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Microbiological analysis of a synbiotic gut health supplement capsule by metagenomics

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ABSTRACT: Probiotics i.e. the use of living microorganisms for health benefits has been known since ancient times. There is an increasing awareness and concern among the consumers, regulators, medical fraternity, researchers, etc. about these products/supplements formulated to contain single or more beneficiary microorganisms. These microbes may be beneficial independently or synergistically for an individual's health by protecting against pathogens, reduce metabolic disorders and boost immunity. Nowadays, next generation probiotics are being developed with microbial strains isolated from humans (gut or stool) or genetically engineered microbes for treating specific health condition. Gut microbiome studies have also helped in developing probiotics with novel microorganisms. Synbiotics are products containing prebiotics and probiotics. Food safety agencies provide guidelines to characterize and identify the microbial strains and their role in such products. Traditional, rapid identification and molecular methods as well as clinical trials are included to justify the efficacy and safety of the probiotics and related products. We report here the 16S metagenomics study on a synbiotic supplemental capsule available online containing twenty-five bacterial species. These results reveal the presence of a consortia of bacteria containing many species not mentioned on the product label. It also provides some insights for testing of these products using molecular tools for regulatory purpose.

Keywords: Probiotics; Metagenomics; Prebiotics; Synbiotics; Health supplements.

1. INTRODUCTION

According to Food and Agriculture Organization (FAO) and World Health Organization (WHO) probiotics are defined as "live microorganisms when administered in adequate amounts confer a health benefit on the host". It also provides the guidelines for evaluation of such products [1]. Dahi and fermented milk products have been mentioned in Vedas and Upanishads about 6000 B. C. and known in other civilizations. The average growth of probiotic market in India was about 19.80 percent during 2014-2019 which is growing exponentially [2, 3]. The minimum criteria to be applied for a probiotic strain include: identification using recognized scientific methods, valid current nomenclature, a retrievable strain designation, deposition in international culture collection, safe for its intended use and provide health benefit as demonstrated by at least one scientific study based on accepted scientific standards or regulations. The probiotic strain(s) must be present in sufficient viable numbers throughout the shelf life and should be manufactured according to applicable good

manufacturing practices to assure safety, purity, and stability. The label should communicate essential information on product contents (specific strains, level of live probiotic delivered at end of shelf life, and statements about health benefits to the consumer [4]. Recent research by Chattaraj et al. [5] showed the prospective nature of probiotics to combat cancer and its management. The mechanisms that underly the antioncogenic effect of probiotics include: immune modulation, regulation of gastrointestinal tract microbiota, and production of anticancer compounds. The challenges posed for the use of probiotics for this purpose includes identification of specific strains with antioncogenic activity, effective dosage, duration of treatment, biosafety as well as antibiotic resistance profile of the microorganism(s). Therefore, to enhance the antioncogenic potential of probiotic for the heterogenous types of cancers it is essential to test the formulations/strains for their clinical efficacy, effect on patient's microbiome, by the use of microbial-derived components, genetically modified microbes, and synthetic biology [5]. The various aspects of prebiotics, probiotics and synbiotics for the management of diseases has been described by Palai et al. [6]. Various species of *Bacillus*, *Lactobacillus*, *Bifidobacterium*, yeasts, etc. are the most common microorganisms used in probiotics. A comprehensive list of probiotic microorganisms (bacteria and fungi) commonly used in probiotic formulations has been summarized [7]. The term 'prebiotics' was coined by Gibson and Roberfroid in 1995, they are substances that are "selectively fermented ingredient that allows specific changes; both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" [8, 9]. Plant products like inulin, fructo-oligosaccharides, lactulose, dietary fiber and gums are widely used a prebiotic. Fructo-oligosaccharides (FOS), mannanoligosaccharides (MOS), xylo-oligosaccharides (XOS), etc. are other commonly used prebiotics. The term "synbiotics" is used for products that contain prebiotics and probiotics, which in conjunction provide better health benefits in treatment of gastrointestinal (GI) or other disorders [6]. Psyllium husk (Isabgol in Hindi) (95%) is used as a prebiotic, which is well-known for its mucilaginous properties, glycosides, proteins, polysaccharides, vitamin B1, choline and fiber content (80%). It is also commonly used for the treatment of constipation (laxative), colon cancer, diabetes, diarrhea, inflammatory bowel disease, irritable bowel syndrome (IBS), hypercholesterolemia, ulcerative colitis, etc. [10]. There are various synbiotic formulations available in the market, and its necessary to asses them at the molecular level. Hence, the objective of the research was to determine the diversity of microbial strains present in a synbiotic supplemental capsule by 16S metagenomics analysis and compare with the microbes indicated on the label.

