacterial strains, and prebiotics added). Because patients use probiotics and synbiotics as a whole product, it seems to be obvious that microbiological tests comparing market products in that form should be conducted to give an indication for family practitioners which product is best for their patients. Investigation of the kinetics of bacterial growth *in vitro* is a reliable method determining bacterial proliferative activity reflecting their ability to reproduce and survive. This, in turn, may influence the ability to colonize gastrointestinal tract. Proposed experimental design appears to be reasonable because the method takes into consideration possible interactions between respective bacterial strains and additional substances working as a prebiotics in tested product, moreover anaerobic conditions of cultivation accurately reflect *in vivo* conditions found in the colon.

Objectives

The purpose of the study was to evaluate kinetics of bacterial growth in selected pro- and synbiotics containing: (1) single bacterial strain, (2) combination of bacterial strains, (3) probiotics with prebiotic substance(s).

Material and methods

The 5 different types of commercially available products were used in the present study. The content of tested material presents Table 1. Because trade names are not presented, each examined product is assigned to the number in Table 1.

All tested products were stored according to the manufacturer's recommendations. The experiment was conducted according to the own method designed for this study. Growth kinetics of bacterial strains were estimated using CFU value for tested bacterial cell cultures.

1. Bacteria from tested commercial products were suspended in 100 mL of liquid MRS medium.
2. Bacteria were incubated in 37°C under anaerobic conditions using anaerobic chamber (GENbag anaer; Bio Merieux) over 48 hours.
3. A sample of 1 mL of incubated suspension was taken every 8 hours and according to the serial dilution method of Koch and the amount of bacterial inoculum was measured.

Statistics

Values are expressed as means ±SD. Statistical analysis of data was performed using the STATISTICA statistical package program, version 9. Differences were considered statistically significant when $p < 0.05$.

Table 1. Tested products

No.	Tested products	Types of bacterial strains in tested products	Amount of bacteria declared by manufacturer (CFU)
1.	Synbiotic containing 9 bacterial strains and oligofructose as a prebiotic, encapsulated according to the MURE technology	*Lactobacillus rhamnosus* *Lactobacillus casei* *Lactobacillus plantarum* *Lactobacillus Helvetius* *Lactococcus lactis* *Bifidobacterium longum* *Bifidobacterium bifidum* *Bifidobacterium breve* *Streptococcus thermophilus*	4.5×10^9
2.	Probiotic containing 1 bacterial strain; encapsulated in traditional capsule	*Lactobacillus rhamnosus*	2×10^9
3.	Probiotic containing 3 bacterial strains; encapsulated in gelatin capsule	*Lactobacillus acidophilus* *Lactobacillus bulgarius* *Bifidobacterium animalis*	1.6×10^9
4.	Probiotic containing 2 bacterial strains	*Lactobacillus rhamnosus* *Lactobacillus helveticus*	2×10^9
5.	Synbiotic containing 2 bacterial strains and inulin and oligofructose as a prebiotic	*Lactobacillus acidophilus* *Bifidobacterium animalis*	2×10^9

Results and discussion

Because interest in probiotic use has grown recently, family practitioners should stay up-to-date by reading new studies that produce new recommendations. To make a proper choice and to prescribe most effective probiotic for particular disorder, family practitioners should be familiar with some basic facts about probiotics, and also should recognize that clinical recommendations are based on studies examining specific probiotic strains, their microbiologic and functional characteristics, kinetics of bacterial growth and bacterial viability.

Comparative studies on the available probiotic products should describe the differences in quality and quantity of the probiotic content and their therapeutic value, which is related mostly to the bacterial viability and colonizing properties in the gastrointestinal tract. The final effect of probiotic product depends on quantity and combination of bacterial strains found in it.

To meet the consumer demands Polish pharmaceutical market offers variety of drugs or diet supplements containing pro-, pre- and synbiotic substances. The final doctor's and patient's choice depends on many factors including price and effectiveness of particular products [13–16]. To evaluate the optimal content of market products more independent studies are necessary to be conducted.

Considering that a wide range of substances are commercially available, our study compared kinetics of bacterial growth in five of them and tested products were analyzed as a whole, without preparation of particular components, which means in a form ready to be used by the consumer and, obviously, in a form prescribed by the family practitioner.

It is well known that many important biological attributes may affect individual growth rates of the microbial strains and the mutual interactions or influences among species in mixed populations [17]. The model of current study includes possible interactions between respective cultures of probiotic bacteria and additional prebiotics.

Stationary culture conditions provide five phases of bacterial growth: (1) lag phase – bacteria adapt themselves metabolically to the new conditions of growth, (2) log or exponential phase – bacteria undergo rapid reproduction, cell doubling occurs every few minutes, (3) declining phase – because of depletion of nutrients and accumulation of waste products bacterial reproduction slows, (4) stationary phase – the number of alive bacteria is constant and death rate equals the growth rate, (5) death phase – when the death rate is greater than the growth rate and all nutrients are exhausted [18].

