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The factors determining effective probiotic activity – evaluation of survival and antibacterial activity of selected probiotic products: an *in vitro* study

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ABSTRACT

Introduction: The purpose of this study was to evaluate osmotic stress resistance and survival during incubation in hydrochloric acid of bacteria contained in various market products of paediatric probiotics.
Material and methods: In the study first phase, the test specimens were incubated in different concentrations of hydrochloric acid simulating the stomach environment of infants and older children. The second part of the study involved exposing the probiotic microorganisms to osmotic stress conditions (incubation in 4% NaCl solution). In both phases of the study, the decrease in the number of viable microorganisms during the experiments was determined on a logarithmic scale and the survival rate was calculated.
Results: The best survival rate of probiotic bacteria was found in probiotic specimens containing the greatest diversity of bacterial strains and produced by microencapsulation technology. The lowest survival rate was found in probiotic specimens containing a single probiotic strain produced by traditional technology. Unquestionable differences in the survival rate of probiotic bacteria in specimens with the same diversity of probiotic strains produced by different technologies were shown.
Conclusions: The highest possible therapeutic effect can be expected from probiotic market specimens containing the highest diversity of bacterial strains and produced by microencapsulation technology. This technology guarantees probiotic bacteria's greatest survival under negative acidic and osmotic stress conditions.

KEY WORDS: probiotic products, chloric acid, osmotic stress, survival of probiotic bacteria.

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INTRODUCTION

According to the FAO/WHO definition, probiotics are live microorganisms that, when consumed in adequate amounts, provide health benefits to the host [1]. The key to the effective action of probiotics is the colonization of the host gut by these microorganisms [2, 3]. Despite the growing knowledge of probiotics, enough attention is not always paid to the quality aspects; in addition, the choice is very large. Not many patients are motivated in their search for a probiotic by analysing the function and action of a particular strain of bacteria, the number of bacterial strains in a capsule, or the pharmaceutical technology used by the manufacturer. These are the essential factors determining the optimal performance of a given probiotic market product. The gut microbiota can even be considered a key "organ" of the human body due to its role in maintaining the balance between health and disease [4, 5]. It also plays an essential role in maintaining the proper homeostasis of the host organism thanks to its many metabolic functions. It consists of not inducing or inhibiting the immune response against microorganisms while identifying invasive pathogens and eliminating them by stimulating the appropriate immune response. Several mechanisms are

responsible for this, among which the correct structure and function of the intestinal epithelium, the regulatory mechanism of the immune response, and the proper recognition of individual bacterial antigens is particularly important [6, 7]. In turn, the occurring disorders in the functioning of the digestive system and intestinal barrier, metabolic, autoimmune, and mental disorders are often caused by intestinal microbiota disorders called intestinal dysbiosis [8]. The gut microbiota is mainly found in the small and large intestines, while the stomach was long considered sterile, especially due to its high acid production. In particular, the stomach was considered a "hostile place" for bacterial growth until *Helicobacter pylori* was identified [4, 5]. The pharmaceutical market offers a wide range of products containing various bacterial strains belonging to the so-called probiotics. These products include drugs, dietary supplements, and special food products. These products additionally contain agents that influence the growth of beneficial microorganisms. These factors are called prebiotics. The combination of a probiotic with a prebiotic is defined as symbiotic. Products offered on the market containing probiotic microorganisms differ in the variety and number of bacterial strains contained in them, the addition of substances influencing the development of beneficial bacteria, as well as the technology of their production [9, 10]. The environmental conditions existing in the various sections of the digestive tract determine the amount of live beneficial microorganisms reaching the site of their action. [11-13]. Traditionally, the stomach was thought to be a sterile organ, unsuitable for microbiota growth. However, the discovery of *H. pylori* disproved this concept. With the development of molecular techniques, an abundance of microbiota with high diversity has been found in the stomach. Moreover, various lines of evidence suggest that the gastric microbiota plays a critical role in the development and progression of gastric disease. The gastrointestinal microbiome plays an important role in various physiological and pathological processes [14]. The low pH of the stomach contributes to the partial sterilization of food reaching further parts of the gastrointestinal tract. At the same time, it is a protective mechanism against pathological bacteria [15, 16].

