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Using real-time polymerase chain reaction for taxonomic and quantitative analysis of multicomponent probiotics that are used in pediatrics

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The methods for determining the qualitative and quantitative composition of multicomponent probiotics play an important role in their development and investigation. Both taxonomic identification and quantitative analysis of such multisymbiosis components can be significantly accelerated with application of modern methods based on the detection and identification of DNA sequences by polymerase chain reaction (PCR). One of the main requirements for this method is the purity of the isolated DNA and the absence of polymerase chain reaction inhibitors in its content.

In the course of study, we developed a method for using polymerase chain reaction in real time for qualitative and quantitative determination of the species composition in multicomponent probiotic preparations containing representatives of 18 species of genera *Bifidobacterium, Lactobacillus, Propionibacterium*, *Lactococcus, Streptococcus* and *Acetobacter*. This method allows rapid and accurate research of samples in the presence of various inhibitors of polymerase chain reaction.

No conflict of interest was declared by the authors.

Key words: real-time polymerase chain reaction, DNA, multicomponent probiotic preparations, microorganism.

Introduction

Last years, there has been persistent interest of the scientific community to the exploration of the human microbiome (the totality of microbiocenoses colonizing all human body surfaces that contact with the environment, including skin, respiratory system, gastrointestinal tract and urogenital system) [5,7,8]. Large number of evidences show that the microbiome influences different processes of the human organism, including behavior and brain biochemistry [7,13,20].

The microbiome is playing a special role in the child health. It is well known that microflora created on the first year of life forms a fundament for maintaining child health with normal growth and development. It should be noted that the progress in the field of the microbiome study changed deep-rooted notions about its role in maintaining children health. It turned out that the microbiome formation may begin still before the childbirth due to specific placental microbiome, which includes microorganisms from different female biotopes [8,16,21].

Therefore, the great importance of microbiota for the child health is convincingly proven; however, under present-day conditions, the pattern of initial microbe colonization critically changed. This, on a large scale, led to the worsening of reproductive health of the young generation, heightened contingent of women with perinatal risk factors, unreasonable medicamentous therapy and so on [5,7,8,20,21]. That is why prophylactic measures, which promote formations of healthy microbiome and prevent its pathological changes in both woman and her child, play a fundamental role in establishing and maintaining child health.

Recognition of the microbiome importance for the child vital functions has led to a wide usage of probiotics in the practice of neonatology, pediatrics and gynecology; living microorganisms of probiotics positively influence human health due to rehabilitation of microflora.

In particular, for today a great positive medioprophylactic experience has been accumulated in the usage of multiprobiotics of Symbiter® series for prophylaxis and elimination of dysbiotic disturbances in women during pregnancy and puerperal period, as well as in all-age children beginning from neonate period. Long-standing practice of using multiprobiotics showed their good tolerance and safety, even when applied to small premature infants [8,21].

The development and manufacture of multicomponent probiotics require continual control of their quality and quantity composition. Standard microbiological approaches, such as isolation of separate cultures and their taxonomic identification based on morphological and biochemical properties, do not always allow correctly appreci-

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ating various microbial taxa in the content of multisymbioses. Moreover, to a large degree such methods are time-consuming and labor-intensive. Not only taxonomic identification but also quantitative analysis of such multisymbiosis components can be significantly accelerated with application of modern methods based on the detection and identification of DNA sequences by polymerase chain reaction (PCR), and especially its modification — quantitative polymerase chain reaction in real time (RT-PCR).

One of the main requirements for accurate quantitative evaluation of the microbiological composition of various sour-milk products, as well as multisymbioses cultivated in milk-rich nutrient media, is the purity of the isolated DNA and the absence of polymerase chain reaction inhibitors in its content. The presence of proteases, calcium ions (Ca^{2+}) and various polysaccharides [1] complicates the reaction, and makes it impossible to carry out in some cases.