2. MATERIALS AND METHODS

2.1. Synbiotic product

A synbiotic (probiotic + prebiotic) product from India was purchased online, and stored in cool and dry place away from sunlight (without refrigeration) as per manufacturer's instructions. It was analyzed before the expiry date on the label. The product label mentioned it as a spore based formulation of twenty-five bacterial strains and prebiotic (psyllium husk) as in Table 1.

2.2. 16S rRNA Metagenomics library preparation

The ingredients of the capsule were suspended in sterile Phosphate Saline Buffer (PBS) and mixed thoroughly. This suspension was directly used for DNA isolation.

2.3. Sample Preparation and DNA Extraction

Microbial genomic DNA was extracted from the environmental or biological samples using a

QIAamp® DNA Mini Kit kit (cat. nos. 51304) following the manufacturer's protocol. The quality and concentration of extracted DNA were assessed using an Epoch spectrophotometer and a Qubit fluorometer. DNA samples with an A260/A280 ratio of 1.8–2.0 were considered for downstream analysis. The DNA integrity was evaluated by 0.8% agarose gel electrophoresis.

Table 1. Specifications of the synbiotic product (spore based formulation) as per the label*.

Bacterial species (25)	Bacterial count	Health benefits	Remarks
Arthrobacter agilis *A. globiformis* *Azospirillum brasilens* *A. lipoferum* *Azotobacter chroococcum* *A. paspali* *Bacillus amyloliquefaciens* *B. clausii* *B. coagulans* *B. indicus* *B. licheniformis* *B. polymyxa* *B. pumilis* *B. subtilis* *Brevibacillus brevis* *Clostridium butyricum* *Enterococcus faecium* *Lactobacillus spp.* *L. lactis* *Leuconostoc mesenteroides* *Paenibacillus macerans* *Streptococcus thermophilus* *Streptomyces cellulosae* *S. fradiae* *S. griseoflavus*	Approx. 100 billion CFU/g	1. Relief from bloating and gas 2. Improves bowel movement 3. Reduces side-effects of antibiotics 4. Enhances gut barrier function 5. Reduces IBS syndrome 6. Reduces abdominal pain	Prebiotic: Psyllium Husk 95%

*Note: All bacteria mentioned are not spore-forming e.g., *Enterococcus, Lactobacillus, Leuconostoc, Streptococcus,* etc.

2.4. 16S rRNA Gene amplification

The amplification of the 16S rRNA gene was performed using the Ion 16S Metagenomics Kit (cat. nos. A26216, Thermo Fisher Scientific, MA, USA), which employs two distinct primer sets targeting the hypervariable regions of the 16S rRNA gene in prokaryotes. Primer Set 1 amplifies regions V2-4-8, while Primer Set 2 targets regions V3-6, 7-9. DNA sample was amplified separately using these two primer sets to ensure comprehensive coverage of the bacterial diversity. The PCR amplification was carried out in a thermal cycler (Veriti™ Thermal Cycler, Thermo Fisher Scientific, MA, USA) following the parameters recommended by the kit's protocol: Hold 95^{o}C, 10 min; Cylces: 25 cycles (95^{o}C 30 sec.; 58^{0}C 30 sec.; 72^{o}C 20 sec.); Hold 72^{o}C 7 min; Hold 4^{o}C to ∞.

2.5. Pooling and purification

After amplification, the PCR products from the two primer sets were pooled for each sample. The

pooled products were subjected to purification using Agencourt AMPure XP reagent (Beckman Coulter, CA, USA) according to the manufacturer's instructions. Briefly, the pooled products were mixed with the AMPure XP reagent and allowed to bind to the magnetic beads. The beads were washed multiple times to remove any impurities, and the purified amplicons were eluted in nuclease-free water.

