High-Throughput Sequencing as a Tool for the Quality Control of Microbial Bioformulations for Agriculture

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Keywords: bioformulations, bacteria, high-throughput sequencing, agriculture, quality control

Abstract:

Microbial bioformulations, due to their positive impact on the growth and development of plants, as well as the absence of harmful effects on the environment and humans, have a vast potential for mass introduction into agriculture. Assessing the quality of bioformulations, especially complex ones, is a difficult task. In this study, we show that high-throughput sequencing can be an effective tool for the quality control and safety of microbial bioformulations. By the method of high-throughput sequencing on the MiSeq platform, we studied 20 samples of commercially available microbial bioformulations. In parallel with this, bioformulations were studied by classical microbiological methods. The analysis showed the presence of extraneous undeclared bacterial genera by the manufacturer. Only 10% of the bioformulations fully corresponded to the commercial composition, and another 10% of the bioformulations did not contain the bacteria declared by the manufacturer in their composition at all. The bacterial composition of 80% of the bioformulations partially corresponded to the composition indicated on the package. The most frequent microbial bioformulations contaminants were Enterococcus, Lactobacillaceae, Klebsiella, Escherichia-Shigella and Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium. Universal methods for the quality control of bioformulations are needed. The advantages of high-throughput sequencing for the evaluation of bioformulations are considered in this work.

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Keywords: bioformulations; bacteria; high-throughput sequencing; agriculture; quality control

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1. Introduction

The excessive use of synthetic agrochemicals (pesticides and fertilizers) poses a threat to soil fertility, and, consequently, the sustainable development of crop production [1–3]. The Commission on Development and Agriculture has banned some commercially available chemical plant protection products. Instead, it recommends using natural fertilizers—biological products that can increase the resistance of cultivated plants to abiotic stresses and phytopathogens [4]. Microbial bioformulations include bacteria, fungi, and sometimes microalgae, whose vital products have similar actions to artificial fertilizers and pesticides, but at the same time do not cause such harm to the environment as chemical pesticides and fertilizers [5–8]. Bioformulations may not be as effective as chemical pesticides and fertilizers [9,10]. The reasons for this may be different. One such reason may be the lack of matching of the actual bioformulations' microbiological composition and the declared one.

The introduction of innovative technologies in agriculture, in particular crop production, is necessary to improve the efficiency of production processes at all stages, from tillage to harvest. Environmental friendliness, economic benefits, and easy reproduction technology put the use of microbial bioformulations in the first place in agriculture. It is known that bioformulations increase productivity by 10–40% and nitrogen fixation by plants up to 50%, and fertilize soils in the long term [11]. A large number of manufacturers of this

product around the world are looking for the optimal formula that can comprehensively protect crops from pests and diseases at all stages of agricultural production as well as stimulate plant growth [12–15].

The use of bioformulations based on microorganisms contributes to the growth of the green mass of crops that is important for agriculture [16]. Due to resistance to chemical stress, the main genera of bacteria included in the bioformulations are *Arthrobacter*, *Bacillus*, *Pseudomonas*, and *Rhodococcus*. The species *Rhizophagus irregularis*, *Funneliformis mosseae*, and *Claroideoglomus etunicatum* increase the total number of soil bacteria, mycorrhizae in the roots of the plant, resulting in an increase in the concentration of chlorophyll in the plants, a higher CO_2 assimilation rate, and increase in the total number of soil bacteria and fungi and root mycorrhizal frequency [17]. Bacteria of the genus *Bacillus* have a high cellulolytic potential and are used as agents to reduce the decomposition time of post-harvest residues [18–20]. *Bacillus thuringiensis* have an antagonistic effect on invertebrate pests of agricultural crops of the *Lepidoptera* order [18]. *Beauveria bassiana* and *Bacillus subtilis*, which are part of bioformulations, affect phytopathogens of tomato–Fusarium wilt and moth–*Helicoverpa armigera* [21]. *Rhizobia* are involved in the process of nitrogen fixation, and also contribute to the growth and reduction in the incidence of plant crops [22]. Bioformulations based on *Trichoderma harzianum* and *Bacillus subtilts*, which are used to treat soils, seeds, and adult plants, increase the yield of spring barley and winter wheat by 3–5%, as they inhibit the growth of phytopathogenic fungi of the genera *Fusarium*, *Drechslera (Helminthosporium)*, *Pseudocercosporella (Tapesia)*, *Gaeumannomyces*, and partially *Rhynchosporium* [23–25].