Recently, the authors developed a new series of multicomponent probiotics of the series Symbiter® forte that contain, besides probiotic biomass, also smectite gel (bentonite) [2–4]. High smectite adsorptive activity is an additional factor that also complicates the application of the method.

In the course of our work, a method for qualitative and quantitative evaluation of the taxonomic composition of multicomponent symbioses of probiotic bacteria was developed. Such multicomponent symbioses are the basis of multispecies probiotics, including the multiprobiotics «Symbiter® Forte-M», «Symbiter® Forte-omega» and «Symbiter® Forte with Propolis» (manufactured by Scientific-production company OD Prolisok). For this purpose, because of high content of polysaccharides, smectite and other PCR inhibitors in samples, the method of DNA separation and purification from bacterial cells was modified. In addition, an approach for quantitative estimation of the species composition of multiprobiotics was developed with the use the RT-PCR method [6].

Materials and methods

To isolate DNA from bacterial cells, the last were previously washed from the casein remnants, calcium ions, smectite and exopolysaccharides. Thereto, the contents of a single package /sachet of the multiprobiotic were diluted in a 2:3 ratio with 0.1N NaOH solution, and 450 μl of the sample were then centrifuged for 10 min at 10,000 rpm. (MiniSpin Eppendorf, Germany). The precipitate was diluted in 0.5 ml of TE buffer (pH 8.0) and as well centrifuged. The washed precipitate was again diluted with 250 μl of TE buffer (pH 8.0) with addition of lysozyme (5 μg/ml) and incubated for 1 hour at 37°C. The bacterial cell lysis was performed by adding 0.5 ml of lysis buffer BQ1 (Macherey-Nagel, Germany) and incubating for 1 hour at 70°C. Then the equal volumes of chloroform were added to the samples that were left for 15 minutes with gentle periodical mixing. Phase separation was carried out by centrifugation for 10 minutes at 10,000 rpm. The upper transparent phase was carefully sampled in a clean tube and equal volume of isopropanol was added. After careful mixing and incubation for 15 minutes at room temperature DNA was sedimented by centrifugation (10 minutes at 10,000 rpm). The precipitate was successively washed with 100% and 70% ethyl alcohol solution, with respective centrifugation for 10 minutes at 10,000 rpm. The washed precipitate was further dried and stored at -20°C; it was diluted in 200 μl of TE buffer immediately before analysis.

Primers were selected using PrimerBlast program (http://www.ncbi.nlm.nih.gov/tools/primer-blast) in accordance with the rules of molecular design, based on complete genome sequencing data from the GenBank database. Thus, species-specific primers (Table) for *Lactobacillus casei, Lactobacillus salivarius, Bifidobacterium adolescentis and Bifidobacterium bifidum* were created against the *dnaA* region (1..1500). This gene encodes the chromosome replication initiation protein, characterized by high species-specificity, is present in all strains and has no repeats in genome. This allowed us to precisely quantitatively analyze bacterial cells of the above species.

The species-specific primers (Table) for *Bifidobacterium longum* subsp. infantis were created against the β-galactosidase gene (400500..402500). The absence of this gene repeats in the bacterial genome allowed us to perform precise quantitative analyzes of Bifidobacterium longum subsp. infantis cells in the sample.

Using Blast program (http://www.ncbi.nlm.nih.gov/blast), comparative analysis of the selected oligonucleotide primers was performed to exclude fragments that were homologous to DNA of related and unrelated species. This analysis was performed against the full bacteria genome sequences available in GenBank.

To identify all other indicated species (Table) we used primers made during our previous studies [5].