2.6. Library preparation

Purified amplicons were then used to construct the sequencing libraries. The Ion Plus Fragment Library Kit (Thermo Fisher Scientific, MA, USA) was utilized for the library preparation. The protocol involved end repair and adapter ligation steps, followed by size selection and final purification. The quality and size distribution of the prepared libraries were assessed using an Agilent Bioanalyzer.

2.7. Template preparation and sequencing

The prepared libraries were diluted to the appropriate concentration and processed for template preparation using the Ion 550 Kit-Chef (Thermo Fisher Scientific, MA, USA). This step was conducted on the Ion Chef Instrument, which automates template preparation and chip loading. The resulting template-loaded Ion 550 chip was then sequenced on the Ion S5 Plus system (Thermo Fisher Scientific, MA, USA) according to the manufacturer's guidelines. Sequencing was performed with 200 base-pair read lengths, ensuring high coverage and depth for accurate microbial community profiling.

2.8. Data analysis

The raw sequencing data were processed using the Ion Torrent Suite Software (Thermo Fisher Scientific, MA, USA) for base calling, quality filtering, and alignment. The resulting high-quality reads were then classified using a reference database for taxonomic identification. The relative abundance of bacterial taxa was determined, and further downstream analyses were performed for microbiome profiling and diversity assessment. The unaligned Binary Alignment Map (BAM) files generated by the Ion Torrent PGM system were uploaded to Ion Reporter Software v5.20 for downstream analysis. The analysis was performed using the software's default settings, employing the curated Greengenes and premium curated MicroSEQ ID 16S rRNA reference databases for microbial identification. The Ion Reporter metagenomic workflow provided detailed information, including reads assigned to each hypervariable region, taxonomic classification (read count, percentage of mapped reads, and percentage of mapped reads per primer), sequence match percentage, and mapping information.

The Metagenomics 16S w1.1 pipeline was used to assess population diversity and taxonomic assignment, facilitating a comprehensive evaluation of microbial communities from the Ion semiconductor reads. The resultant BAM files were further analyzed using the QIIME software for phylogenetic analysis and operational taxonomic unit (OTU) clustering. OTU tables generated by QIIME were utilized for alpha and beta diversity analyses, yielding metrics such as species richness, evenness, and community dissimilarity indices. This integrated workflow provided a robust framework for understanding microbial diversity and population dynamics within the samples.

3. RESULTS

A diverse bacterial community was detected in the sample. The top ten bacteria identified and their relevance are listed in Table 2. The metagenomics analyses from PBS revealed the presence of bacterial species that were not listed on the product label, for example: *Brevibacillus borstelensis, Clostridium cochlearium, Rummeliibacillus pycnus, Staphylococcus cohnii, Serratia rubidaea, etc.* Some of these are pathogenic species.

None of the nitrogen-fixing bacteria (*Azospirillum brasilens, A. lipoferum, Azotobacter chroococcum, A. paspali*), and *Leuconostoc* sp. mentioned in table 1 (product label) were detected.

The results of Krona charts show more diversity in the analysis carried out with PBS. The PBS sample contained Firmicutes (39%), Proteobacteria (38%) and Cyanobacteria (21%) (Fig. 1).

Table 2. List of top ten (10) bacteria identified from PBS by metagenomics (non-isolated).

No.	Bacterial species	% ID	%	Mentioned on label	Remarks
1.	*Clostridium* sp.	99.09-100	6.94%	Yes	*Cl. butyricum* mentioned on label
2.	*Brevibacillus borstelensis*	99.44-100	1.98%	No	*Brevibacillus brevis* mentioned on label
3.	*Bacillus thermoamylovorans*	99.10-100	1.59%	No	Emerging as a threat in causing spoilage of dairy products due to heat resistance
4.	*Clostridium cochlearium*	99.04-100	1.21%	No	May prevent or alleviate obesity and diabetes in humans
5.	*Rummeliibacillus pycnus*	99.11-100	1.19%	No	Gram-positive, spore-forming
6.	*Paenalcaligenes hermetiae*	99.44-100	1.08%	No	Gram-negative, facultative anaerobic
7.	*Staphylococcus cohnii*	99.55-100	1.02%	No	Gram-positive coccus, an opportunistic pathogen
8.	*Lactobacillus fermentum*	99.11-100	0.09%	No	*Lactobacillus spp.*, *L. brevis* mentioned on label
9.	*Enterococcus cecorum*	99.44-100	0.74%	No	Gram-positive cocci, facultatively anaerobic or anaerobic; can cause infections in poultry and humans
10.	*Serratia rubidaea*	99.38-100	0.66%	No	Associated with infections of bile and blood