As we know, the environment of the stomach is not completely sterile. In recent years, bacteria have been detected living in these unfavourable conditions. Many bacterial pathogens, such as *Escherichia coli, Salmonella typhimurium*, and *H. pylori*, can avoid the acidic conditions of the stomach by developing adaptive mechanisms that allow these bacteria to survive in an acidic environment. Consequently, these bacteria can survive the acidic environment of the stomach and enter the intestine, where they can cause gastroenteritis [17].

In addition, the correlation between gastric ulcer disease and *H. pylori* bacteria has been known for years [18]. Several methods are used to improve the biological effect of administered probiotics. One of them is an attempt to grow stable acid-resistant *Bifidobacterium* strains isolated from humans and characterize their phenotypic features. The successful use of prolonged exposure to acid stress to improve the stability of human bifidobacteria indicates that this strategy may be useful in the future for producing low pH tolerant probiotic strains. These trials are currently in the research phase [19, 20].

In the case of probiotics, the effect of hydrochloric acid on the microorganisms in them is an adverse phenomenon reducing their survival rate. In our previous studies, we found reduced survival of probiotic bacteria under conditions simulating the human stomach environment for adult capsule probiotic products [21].

To protect the bacterial strains found in probiotic products, various types of technologies have been introduced to protect the microorganisms found in these specimens. These technologies involve protecting the bacterial strains in their specimens from the destructive effects of hydrochloric acid and other digestive and environmental factors. One technology for such protection is microencapsulation [22].

Microencapsulation is used to increase resistance and enhance the stability and survival of probiotic bacterial strains by protecting them from adverse physicochemical factors such as high temperature, oxygen, osmotic pressure, relative humidity, digestive tract enzymes, and finally, low pH prevailing in the stomach. Microencapsulation technology involves coating the bacteria with 2 protective coatings: protein and polysaccharide-hydrocolloid. This layer is designed to protect the bacteria from the destructive effects of gastric juice, which increases the survival rate of bacteria entering the intestinal tract lumen, i.e. their target site [23, 24].

Formulation of probiotics into microcapsules is a relatively new method of reducing cell death during their passage through the stomach. This method also offers the possibility of controlled release of bacterial cells in the intestinal tract lumen itself [25, 26]. The nature of this technology is based on the immobilization of bacteria in a polymer matrix that retains its structure in the stomach. Degradation and dissolution of the matrix take place in the intestinal lumen. In this way, the bacteria in an undisturbed state are expected to reach the site of action [25-27]. This technology is said to allow for the survival of most of the probiotic bacteria during their transport, storage, as well as passage through the gastrointestinal tract [28]. Previous studies have shown that microencapsulation provides higher protection for probiotics during storage or thermal processing. The method also increases the survival rate of probiotics during refrigerated storage compared to bacterial cells without protection. The chitosan coating was also shown to increase the survival rate of probiotics in vegan milk during refrigerated storage. Microencapsulation by external ionic gelation in alginate has

been found to be an effective technique applicable to improving probiotic survival during storage and under temperature-, pH-, and NaCl-induced stress conditions [29]. Among other things, our research is expected to show how effective microencapsulation technology is for probiotic products used in paediatrics. The pH of an adult's stomach is about 1. This value stabilizes at about 3 years of age. In younger children, the pH is higher. After the first year of life, the pH of a child's gastric juice is about 3. Probiotic products used in paediatrics contain both single bacterial strains and their mixtures. In addition to qualitative differences, these specimens also have differences in the amounts of live microorganisms contained in them. To this should be added the use of different technologies in their production. In medical practice, we are faced with the question of which probiotic specimens are the most biologically effective. Based on the available scientific literature, it should be noted that some of the most important criteria for the therapeutic suitability of pharmaceutical specimens containing live microorganisms are their survival rate at low pH simulation of the stomach environment and resistance to osmotic stress. It seems obvious, therefore, that the specimens of probiotics used in paediatrics present on our market should be tested to determine their therapeutic usefulness. In our study, we took into account the physiological specificities of the digestive tract of infants and children (different pH values of gastric juice compared to adults). Taking into account the above facts, our research model seems to be the most reasonable. Therefore, the aim of our study was to evaluate the bacterial survival of several commercially available probiotic products for children under osmotic stress and simulating a gastric juice environment. The study evaluated specimens of different qualitative compositions and produced with different technologies.