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Table

Used primers and sizes of appropriate amplification products for every tested species in multisymbiosis

Species	Pair of primers	Amplicons (bp)
B. adolescentis	F 5'-GTGGCTGATAACACGACAACAGATCC-3' R 5'-TTTTGAAGGCGGGGAAGATGTCCT-3'	268
B. bifidum	F 5'-ACAAGAGCTGGCTTGAAGGAGTCGTA-3' R 5'-ATGTAGGATTCCTGAGCCAGATCG-3'	304
B. breve	F 5'-CCGGATGCTCCATCACAC-3' R 5'-ACAAAGTGCCTTGCTCCCT-3'	288
B. longum	F 5'-TTTCTATTGAACAGACACAGGTTTGCCC-3' R 5'-AAACTGATTTGCCGATTTTGCC-3'	268
B. longum subsp. infantis	F 5'- TGGGGTATTATCAACCCGGC -3' R 5'- CGTCAACGATTCCAACCACG -3'	284
L. acidophilus	F 5'-TGCAAAGTGGTAGCGTAAGC-3' R 5'-CCTTTCCCTCACGGTACTG-3'	207
L. brevis	F 5'-TTTGACGATCACGAAGTGACCG-3' R 5'-GCCTTGAGAGATGGTCCTC-3'	495
L. casei	F 5'-GAAACGTGGACCTGCTGTTG-3' R 5'- CAGCATCGGCTTTATTCCGC-3'	258
L. fermentum	F 5'-AAGAATCAGGTAGTCGAAGTG-3' R 5'-GCCTTGAGAGATGGTCCTC-3'	147
L. helveticus	F 5'-GAAGTGATGGAGAGTAGAGATA-3' R 5'-CTCTTCTCGGTCGCCTTG-3'	179 та 429
L. gasseri	F 5'-GAGTGCGAGAGCACTAAAG-3' R 5'-CTATTTCAAGTTGAGTTTCTCT-3'	198 та 423
L. plantarum	F 5'-GCCGCCTAAGGTGGGACAGAT-3' R 5'-TTACCTAACGGTAAATGCGA-3'	283 та 512
L. salivarius	F 5'- TTCTCGCTTTAAATGGGGGCT -3' R 5'- GCTGGATTTGCCACTGACTTT -3	271
L. lactis	F 5'-GTACTTGTACCGACTGGA-3' R 5'- GGGATCATCTTTGAGTGAT-3'	163
P. acidopropionici	F 5'-CTGGAAGCTGGCCGTCG-3' R 5'-CTTGCAACACAACACATTAC-3'	304
P. freudenreichii ssp. shermanii	F 5'- GACTCGGGCTACAGACAGTG -3' R 5'- TTCTCGCGCGTGTAGTCATT -3'	171
S. salivarius subsp. thermophilus	F 5'-CACTATGCTCAGAATACA-3' R 5'-CGAACAGCATTGATGTTA-3'	968
A. aceti	F 5'-TGGTACGGCATTCCGGG-3' R 5'-ACGCTCAATGGACCACTG-3'	285

Quantitative analysis of the multiprobiotic composition was performed with real-time PCR using DT-322 amplifier (DNA-technology, Russia). Composition of the PCR mixture: PCR buffer (Amplisens, Russia) — 10 μl, nucleotide mixture — 2.5 μl, forward and reverse primers — 1 μl, Taq polymerase (Amplisens, Russia) — 2.5 μl, DNA — 8 μl The reaction was visualized by addition of ZUBRgreenI (Belarus) to the PCR buffer at a final concentration of 1x. The reaction was carried out according to the following program:

1. 1 cycle: 94°C — 5 minutes;
2. 70 cycles: 94°C — 15 s
   58°C — 30 s (fluorescence was read)
   72°C — 1 minute 30 s;
3. 1 cycle: 72°C — 5 minutes;
4. Melting curve (from 94°C to 50°C in a 1°C step and 45s delay for fluorescence read);
5. Storage at 10°C.