In addition to all the above advantages, there are disadvantages in using live microorganisms. These include the unpredictability of the effect due to the dynamics of field conditions, as well as the possibility of reducing their viability as a result of competition with the local microflora of the soil [26–28]. However, these disadvantages are minor compared to the disadvantages of synthetic fertilizers and pesticides, which can have serious side effects. Therefore, the use of microorganisms as biostimulators of plant growth and development, as well as its protection from pathogens, is a promising method [3,6]. At the same time, at the moment, there are no universal methods for assessing the quality of such bioformulations. There are no data on the typical microbiological pollutants of bioformulations. Therefore, there are many bioformulations on the market, whose the quality is sometimes impossible to assess.

The mass production of bioformulations can lead to deterioration in their composition, and in turn reduces the effectiveness of its impact on the soil and the growth of cultivated plants. Therefore, it is necessary to introduce effective methods for the quality control of bioformulations; one of such methods can be high-throughput sequencing. The purpose of this work is to study the composition of the microbial bioformulations used to protect and stimulate plant growth and development using high-throughput sequencing, as well as a comparative analysis of two approaches to identify microorganisms: the classical microbiological method and high-throughput sequencing.

2. Materials and Methods

2.1. Objects

The object of the study was 20 samples of commercially available microbial bioformulations.

2.2. DNA Isolation

The DNA was extracted using a FastDNA(TM) Spin Kit (MP Biomedicals, Solon, OH, USA), according to the manufacturer's instructions.

2.3. High-Throughput Sequencing

The libraries were prepared by PCR using universal primers for the V4 region of the 16S rRNA gene [29]. The following pairs of primers were used: 515F (5′-GTGBCAGCMGCCGCGGTAA-3′) [30] and Pro-mod-805R (5′-GACTACNVGGGTMTCTAATCC-3′) [31]. At the stages of

preparation of samples for sequencing (DNA isolation and PCR), negative controls were used to exclude the factor of internal laboratory contamination of samples. Two libraries were prepared for each DNA sample, which were sequenced in parallel using the MiSeq Reagent Micro Kit v2 (300 cycles) MS-103-1002 (Illumina, San Diego, CA, USA) on a MiSeq sequencer (Illumina, San Diego, CA, USA), which allows reading 150 bp from each end. After sequencing, fastq files were obtained at the output. After preliminary bioinformatic processing, which consisted in combining forward and backward reads, filtering sequences with low readings of individual nucleotides, filtering chimeric sequences, distributing reads based on barcode sequences and removing technical sequences (including primer sequences for the 16S rRNA gene), the resulting sequences were allocated to operational taxonomic units (OTUs) based on sequence similarity of more than 97%. OTU identification was performed with SILVAngs 1.3 [32]. Raw sequencing data can be seen in Table S1 (Supplementary Materials).

2.4. Microbiological Analysis

For microbiological analysis, tenfold dilutions of bioformulations up to 10^{-8}–10^{-10} were prepared, depending on CFU. From each dilution, 3 Petri dishes were seeded as follows: 0.1 mL of the bioformulations, taken from each dilution, was placed in a sterile Petri dish and filled with agar. Table S2 (see Supplementary Materials) shows the composition of the microbiological media. After 24–120 h of incubation, the Petri dishes were examined. The cultural and morphological traits of the microorganisms and their compliance with the declared composition were evaluated.

3. Results

The classical microbiological analysis revealed that only 40% of bioformulations contained the microorganisms declared by the manufacturers. The results are shown in detail in Table 1.