MATERIAL AND METHODS

A parameter measuring the resistance of bacteria to osmotic stress is their survival rate in an environment of high NaCl concentration. In our study, we evaluated the survival rate of microorganisms contained in market products (Table 1) of paediatric probiotics after a 12-hour incubation in a 4% NaCl solution. 4% NaCl was chosen based on the results obtained by Silva *et al.* [30], who indicated that the concentration of 4% was the most favourable. During the experiments, the number of colony-forming units (CFU) per mL after (CFUA) and before (CFUB) osmotic stress was determined by the plate dilution method in MRS agar plates. The survival rate (SR) was calculated as CFUA/CFUB [30].

In the second part of the research, an experimental model was created *in vitro* to imitate the *in vivo* acidic conditions existing in the stomach lumen of adults and children. Under these conditions (hydrochloric acid solution at pH-1.2, 2, and 3), the SR and CFU reduction on a logarithmic scale of bacterial strains found in various market products were examined after an hour's incubation. This time roughly corresponds to the residence time of the food in the stomach. The amount of CFU was examined using the plate method [31].

TABLE 1. Probiotic samples were used in the study

Product	Content of bacterial strains in the product	Production technology
Product A	*Lactobacillus acidophilus, Lacticaseibacillus casei, Lacticaseibacillus paracasei, Lactiplantibacillus plantarum, Lacticaseibacillus rhamnosus, Ligilactobacillus salivarius, Bifidobacterium bifidum, Bifidobacterium lactis, Bifidobacterium longum*	Microencapsulation
Product B	*Lactobacillus rhamnosus, Lactobacillus plantarum, Bifidobacterium breve*	Traditional
Product C	*Lactobacillus rhamnosus, Lactobacillus plantarum, Bifidobacterium breve*	Microencapsulation
Product D	*Saccharomyces boulardii*	Traditional

For the study, 4 commercially available probiotic products for children were used (Table 1):

- 2 products produced by microencapsulation technology;
- 2 products produced by traditional technology.

Probiotic products were incubated in hydrochloric acid at pH 1.2, 2, and 3 found in the gastric juice of adults and children between 1 and 3 years of age, respectively.

MICROORGANISM SURVIVAL STUDY

Survival studies of probiotic microorganisms were carried out on a model imitating *in vitro* acidic conditions in the stomach of children and adults. Under these conditions (a solution of pH 1.2 corresponding to the acidity of the gastric juice of an adult, and 2 and 3 corresponding to the acidity of children up to the age of 3 years), the probiotic products tested were incubated for a period of 90 minutes. After this time, the number of live microorganisms present in the solution was determined.

The study was conducted according to the method described in "Enumeration of Probiotic Microorganisms Exposed to Acid Conditions" [32, 33].

INVESTIGATION OF THE NUMBER OF CFU BACTERIA BEFORE AND AFTER EXPOSURE TO HYDROCHLORIC ACID

In the experiments conducted, the initial and final numbers of probiotic bacteria in the tested products were determined using the plate method [31].

STATISTICAL ANALYSIS

Statistica 12.5 (StatSoft, Kraków, Poland) was used for statistical analyses, including one-way ANOVA and Tukey's HSD test to assess significant differences ($p \leq 0.05$) between different products.