Results and discussions

Correct methods for determining the qualitative and quantitative composition of multispecies symbioses play an important role in their development and investigation. Such methods are particularly important for controlling the manufacture of both probiotic and multiprobiotic products that contain a broad spectrum of bacteria with declared probiotic effects. Our long-term studies have shown that close symbiotic relationships between members of multicomponent probiotics greatly complicate the possibility of using standard microbiological methods for the identification and quantification of different bacterial species that frequently form common colonies on nutrient medium [2,4,7,8]. Moreover, differential cultivation and microscopy are time-consuming methods and do not always allow to carry out precise quantitative analysis and fully

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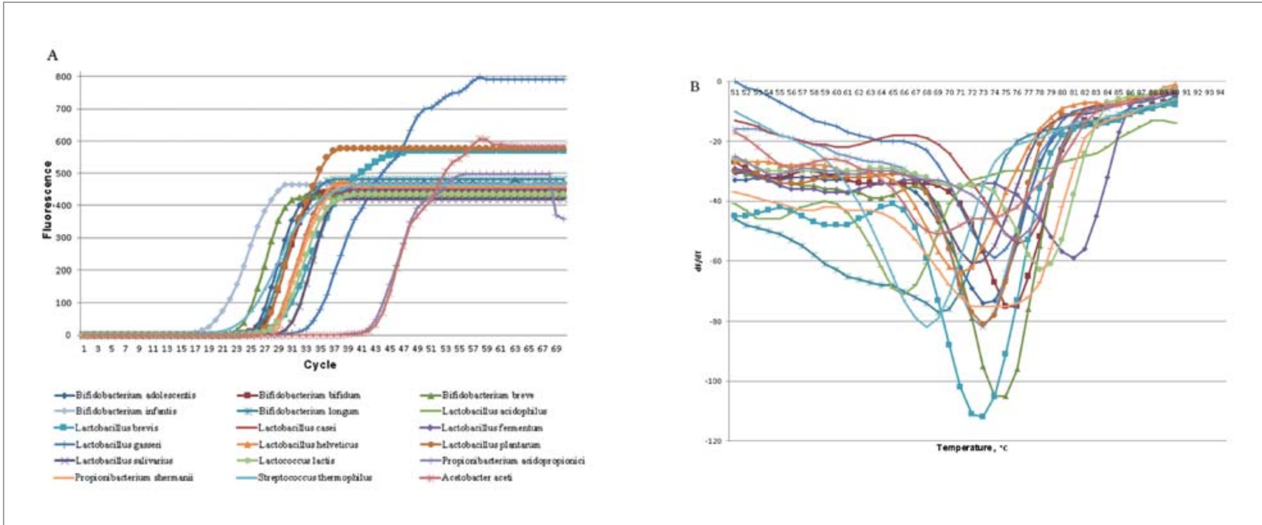

Fig. 1. Results of RT- PCR for Symbiter® Forte-M. A — Fluorescence dependence on cycle. B — Melting curves of amplification products (dependence of rate of fluorescence change on temperature)