The microbiological analysis did not reveal any expected bacterial taxa for 20% of the samples. A total of 35% of the bioformulations was characterized by a partial coincidence of the declared and detected bacteria. For example, *Enterobacter* spp. and *Paenibacillus polimyxa* were not detected in sample 7, and sample 20 did not contain the expected species *Bacillus megaterium* and *Azospirillum brasilense*.

The analysis of the sequencing data revealed extraneous microorganisms that were not declared by the manufacturers (Table 2). Thus, more than half of the microbial composition of the bioformulations (55%) had microorganisms not included in the declared composition of the bioformulations by the manufacturers. The bacterial composition of 50% of the studied samples of bioformulations, partially, did not include the declared genera in their compositions. For example, the genera *Bacillus*, *Azospirillium*, *Bradyrhizobium*, and *Mesorhizobium* were not found in sample number 20, and the genera *Azotobacter*, *Enterobacter*, and *Paenibacillus* were not found in sample number 7. In samples 5 and 14, the genera *Pseudomonas* (99%) and *Bacillus* (100%) were detected, respectively, which coincide with the formulations indicated by the manufacturers. The bacterial composition of samples 2, 4, and 9 was dominated by the declared genera *Bacillus* (94%), *Bradyrhizobium* (76%), and *Bacillus* (73.5%), respectively.

We discovered that two bioformulations contain a different bacterial composition to the declared one. Thus, the genus *Pseudomonas* declared by the manufacturer was not identified in sample 19. In turn, the genera were found *Escherichia-Shigella* (2.5%), *Proteus* (18%), *Providencia* (1%), *Aeromonas* (1.5%), *Bacteroides* (2.5%), *Enterococcus* (2%), *Acinetobacter* (38%), and *Klebsiella* (29%).

Table 1. Results of microbiological examination of bioformulations.

Bioformulations	Medium	Common Traits	Morphological Traits	Declared Microorganism	Correspondence of Signs to the Microorganism Declared in the Composition
1	Meat peptone agar	white, finely wrinkled colonies with scalloped margins	Gram-positive spore-forming rods	*Bacillus subtilis*	Present
2	Meat peptone agar	small, smooth-edged, round, opaque, yellowish-white colonies	large Gram-positive spore-forming rods in short chains	*Bacillus megaterium*	Present
3	Meat peptone agar	small, smooth-edged, round, opaque, yellowish-white colonies	large Gram-positive spore-forming rods in short chains	*Bacillus megaterium*	Present
	Potato dextrose agar	round, convex, smooth colonies with smooth edges, white	Gram-positive spore-forming rods	*Bacillus mucilaginosus*	Present
	Ashby's medium	no growth	-	*Azotobacter chrococcum*	Not
4	Yeast mannitol agar	round small shiny colonies stained red	Gram-negative non-spore-forming rods	*Bradyrhizobium japonicum*	Present
5	Meat peptone agar	round, smooth-edged, creamy colonies	Gram-negative non-spore-forming rods	*Pseudomonas aureofaciens*	Present
6	Meat peptone agar	white, finely wrinkled colonies with scalloped margins	Gram-positive spore-forming rods	*Bacillus subtilis*	Present
	Ashby's medium	no growth	-	*Azotobacter* spp.	Not
	Medium for Paenibacillus polymyxa	colorless flat mucous colonies with a serrated edge	Gram-positive, lemon-shaped spore-forming cells	*Paenibacillus polymyxa*	Present
	Enterococcus Agar	concave, round colonies	Gram-positive non-spore-forming rods in chains	*Enterococcus* spp.	Not
	MRS	no growth	-	*Lactobacillus* spp.	Not