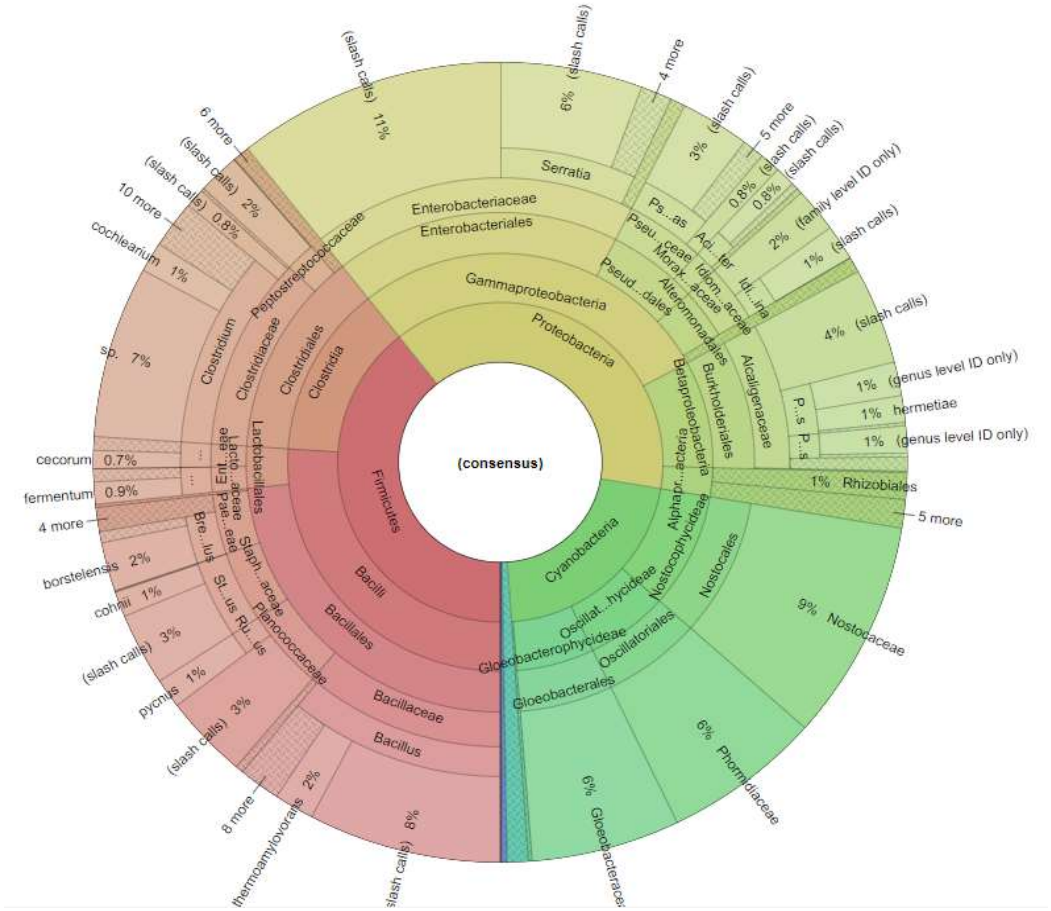


Figure 1. Krona chart showing bacterial diversity from PBS suspension.

4. DISCUSSION

There are many probiotic, synbiotic and other nutraceutical products available as liquid, semi-solid or solid (powder or capsule) in India. The ICMR-DBT has provided guidelines for the evaluation of probiotics in food, which are very much in line with those laid down by international regulatory agencies such as FAO-WHO [11]. It includes strain identification, deposition in international culture collection center, *in vitro* and *in vivo* tests (animals and human), labelling, etc. It also mentions that the probiotic strains should be evaluated for safety for human consumption, even if it falls in the Generally Recognized as Safe (GRAS) category. The strains should not produce toxins, have virulence or other potential disease-causing traits, and drug resistance genes. The content of a probiotic food or supplement should be consistent as per the label and the information provided must not be deceptive for the consumer. There are several reports on the inconsistencies of such products with reference to the label, viable count of cells, strain or species or genus has been found to be different from the one claimed on the label or the presence of contaminants as given below.