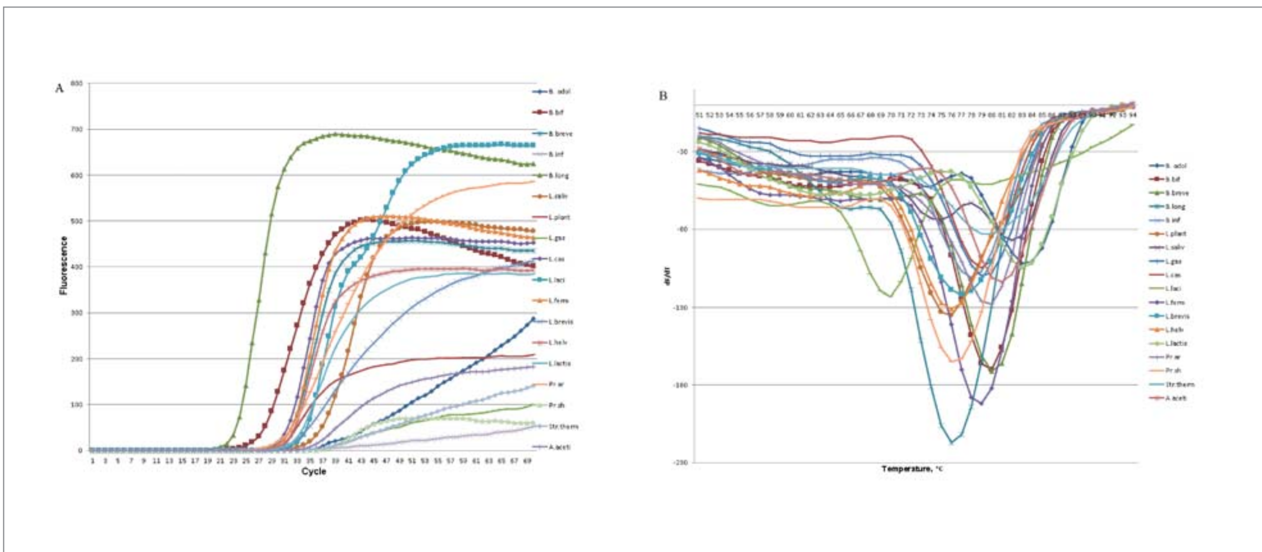

Fig.2. Results of RT- PCR for Symbiter® Omega. A — Fluorescence dependence on cycle. B — Melting curves of amplification products (dependence of rate of fluorescence change on temperature)

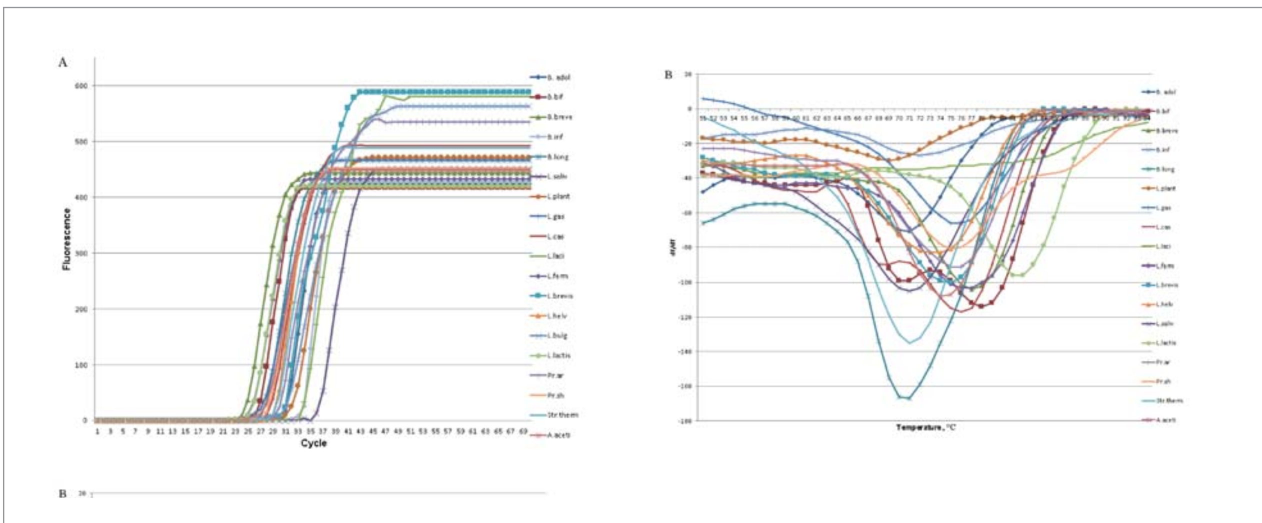

Fig. 3. Results of RT- PCR for Symbiter® Forte with propolis. A — Fluorescence dependence on cycle. B — Melting curves of amplification products (dependence of rate of fluorescence change on temperature)

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separate members of a persistent mutualistic symbiosis into definite species, and, furthermore, strains. A perspective possibility for solving this problem is in using molecular biology methods, such as PCR and its modifications.