Table 1. *Cont.*

Bioformulations	Medium	Common Traits	Morphological Traits	Declared Microorganism	Correspondence of Signs to the Microorganism Declared in the Composition
7	Meat peptone agar	white, finely wrinkled colonies with scalloped margins	Gram-positive spore-forming rods	*Bacillus subtilis*	Present
	Ashby's medium	flat mucous colonies with a smooth edge, colorless colonies, later-stained brown	Gram-negative non-spore-forming rod-shaped and spherical cells	*Azotobacter chrococcum*	Present
	Endo's medium	matte flat colonies	Gram-negative non-spore-forming rods	*Enterobacter* spp.	Not
	Medium for Paenibacillus polymyxa	white, small wrinkled colonies with a scalloped edge	Gram-positive spore-forming rods	*Paenibacillus polimyxa*	Not
8	L	round, smooth-edged, creamy colonies	Gram-negative non-spore-forming rods	*Pseudomonas fluorescens*	Present
	Meat peptone agar	small, smooth-edged, round, opaque, yellowish-white colonies	large Gram-positive spore-forming rods in short chains	*Bacillus megaterium*	Present
	Meat peptone agar	white, finely wrinkled colonies with scalloped margins	Gram-positive spore-forming rods	*Bacillus subtilis*	Present
	Potato dextrose agar	round, convex, smooth colonies with smooth edges, white	Gram-positive spore-forming rods	*Bacillus mucilaginosus*	Present
	Endo's medium	round stained colonies	Gram-positive cocci	*Enterobacter* spp.	Not
	ISP	small rough white with uneven edge convex	Gram-positive cocci	*Streptomyces* spp.	Not

Table 1. *Cont.*

Bioformulations	Medium	Common Traits	Morphological Traits	Declared Microorganism	Correspondence of Signs to the Microorganism Declared in the Composition
9	Meat peptone agar	white, finely wrinkled colonies with scalloped margins	Gram-positive spore-forming rods	*Bacillus subtilis*	Present
	Ashby's medium	flat mucous colonies with a smooth edge, colorless colonies, later-stained brown	Gram-negative non-spore-forming rod-shaped and spherical cells	*Azotobacter* spp.	Present
	Endo's medium	small round crimson colonies	Gram-negative non-spore-forming rods	*Enterobacter* spp.	Present
	Enterococcus agar	white with jagged edges	Gram-positive non-spore-forming rods	*Enterococcus* spp.	Not
10	Meat peptone agar	white, finely wrinkled colonies with scalloped margins	Gram-positive spore-forming rods	*Bacillus subtilis*	Present
11	Meat peptone agar	small, smooth-edged, round, opaque, yellowish-white colonies	large Gram-positive spore-forming rods in short chains	*Bacillus megaterium*	Present
	L	white, finely wrinkled colonies with scalloped margins	Gram-positive spore-forming rods	*Azospirillum brasilense*	Not
	Meat peptone agar	white, finely wrinkled colonies with scalloped margins	Gram-positive spore-forming rods	*Bacillus subtilis*	Present
12	Meat peptone agar	small, smooth-edged, round, opaque, yellowish-white colonies	large Gram-positive spore-forming rods in short chains	*Bacillus megaterium*	Present
	L	small, round, colorless colonies	Gram-positive cocci	*Azospirillum brasilense*	Not
13	Meat peptone agar	round white shiny colonies	Gram-negative non-spore-forming rods	*Bacillus megaterium*	Not
	Ashby's medium	flat mucous colonies with a smooth edge, colorless colonies	Gram-positive spore-forming rods	*Bacillus azotofixans*	Not
14	Meat peptone agar	dull, flat with rhizoidal margin, white colonies	Single and lined in chains, long, thin Gram-positive spore-forming rods	*Bacillus thuringiensis*	Present