RESULTS

Based on our experiments, we found that the greatest reduction in the amounts of live microorganisms in the tested probiotic market products occurred when they were incubated in a hydrochloric acid solution at pH 1.2 (Table 2). These conditions correspond to the acidity of the stomach of an adult human or a child over 3 years old. An increase in the pH of the hydrochloric acid used for incubation results in a decrease in the reduction of live microorganisms in the solution and thus an increase in the survival rate of probiotic bacteria of the probiotic market products tested. Similar values of reduction in the number of living microorganisms were recorded in the case of osmotic stress induced by incubation of bacteria in the concentrated NaCl solution. Particularly interesting is the fact that there were significant differences in the reduction of the number of live bacteria and therefore their survival rate in the case of specimens produced by traditional technology compared to those in which microorganisms were microencapsulated. In the case of market products containing the same number of probiotic bacterial strains in their composition but produced with different technologies, the survival rate was significantly higher when microencapsulation technology was used. Our results also showed that the lowest survival rate and thus the greatest reduction in live bacteria was found when incubating a market product containing only one bacterial strain and produced using traditional technology without microencapsulation. We found the best survival rate results and the smallest reduction of live microorganisms in the multi-strain specimen produced with microencapsulation technology. In our study, we also noted differences between the survival rate in products produced with the same microencapsulation technology. In this case, the smallest reduction of live bacteria and the highest survival rate was found for the product containing the greatest variety of bacterial strains contained in it. The tendency to reduce live microorganisms applies both to their incubation in hydrochloric acid and under osmotic stress conditions induced by incubation in a 4% NaCl solution (Tables 3 and 4).

TABLE 2. CFU of bacterial strains contained in the tested probiotic market products [log 10(N)]

Product	CFUB	CFUA, pH = 1.2	CFUA, pH = 2	CFUA, pH = 3	CFUA, 4% NaCl
Product A	$8.52 \pm 0.12^{a*}$	7.43 ± 0.13^{a}	7.54 ± 0.14^{a}	7.62 ± 0.14^{a}	7.30 ± 0.13^{a}
Product B	8.48 ± 0.15^{a}	$5.36 \pm 0.10^{a,b}$	$5.62 \pm 0.10^{a,b}$	$5.84 \pm 0.11^{a,b}$	$5.62 \pm 0.10^{a,b}$
Product C	8.24 ± 0.11^{a}	$6.09 \pm 0.11^{a,b}$	$6.13 \pm 0.11^{a,b}$	$6.27 \pm 0.11^{a,b}$	$6.21 \pm 0.11^{a,b}$
Product D	8.30 ± 0.15^{a}	4.58 ± 0.08^{b}	4.65 ± 0.08^{b}	4.76 ± 0.09^{b}	4.70 ± 0.08^{b}

**Mean (n = 3) values denoted by the same letter in the horizontal line do not differ statistically significantly at 0.05 according to Tukey's test.*

TABLE 3. Reduction on the logarithmic scale of CFUs after 90 minutes of exposure to hydrochloric acid and 12 hours of incubation under osmotic stress (4% NaCl solution)

Product	HCl^-, pH = 1.2	HCl^-, pH = 2.0	HCl^-, pH = 3.0	OSC**
Product A	$1.09 \pm 0.02^{b*}$	0.99 ± 0.02^{b}	0.89 ± 0.02^{b}	1.24 ± 0.02^{b}
Product B	$3.12 \pm 0.06^{a,b}$	$2.86 \pm 0.05^{a,b}$	$2.63 \pm 0.05^{a,b}$	$2.88 \pm 0.05^{a,b}$
Product C	$2.15 \pm 0.04^{a,b}$	$2.09 \pm 0.04^{a,b}$	$1.98 \pm 0.04^{a,b}$	$2.05 \pm 0.04^{a,b}$
Product D	3.72 ± 0.07^{a}	3.63 ± 0.07^{a}	3.52 ± 0.06^{a}	3.58 ± 0.06^{a}

**Mean (n = 3) values denoted by the same letter in the horizontal line do not differ statistically significantly at 0.05 according to Tukey's test.*
***Osmotic stress conditions.*

TABLE 4. Survival rate (SR) of probiotic bacteria in market specimens after 90-minute exposure to hydrochloric acid and 12-hour incubation under osmotic stress (4% NaCl solution)