Studies *in vitro s*howed that polymerase chain reaction is based on multiple selective replication of specific DNA regions by specific enzymes [6]. Thus, only the area, which satisfies the given conditions, is copied (even when present in the sample in extremely low concentrations). Such selectivity and specificity of PCR can be achieved through carefully selected primers (usually short, chemically synthesized DNA molecules, 20–30 nucleotide residues in length) that limit the multiplicated / copied / amplificated sequence on both sides. Nowadays this method is widely used to identify and detect species-specific genes. However, it should be noted that used enzymes require accurate maintenance of physical and chemical conditions for their effective functioning. Thus, the polymerase chain reaction itself is extremely sensitive to the presence of a wide range of inhibitors such as, for example, calcium ions, phenols, alcohols, polysaccharides, some proteins etc. Such sensitivity imposes certain requirements not only to the purity of DNA obtained but in total to the process its isolation.

Multiprobiotics «Symbiter® Forte», used in this study, are complex preparations characterized by the rational combination of living biomass of probiotic bacteria with smectite gel of deep cleaning and other biologically active products of natural origin. Introduction of smectite gel into the multiprobiotic reasonably supplements all spectrum of its properties with new physiological activities and significantly increases shelf life of the live probiotic preparation due to protective effect on anaerobic bacteria [3,7,8]. The bacterial base of this probiotic series is the multispecies symbiosis of the following bifidobacteria species: *Bifidobacterium bifidum, B. longum, B. breve, B. longum* ssp. infantis, *V. adolescentis*; lactobacillus species: *Lactobacillus acidophilus, L. casei, L. brevis, L. gasseri, L. helveticus, L. plantarum, L. fermentum, L. salivarius*; lactic acid streptococci species: *Lactococcus lactis* and *Streptococcus salivarius* ssp . thermophiles; and propionic acid bacteria species: *Propionibacterium freudenreichii* ssp. shermanii and *P. acidipropionici*. As mentioned above, these preparations contain a number of other biologically active products of natural origin. Thus, Symbiter®Forte-M additionally contains the suspension of wheat germs, Symbiter® Omega contains flaxseed oil and wheat germ oil, and Simbiter® Forte with propolis includes propolis [3,7].

The presence of sorbent, suspension of wheat germs, fats and propolis in the multiprobiotics of the Symbiter® forte series greatly complicates the processes of bacterial cells washing with further separation and purification of DNA. In addition, smectite was shown to increase significantly synthesis of exopolysaccharides by the multisymbiosis members.

The primary task of our work was to develop a unified method for washing microbial cells and isolating pure DNA suitable for subsequent PCR. Some of the main components of multiprobiotic preparations may complicate DNA extraction and /or act as PCR inhibitors. Therefore, the presence of milk and high level of polysaccharides, including those associated with the bacterial cell wall, almost eliminates the separation of this cells from the nutrient medium by centrifugation. In addition, the high content of short chain fatty acids in the multiprobiotics «Symbiter® Forte» causes casein to precipitate. In order to dissolve such milk proteins we increased pH of the sample to 8.5–9.5. Subsequently, the calcium ions were bound with EDTA and pH level was adjusted to 8.0.

Bacterial cells are characterized by a high content of polysaccharides, which complicates both further cell lysis and DNA extraction. During our previous studies, DNA was isolated usually in a mixture with these polysaccharides, making it impossible to perform PCR. The problem was solved by additional processing of the sample with lysozyme, which enzymatically separated the polysaccharides from the cell wall, so that further centrifugation allowed separating bacterial cells. During further extraction of DNA from bacterial cells, it is advisable to use chloroform for additional purification (not obligatory) of the protein residue lysate (including lysozyme itself) and polysaccharides. Obtained purified DNA has been subsequently used for qualitative and quantitative evaluation of the bacterial composition of multiprobiotic preparations.