Table 1. *Cont.*

Bioformulations	Medium	Common Traits	Morphological Traits	Declared Microorganism	Correspondence of Signs to the Microorganism Declared in the Composition
15	Meat peptone agar, pH = 5.0	round cream colonies with a smooth edge	Gram-positive cocci	*Paenibacillus macerans*	Not
	Meat peptone agar	round cream colonies with a smooth edge	Gram-positive cocci	*Bacillus pumilus*	Not
	Meat peptone agar, 50 °C	no growth	-	*Bacillus licheniformis*	Not
	Meat peptone agar, 40 °C	no growth	-	*Bacillus stearothermophilus*	Not
	Medium for Paenibacillus polymyxa	round cream colonies with a smooth edge	Gram-positive cocci	*Paenibacillus polymyxa*	Not
16	Yeast mannitol agar	no growth	-	*Bradyrhizobium japonicum*	Not
17	Meat peptone agar	colonies dull, flat with rhizoidal margin, white	single and lined up in chains long, thin Gram-positive rods, spore-forming	*Bacillus thuringiensis*	Present
18	L	creamy, rough colonies with a jagged edge	Gram-positive spore-forming rods	*Bacillus amyloliquefaciens*	Present
19	Meat peptone agar	colonies are heterogeneous, there are different species	Gram-negative and Gram-positive rods, spores	*Pseudomonas aureofaciens*	Not
20	Meat peptone agar	small, smooth-edged, round, opaque, yellowish-white colonies	large Gram-positive non-spore-forming rods	*Bacillus megaterium*	Not
	L	round, smooth-edged mucoid colonies	long Gram-positive non-spore-forming rods in chains	*Azospirillum brasilense*	Not
	Pea medium	rounded, colorless, slimy, shiny colonies	Gram-negative non-spore-forming rods	*Risobium leguminosarum*	Present
	Meat peptone agar	white, finely wrinkled colonies with scalloped margins	Gram-positive spore-forming rods	*Bacillus subtilis*	Present
	Yeast mannitol agar	no growth	-	*Bradyrhizobium japonicum*, *Mesorhizobium ciceri*	Not

Table 2. Comparison of the declared bacterial taxa with the identified genera.

№	Unidentified but Declared Genera of Bacteria	Match with the Declared Bacteria		Extraneous Bacteria	
		Genus of Bacteria	Abundance	Genus of Bacteria	Abundance
1	-	*Bacillus*	49%	*Brevibacillus* *Lacticaseibacillus* *Levilactobacillus* *Secundilactobacillus* *Latilactobacillus* *Enterococcus* *Lactobacillales*	4.5% 25.5% 5% 3.5% 0.5% 6% 3%
2	-	*Bacillus*	94%	*Staphylococcus* *Enterococcus* *Vagococcus*	1% 2% 0.5%
3	*Azotobacter*	*Bacillus*	35.5%	*Lachnospiraceae* *Eubacterium* *Ruminococcaceae* *Oscillibacter* *Clostridiaceae* *Enterobacteriaceae* *Morganella* *Providencia* *Prevotella* *Prevotellaceae* *UCG-004* *Bacteroides* *Latilactobacillus* *Lactiplantibacillus* *Enterococcus* *Vagococcus* *Lactococcus*	4% 0.95% 2% 2.5% 1% 1% 4% 1% 1.5% 9.5% 3% 4% 5.5% 2% 15.5% 2% 4%
4	-	*Bradyrhizobium*	76%	*Pseudomonas*	23.5%
5	-	*Pseudomonas*	99%	-	-
6	*Azotobacter* *Enterococcus* *Paenibacillus* *Lactobacillus*	*Bacillus*	29.5%	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* *Secundilactobacillus* *Loigolactobacillus*	52% 2% 13%
7	*Azotobacter* *Enterobacter* *Paenibacillus*	*Bacillus*	12%	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* *Pediococcus* *Enterococcus* *Klebsiella* *Escherichia-Shigella*	57.5% 6% 1.5% 20.5% 1%
8	*Streptomyces* *Enterobacter*	*Bacillus* *Pseudomonas*	54.5% 24%	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	20%
9	*Azotobacter* *Enterobacter* *Enterococcus*	*Bacillus*	73.5%	*Klebsiella* *Escherichia-Shigella* *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* *Lacticaseibacillus* *Lactiplantibacillus* *Levilactobacillus*	10.5% 2% 7.5% 0.5% 1.5% 45.5%