Product	HCl^-, pH = 1.2	HCl^-, pH = 2.0	HCl^-, pH = 3.0	OSC**
Product A	$0.87 \pm 0.02^{a*}$	0.88 ± 0.02^{a}	0.89 ± 0.02^{a}	0.86 ± 0.02^{a}
Product B	0.63 ± 0.01^{a}	0.66 ± 0.01^{a}	0.69 ± 0.01^{a}	0.66 ± 0.01^{a}
Product C	0.74 ± 0.01^{a}	0.74 ± 0.01^{a}	0.76 ± 0.01^{a}	0.75 ± 0.01^{a}
Product D	0.55 ± 0.01^{a}	0.56 ± 0.01^{a}	0.57 ± 0.01^{a}	0.57 ± 0.01^{a}

**Mean (n = 3) values denoted by the same letter in the horizontal line do not differ statistically significantly at 0.05 according to Tukey's test.*
***Osmotic stress conditions.*

DISCUSSION

Despite the growing understanding of the use of probiotics, including among doctors and pharmacists, in many situations, the choice of probiotics is still determined by price, package size, or advertising. A small number of patients are guided in their search for the right probiotic by analysing the function and action of a particular bacterial strain, the number of bacterial strains in a capsule or the pharmaceutical technology used by the producer. We must also remember the physiological differences in the digestive tract of infants, children, and adults [34]. These are essential factors for the optimal performance of a given probiotic market product, in particular just in children and infants [10]. It is well known that orally ingested food enters the stomach via the oesophageal tract. Enzymes and hydrochloric acid produced by the stomach are part of the gastric juice, which in children has a pH slightly higher than in adults. Under physiological conditions, food in the stomach is exposed to digestive juices and gastric motility for about 90 minutes [35, 36]. These extreme environmental conditions are a requirement for proper digestion and act as a barrier to microorganisms. The low pH of gastric juice disinfects ingested food and activates proenzymes produced in the gastrointestinal tract [37, 38]. In some situations, this stage of digestion proves detrimental to the human body. In cases where we want to intentionally implement health-promoting substances, the process of gastric digestion can reduce the expected effects of the administered specimens. An attempt to colonize the mucosa of the gastrointestinal tract with microflora administered in probiotics can serve as a typical example of these adverse interactions. To colonize further parts of the intestine, it is necessary to keep in mind the process of "passage" through the stomach and design the specimen in such a way that the microorganisms survive and can colonize within the intestine. Recently, several studies [37-39] have been conducted to clarify the resistance of probiotic microorganisms to hydrochloric acid [39]. The survival of microorganisms in the gastrointestinal tract has been shown to be largely dependent on the type of bacterial strain. Some authors [39, 40] have suggested that different bacterial strains will exhibit different functional behaviour after passing through the gastric tract. A test has even been developed to initially evaluate strains for use as probiotic cultures before selecting them for further *in vivo* studies [40]. In the large intestine, there are 1012 live microorganisms per gram of intestinal juice content. It is the most colonized section of the gastrointestinal tract [41]. The bacteria inhabiting the intestine make up a complex ecosystem that has a huge impact on the child's body [42]. Hence, providing an adequate supply of live microorganisms is very important. Maintaining the efficacy of probiotic bacteria is the most important challenge to address when developing functional food products. Several factors have been found to be responsible for reducing the viability of probiotics, including the acidity of the matrix, oxygen levels in the products, the presence of other lactic acid bacteria, and sensitivity to metabolites produced by other competitive bacteria. Several measures are taken to improve and maintain the viability of bacterial cells [43]. The second very important factor determining the therapeutic use of products containing probiotic microorganisms is their resistance to stress. This parameter indicates the viability and biological utility of a given bacterial strain. Studies are currently being conducted to clarify the cellular mechanisms responsible for bacterial stress resistance [44].