A study on seven probiotic products from the UK market revealed that only three had adequate culture count, products labelled with multi-species did not contain all the species listed and some were misidentified. A potential pathogen *Enterococcus faecium* was present in one of the products. A proper global legislation for quality and efficacy of probiotics is necessary [12]. The presence of two major pathogenic *Bacillus* spp. namely, *B. anthracis* and *B. cereus*, which were unrelated to the *Bacillus* spp. commonly used probiotics has been reported [13]. The pathogenicity of *B. cereus* is strain specific related with production of several extracellular factors viz. (phospholipase, cereulide (emetic toxin), enterotoxin Hbl, non-haemolytic toxin (Nhe), haemolysin IV), etc. Microbiological analysis of 25 probiotic products available in Polish market. Their study was based on isolation of bacteria on different media and identification of the strains by API 50 (CHL), API 20 A and MALDI-TOF MS. They concluded that some of the products did not contain adequate number of viable bacteria, lacked the bacterial strain mentioned on the label and contained bacterial strains not mentioned by the manufacturer [14]. Many of the strains were resistant to common antibiotics. Assessment of twenty probiotics products (containing single and multiple strains) from India was carriedout to determine the quality as per the contents on its label by culture, viable count and targeted metagenomics using Illumina's MiSeq platform. There was poor correlation between the results observed and contents of the product label [15]. The microbiome of raw goat milk by next generation sequencing showed that the phylum *Acinetobacter* dominated (62.8%), whereas the phyla Firmicutes, Proteobacteria and Bacteroidetes represented 20.5, 7.4 and 6.4%, respectively. It was suggested that such study would be useful for assessing the role of microbiota on the quality of milk [16]. The guidelines for judging the biosafety of probiotics has been provide in a review by Merenstein et al. [17]. Whole genome sequencing data of the strains used would provide information about their identity, presence of genes related to virulence, toxin production, antibiotic resistance, etc. It was recommended to impose stringent testing of probiotics products used for vulnerable populations (infants, immunosuppressed, critically ill patients, etc.) [17]. Fusco et al. in their review have highlighted the use of DNA based techniques such as PCR-based methods, PCR-DGGE, WGS, metagenetics, metagenomics, biosensors, biochips, etc. for food authentication of probiotics as well as Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) of fermented foods and beverages [18, 19]. Gibson et al. have concluded that fructo-oligosaccharides, galacto-oligosaccharides and lactulose fulfilled the criteria for use as prebiotics. It was recommended that robust technologies and molecular-based techniques would be necessary to test the functionality and effect on microflora by the prebiotics in a range of synbiotic products [8].

The results presented here on 16S metagenomic analysis of the multi-species synbiotic product reveals

the presence of bacteria not mentioned on the product label and some pathogenic species (*Enterococcus faecium*; *E. cecorum* and *Staphylococcus cohnii*) as mentioned in Table 2. So, the product is not suitable for human consumption, especially in immuno-compromised individuals. The Ion Torrent short read sequencing (around 100-300 bp) is cost-effective as well as efficient, however its limitation is that it cannot distinguish between closely related species, resolve complex community structure, identification of new species, etc. Therefore, in future a similar study with long read sequencing is envisaged for comparative analysis of such products. In a comprehensive review on the use of metagenomics in food microbiome analysis and research states that it is not widely used at present, however its implementation will increase food safety and product quality in the future [20]. It is therefore necessary to develop protocols from DNA isolation to sequencing and analysis for the use of molecular biology tools for evaluating the identity and diversity of microorganisms in such food supplements. This will also help the regulatory bodies to formulate a global legislation for the quality and safety of these products.

5. CONCLUSION

The market for probiotics and other food/health supplements is increasing worldwide due to the rise in life-style diseases that cause digestive and other disorders. Various agencies such as FAO-WHO (2006) and ICMR-DBT (2011) have provided guidelines for the quality of these products. Poor GMP in many countries leads to the presence of undesirable microorganisms in these products that can cause an adverse effect on health. Sterility of the prebiotic(s) added in case of synbiotics is not mentioned on the label, which can also be a cause of presence of contaminants in the product. 16S metagenomic analysis can be an useful tool for the microbiological analysis of the multi-species synbiotic products. Our results showed the presence of bacteria not mentioned on the product label and some pathogenic species. Global consensus on the use of molecular tools and its methodology for such products is recommended.