Our experiment compared microbial growth in five commercially available products that contain from 1 to 9 bacterial strains.

The kinetics of bacterial growth are summarized in Table 2. Figures 1–5 present the curves of bacterial growth for particular tested products.

Table 2. Kinetics of microbial growth during 48 hrs of incubation expressed in $\log_{10}$ CFU ml^{-1}

Tested product	0 h	8 h	16 h	24 h	32 h	40 h	48 h
1	7.58±0.04	7.57±0.03	8.02±0.06	8.71±0.05	9.65±0.06	9.64±0.04	9.64±0.05
2	6.95±0.04	6.95±0.03	6.95±0.04	7.41±0.03	7.92±0.05	8.42±0.04	8.31±0.03
3	7.04±0.03	7.05±0.05	7.06±0.04	7.23±0.04	7.53±0.03	7.92±0.02	8.30±0.04
4	7.06±0.05	7.03±0.02	7.07±0.03	7.24±0.03	7.54±0.03	8.21±0.05	8.17±0.04
5	7.00±0.03	7.05±0.03	7.06±0.04	7.22±0.03	7.52±0.02	7.93±0.04	7.89±0.03

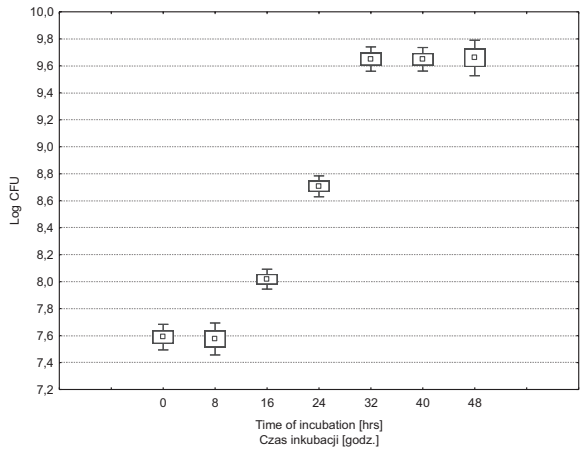

Figure 1. Growth curve of probiotic bacteria in product No 1. CFU – Colony Forming Unit; Time reflects the time of incubation

We have observed particular phases of bacterial growth under stationary anaerobic conditions and noticed the shortest lag phase for the Product No 1, containing nine bacterial strains with prebiotic substance. The longest lag phase was noted for the product containing one bacterial strain without prebiotic. This finding may indicate the greatest viability of bacteria from Product No 1 and, in turn, best possible capacities of that product to colonize gastrointestinal tract.

In this study we have investigated the bacterial ability to reproduce according to different combination of bacterial strains in tested products. Based on our results CFU did not reach the value declared by the manufacturers in all tested products. This may result from the different culture medium (we used MRS liquid medium) and/or from the different storage conditions in a period between the end of product manufacturing and beginning of the cultivation procedures. The smallest difference between obtained and declared values of CFU was found for the Product No 1.

The results of present study showed that the Product No 2 containing only one bacterial strain (*Lactobacillus rhamnosus*) exhibited the lowest rate of bacterial reproduction, while in Product No 1, that includes nine bacterial strains and prebiotic, bacterial growth was very rapid and achieved the

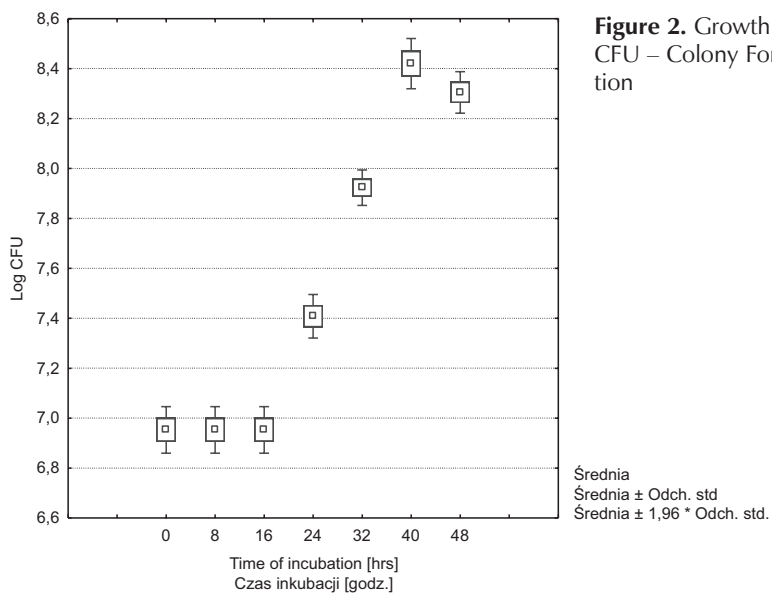

Figure 2. Growth curve of probiotic bacteria in product No 2. CFU – Colony Forming Unit; Time reflects the time of incubation