Table 2. *Cont.*

№	Unidentified but Declared Genera of Bacteria	Match with the Declared Bacteria		Extraneous Bacteria	
		Genus of Bacteria	Abundance	Genus of Bacteria	Abundance
10	-	*Bacillus*	43%	*Enterobacter*	3%
				Escherichia-Shigella	1%
				Providencia	3%
				Comamonas	1%
				Lacticaseibacillus	1%
				Secundilactobacillus	1%
				Latilactobacillus	10%
				Pediococcus	21%
				Paucilactobacillus	0.5%
				Enterococcus	3%
				Lactococcus	4%
				Klebsiella	3.5%
				Loigolactobacillus	0.5%
11	*Azospirillium*	*Bacillus*	26.5%	*Klebsiella*	2%
				Escherichia-Shigella	1%
				Providencia	2.5%
				Aeromonas	5%
				Lacticaseibacillus	2.5%
				Secundilactobacillus	1%
				Latilactobacillus	14.5%
				Loigolactobacillus	2%
				Pediococcus	30%
				Paucilactobacillus	3%
				Enterococcus	2.5%
				Lactococcus	4%
12	*Azospirillium*	*Bacillus*	27%	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	5%
				Pseudarcobacter	7%
				Bacteroides	21.5%
				Clostridium sensu stricto 13	6.5%
				Pseudomonas	20%
				Enterobacteriaceae	1%
				Serratia	5.5%
				Hafnia-Obesumbacterium	2%
				Escherichia-Shigella	0.45%
13	-	*Bacillus*	33.5%	*Rickettsiales*	57.5%
				Acinetobacter	6%
				Lactobacillales	2%
14	-	*Bacillus*	100%	-	-

Table 2. *Cont.*

№	Unidentified but Declared Genera of Bacteria	Match with the Declared Bacteria		Extraneous Bacteria	
		Genus of Bacteria	Abundance	Genus of Bacteria	Abundance
15	-	*Paenibacillus*	1%	*Nocardioides*	1%
				Pseudonocardiaceae	1%
				Promicromonospora	1%
				Micrococcaceae	1%
				Microbacteriaceae	1%
				Streptomyces	1%
				Acidimicrobiia	1%
				Bacteroidia	2%
				Chloroflexi	0.5%
				Nodosilinea PCC-7104	1%
				Cyanobacteriia	11.5%
				Ligilactobacillus	11%
				Lactobacillus	5%
				Limosilactobacillus	5.5%
				Planococcaceae	1.5%
				Burkholderiales	2%
		Bacillus	6%	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	1%
				Devosia	1%
				Sphingomonadaceae	3%
				Rickettsiales	16%
				Arthrobacter	1%
				Serinicoccus	0.5%
				Levilactobacillus	0.5%
16	*Bradyrhizobium*	-	-	*Afipia*	100%
17	-	*Bacillus*	32.5%	*Enterobacter*	12%
				Escherichia-Shigella	3%
				Aeromonas	4.5%
				Janthinobacterium	0.5%
				Rickettsiales	4.5%
				Streptomyces	13.5%
				Latilactobacillus	4.5%
				Paucilactobacillus	0.95%
				Pseudomonas	5%
				Klebsiella	13%
				Pantoea	0.5%
				Oxalobacteraceae	0.5%
18	-	*Bacillus*	24.5%	*Fusobacterium*	9%
				Prevotella_9	3.5%
				Bacteroides	19%
				Dysgonomonas	6%
				Macellibacteroides	2%
				Enterococcus	6%
				Citrobacter	24.5%
				Escherichia-Shigella	2.5%
				Hafnia-Obesumbacterium	0.5%
19	*Pseudomonas*	-	-	*Escherichia-Shigella*	2.5%
				Proteus	18%
				Providencia	1%
				Aeromonas	1.5%
				Bacteroides	2.5%
				Enterococcus	2%
				Acinetobacter	38%
				Klebsiella	29%

Table 2. *Cont.*

№	Unidentified but Declared Genera of Bacteria	Match with the Declared Bacteria		Extraneous Bacteria	
		Genus of Bacteria	Abundance	Genus of Bacteria	Abundance
20	*Bacillus* *Azospirillium* *Bradyrhizobium* *Mesorhizobium*	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	40.5%	*Lapidilactobacillus* *Leuconostoc* *Pediococcus* *Lactococcus* *Clostridium sensu stricto 13* *Clostridium sensu stricto 1* *Pseudomonas* *Enterobacter* *Shewanella* *Aeromonas* *Klebsiella*	2% 15.5% 1% 18% 1.5% 3% 3% 2% 2.5% 3% 2%