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REFERENCES

1. FAO-WHO Food and nutrition Paper 85. Probiotics in food: Health and nutritional properties and guidelines for evaluation, 2006.
2. Prakash O, ed. Food and drinks in ancient India, Delhi (India), 1st edn. Munshi Ram Manoharlal Publication, Oriental Book Sellers and Publishers, India, 1961.
3. Lakshmy B, Seetha DB, Ramesh B. The blooming prospects of probiotic products in India. Int J Recent Technol Eng. 2018; 7(4S2): 253-258.
4. Binda S, Hill C, Johansen E, Obis, D, Pot D, Sanders ME. Criteria to qualify microorganisms as “Probiotic” in foods and dietary supplements. Front Microbiol. 2020, 11: 1662.
5. Chattaraj S, Mitra D, Chattraj A. et al. Antioncogenic potential of probiotics: Challenges and future prospective. Ind J Microbiol Res. 2023; 10(1): 1-10.
6. Palai S, Derecho CMP, Kesh SS, Egbuna C, Onyeike PC. Prebiotics. In: Egbuna C, Dable Tupas G, eds. Functional Foods and Nutraceuticals. Springer, 2020.

7. Zawistowska-Rojek A, Zaręba T, Tyski S. Microbiological Testing of Probiotic Preparations. Health Int J Environ Res Public Health. 2022; 19(9): 5701.
8. Gibson R, Probert HM, van Loo JAE, Rastall RA, Roberfroid, MB. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr Res Rev. 2004; 17: 259-275.
9. Roberfroid M. Prebiotics: the concept revisited. J Nutr. 2007; 137: 830-837.
10. Katke SD, Deshpande HW, Tapre AR. Review on Psyllium husk (*Plantago ovata*): A novel superfood for human health. Int J Curr Microbiol Appl Sci. 2020; 9(12): 1949-1959.
11. ICMR-DBT. Guidelines for evaluation of probiotics in food, New Delhi, India: ICMR, 2011.
12. Fredua-Agyeman M., Parab S, Gaisford S. Evaluation of commercial probiotic products. Brit J Pharm. 2016; 1(1): 84-89.
13. Elshaghabee Fouad MF, Rokana N, Gulhane RD, Sharma C, Panwar H. *Bacillus* as potential probiotics: Status, Concerns, and Future Perspectives. Front Microbiol. 2017; 8: 1-15.
14. Zawistowska-Rojek A, Zareba T, Mrówka A, Tyski S. Assessment of the microbiological status of probiotic products. Pol J Microbiol. 2016; 65(1): 97-104.
15. Kesavelu DS, Rohit A, Karunasagar I. Composition and laboratory correlation of commercial probiotics in India. Cureus. 2020; 12(11): e11334.
16. Lauková A, Micenková L, Grešáková Ľ, et al. Microbiome associated with Slovak raw goat milk, trace minerals, and vitamin E content. Int J Food Sci. 2022; 4595473: 1-8.
17. Merenstein D, Pot B, Leyer G, Ouwehand AC, Preidis GA, Elkins CA, et al. Emerging issues in probiotic safety: 2023 perspectives. Gut Microbes. 2023; 15(1): 1-18.
18. Fusco V, Francesca F, Daniele C. Chapter 3 Authenticity of probiotic foods and supplements: up-to-date situation and methods to assess it. In: de Souza EL, de Brito Alves JL, Fusco V, eds. Probiotics for Human Nutrition in Health and Disease, Academic Press, 2022: 45-74.
19. Fusco V, Fanelli F, Chieffi D. Recent and advanced DNA-based technologies for the authentication of probiotic, protected designation of origin (pdo) and protected geographical indication (pgi) fermented foods and beverages. Foods, 2023; 12: 3782.
20. Sadurski J, Polak-Berecka M, Staniszewski A, Wasko A. Step-by-Step Metagenomics for Food Microbiome Analysis: A Detailed Review. Foods. 2024; 13: 2216.