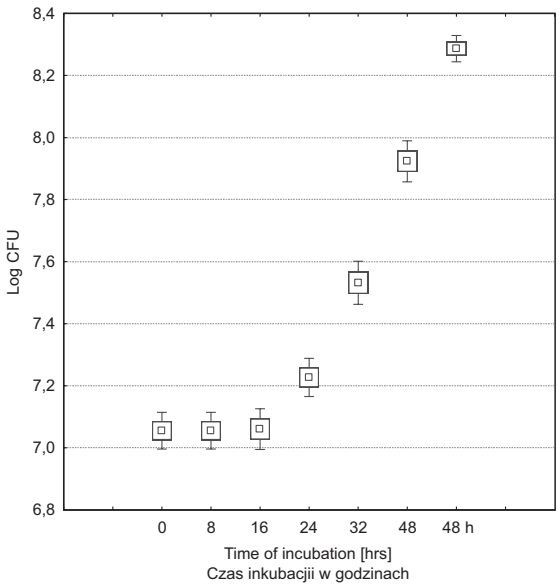

Figure 3. Growth curve of probiotic bacteria in product No 3. CFU – Colony Forming Unit; Time reflects the time of incubation

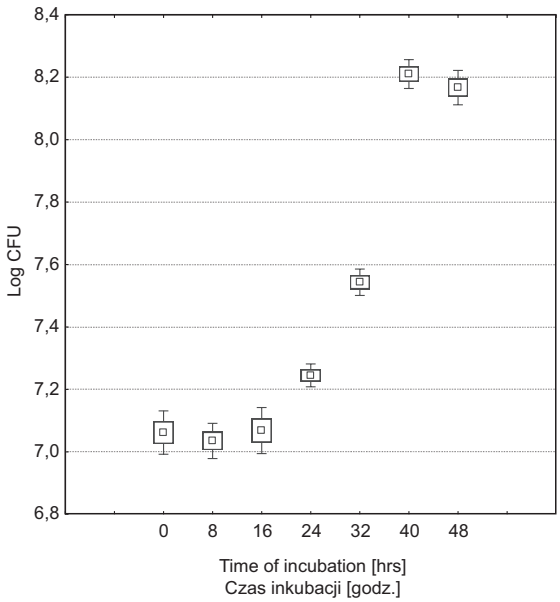

Figure 4. Growth curve of probiotic bacteria in product No 4. CFU – Colony Forming Unit; Time reflects the time of incubation

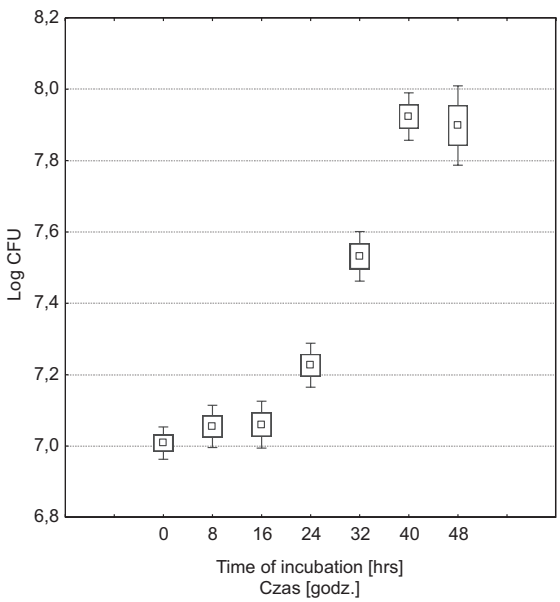

Figure 5. Growth curve of probiotic bacteria in product No 5. CFU – Colony Forming Unit; Time reflects the time of incubation

greatest value. These findings confirm the reports that growth and cell-cell interactions, may play an important role in modifying the population dynamics of bacteria [19] and show that particle like oligofructose, which works as a prebiotic to maintain high bacterial viability in the gastrointestinal tract [20, 21] is very effective *in vitro*. Moreover, one may speculate that probiotics containing more than one bacterial strain may be more effective in bacterial growth and development because of possible synergistic mechanisms.

Many reports indicated that there is poor survival of probiotic bacteria in commercially offered products when administrated to the human gastrointestinal system. Interestingly, we have noticed that Product No 1, which showed the greatest rate of microbial growth, was equipped with modern capsule made according to the MURE technology (Multi Resistant Encapsulation). This is an additional and important benefit of Product No 1, since before reaching the intestinal tract, probiotic bacteria must first survive in the gastric juice and then resist the effects of bile acids.

The effectiveness of probiotics in competitive inhibition of pathogenic microbial growth depends among others on their content, which in turn reflects viability and metabolic activities of probiotics [21, 22]. Testing kinetics of bacterial growth our study indirectly suggests that the best expected effect may be achieved when the preparation is reach in a variety of probiotic bacterial strains and additionally contains prebiotics.

Conclusions

1. Under anaerobic conditions of cultivation the fastest growth rate and the greatest value of CFU is observed for the bacteria found in probiotic products that contain numerous mixed strains.
2. Prebiotics strongly enhance microbial reproduction and growth.

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