There are approaches for identifying representatives of genera *Bifidobacterium Lactobacillus, Propionibacterium, Lactococcus, Streptococcus and Acetobacter* with PCR methods. They are mainly based on the amplification of specific regions of the gene cluster encoding 5S, 16S, 23S subunits of the bacterial ribosome, spacers between them and *lacZ* gene [9–14]. High conservatism of the ribosomal operon allowed develo-

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ping primers to identify not only certain species and subspecies, but also the genera of probiotic bacteria. At the same time, such conservatism may lead to false-positive results for some strains, and different copy numbers of these genes not only in different species, but also in different strains, complicate their use for reliable quantitative analysis of bacterial cells in samples.

Previously, we proposed a method based on polymerase chain reaction for determining DNA in probiotic bacteria of the genera *Bifidobacterium, Lactobacillus, Propionibacterium, Lactococcus, Streptococcus* and *Acetobacter*. This method used primers against the region of ribosomal operon coding 16S sub-particles of ribosomal RNA [15]. Disadvantage of this method is high conservatism of 16S rRNA gene, which may complicate the process of species identification and accurate quantitative analysis of the bacterial content. Genotypes of *Bifidobacterium longum* subsp. longum and *Bifidobacterium longum* subsp. infantis are highly homologous [16]. This makes almost impossible using highly conservative ribosomal operon genes as targets for PCR identification. Proposed in the literature primers for identifying *Lactobacillus casei, Lactobacillus salivarius, Bifidobacterium adolescentis* and *Bifidobacterium bifidum* were also ineffective, especially for quantitative analysis. Therefore, for the above bacterial species, we have designed and synthesized appropriate pairs of specific primers (see Methods and Table for more details).

The results of Real-Time PCR for Symbiter® Forte-M, Symbiter® Omega and Symbiter® Forte with propolis are shown in Figures 1, 2 and 3, respectively. The figures show the dependence of fluorescence on cycles (A) and melting curves of amplification products (B). Polymerase chain reaction passed successfully for all samples with sufficient efficiency. The proposed method for DNA obtaining from these samples allows unifying such studies. It has high repeatability and corresponds to the basic requirements to standard qualitative, and quantitative analyzes of multisymbiosis components in samples with high content of PCR inhibitors. In the case of Symbiter ® Omega, PCR efficiency was somewhat worse (Fig. 2), which can be explained by the presence of oil in the samples. However, the results obtained for this preparation were reliable and characterized by high experimental repeatability.

Quantitative calculation of the content of individual members of multisymbioses in the studied samples was carried out using the methods we developed earlier [5]. The results obtained by the PCR method are in full agreement with the data obtained by microbiological research methods.

Conclusions

Thus, in the course of study, we developed a method for using polymerase chain reaction in real time for qualitative and quantitative determination of the species composition in multicomponent probiotic preparations containing representatives of 18 species of genera *Bifidobacterium, Lactobacillus, Propionibacterium, Lactococcus, Streptococcus* and *Acetobacter*. This method allows rapid and accurate research of samples in the presence of various inhibitors of polymerase chain reaction.

No conflict of interest was declared by the authors.

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УВАГА! ВАЖЛИВА ІНФОРМАЦІЯ!

Зміни в оформленні списку літератури

Перший (основний) варіант наводиться одразу після тексту статті, джерела подаються в алфавітному порядку. Список літератури наводиться латиницею. Джерела українською та російською мовами наводяться у перекладі на англійську мову, але так, як вони показані та реєструються на англійських сторінках сайтів журналів. Якщо джерело не має аналога назви на англійській мові — воно наводиться у транслітерації. Таке оформлення списку літератури необхідне для аналізу статті та посилань на авторів у міжнародних наукометричних базах даних, підвищення індексу цитування авторів.

Другий варіант повторює перший, але джерела українською та російською мовами подаються в оригінальній формі. Цей варіант необхідний для оформлення електронних версій журналу на українській і російській сторінках, цитованості у кирилічних наукометричних базах.

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