In the "in vitro" model, the indicator of stress resistance is the growth and survival of individual bacterial strains in concentrated NaCl solutions. In different studies [45, 46], the authors use different concentrations of, and exposure times to, concentrated salt solutions. In our study, we used a 4% NaCl solution, and the exposure time of the bacteria was 12 hours. Our results showed unquestionably that microencapsulation technology is extremely effective in protecting probiotic microorganisms both from hydrochloric acid and from stress caused by incubation in concentrated NaCl solution. Both in the case of single-component specimens and those containing several bacterial strains, the survival of bacteria was significantly longer. Undoubtedly, products produced by microencapsulation technology have significantly higher biological availability than those produced by traditional technologies [45, 46].

In our opinion, information on the proper criteria for selecting the best probiotic should be spread among both patients and professional health care professionals. On the packaging of probiotics, the most common information given is the concentration of microorganisms in a sachet or capsule. Taking into account the results of our research, we can conclude that this is not the most relevant information. From a pharmacological point of view, what is important is how many live microorganisms reach the site of their action, i.e. the intestine, and not their compactness in the specimen. As we can see, the passage of probiotic microorganisms through the stomach can reduce their amount by up to 1000 times. The situation is similar for protection against stress induced by incubation in a concentrated NaCl solution. Our study also suggested that the survival rate of probiotic microorganisms can depend on the age of the child. This is related to the different hydrochloric acid content of gastric juice depending on the age of the child. The youngest children are the most sensitive to administered probiotics. The low survival rate of microorganisms in products produced by traditional technologies undoubtedly contributes to weaker colonization of the intestine and lower effectiveness of their action. In the case of stress resistance induced by incubation in concentrated NaCl solution, the age of the patient is irrelevant. Stress resistance is associated with better survival of probiotic microorganisms during transport or storage. Thus,

undoubtedly, the stress resistance of microorganisms is a determinant of their viability and therapeutic usefulness. Resistance of *Lactobacillus* bacteria to osmotic stress in the works of other authors was evaluated by determining their growth in MRS (as a control) with the addition of NaCl (2-8%). The most resistant strains were *Lactobacillus gasseri* CRL1509, *L. rhamnosus* CRL1332, and *L. reuteri* CRL1327 [30]. Studies by other authors have found that bifidobacteria are highly heterogeneous in their tolerance to these stress factors such as low pH, oxygen, and osmotic stress. This has led to increased interest in the physiology of stress in these bacteria, and many studies have been undertaken to understand the molecular mechanisms underlying their stability and resistance [47, 48].

Based on the results of our study, when choosing the appropriate paediatric probiotic, the first and foremost consideration should be their production technology. It seems that the mere amount of bacteria in a probiotic product declared by the manufacturer is of secondary importance. An equally important factor determining the possible therapeutic usefulness of marketable paediatric probiotic products is their qualitative composition. Our studies have also shown better survival of microorganisms contained in complex products. The cellular mechanism of this phenomenon has not yet been explained. We know that different bacterial strains have different resistance to both hydrochloric acid and stress conditions induced by incubation in concentrated salt [49]. It can be assumed that the survival rate of complex products is that of the most resistant microorganism included in the market product. However, mechanisms of metabolic interaction between the individual bacterial strains included in a given probiotic cannot be excluded either. In our further research, we plan to perform studies of both survivals in hydrochloric acid and resistance to stress induced by incubation in concentrated NaCl solutions of most microorganisms available in market products.

CONCLUSIONS

The microencapsulation technology used in the production of some probiotic products is effective in protecting probiotics from the destructive effects of hydrochloric acid found in the stomach. This method is also effective for protecting microorganisms from the destructive effects of stress caused by incubation in a concentrated NaCl solution. In our study, we also showed that the survival of microorganisms under conditions simulating gastric juice and stress conditions during incubation in concentrated NaCl solution also depends on the diversity of bacteria contained in market products. The molecular mechanisms leading to increased resistance of probiotics containing different bacterial strains to hydrochloric acid and stress require further study.

DISCLOSURE

The authors report no conflict of interest.

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AUTHORS' CONTRIBUTIONS

MB, HS prepared concept of an article. MB, HS, JP collected data, conducted analysis and wrote the article. All authors critically revised it and approved the final version of publication.