It was shown that the composition of some bioformulations partially coincided with the composition indicated by the manufacturers. For example, the presence of the genera *Bacillus* and *Azotobacter* was declared by the manufacturers as being part of sample 3. The sequencing of this sample revealed only one genus of *Bacillus*, whose percentage was 35.5%. Other identified taxa include the genus *Vagococcus* with 15.5%, the *Prevotellaceae* family UCG-004 9.5%, the genus *Lactiplantibacillus* 5.5%, and 4% each on the taxa *Lachnospiraceae*, *Morganella*, and *Latilactobacillus*. Less than 3% were the taxa *Eubacterium*, *Ruminococcaceae*, *Oscillibacter*, *Clostridiaceae*, *Enterobacteriaceae*, *Providencia*, *Prevotella*, *Bacteroides*, *Enterococcus*, and *Lactococcus*.

We identified the bacterial taxa that contaminated the bioformulations most frequently (Table 3). *Enterococcus* and *Lactobacillaceae* were identified in 40% of the samples. A total of 35% of the bioformulations contained the following extraneous taxa of microorganisms: *Klebsiella*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, and *Escherichia-Shigella*. Additionally, 20% of the samples were contaminated with the bacteria *Providencia*, *Bacteroides*, *Lactococcus*, *Pseudomonas*, *Pediococcus*, and *Aeromonas*. It was revealed that 10% of bioformulations were contaminated with *Clostridium sensu stricto 1*, *Acinetobacter*, and *Streptomyces*. Samples of bioformulations that were not contaminated were also found, as their composition fully corresponding to the microbial composition declared by the manufacturers. These were samples 5 and 14.

The classical microbiological method and the method of high-throughput sequencing allowed us to obtain similar results for 80% of the bioformulations. For example, the declared genus *Bacillus* was identified in sample 2, and sequencing and microbial seeding did not identify *Streptomyces* and *Enterobacter*, but the genera *Bacillus* and *Pseudomonas* were identified in sample 8.

A partial correspondence of the two methods results was revealed in 10% of the analyzed bioformulations. By seeding on Petri dishes, it was determined that, in samples 7 and 9, in addition to the genus *Bacillus* identified by sequencing, the genus *Azotobacter* was found in sample 7, and bacteria of the genera *Enterobacter* and *Azotobacter* were found in sample 9.

The composition of 10% of the commercially available bioformulations was not identified by the classical microbiological method, whereas high-throughput sequencing made it possible to determine the declared genus *Bacillus* in sample 13 and in sample 15 the genera *Bacillus* and *Paenibacillus*.

Table 3. Relative abundance (%) of extraneous bacteria in the bioformulations.

Bacteria	Bioformulation																	
	1	**2**	**3**	**4**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**15**	**16**	**17**	**18**	**19**	**20**
Enterococcus	6%	2%	15.5%	-	-	1.5%	-	-	3%	2.5%	-	-	-	-	-	6%	2%	-
Klebsiella	-	-	-	-	-	20.5%	-	10.5%	3.5%	2%	-	-	-	-	13%	-	29%	2%
Lactobacillaceae	34%	-	7.5%	-	15%	-	-	47%	12%	23%	-	-	21.5%	-	4.5%	-	-	-
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	-	-	-	-	52%	57.5%	20%	7.5%	-	-	5%	-	1%	-	-	-	-	-
Enterobacteriaceae	-	-	1%	-	-	-	-	-	3%	-	1%	-	-	-	12%	-	-	2%
Escherichia-Shigella	-	-	-	-	-	1%	-	2%	1%	1%	-	-	-	-	3%	2.5%	2.5%	-
Providencia	-	-	1%	-	-	-	-	-	3%	2.5%	-	-	-	-	-	-	1%	-
Bacteroides	-	-	4%	-	-	-	-	-	-	-	21.5%	-	-	-	-	19%	2.5%	-
Lactococcus	-	-	4%	-	-	-	-	-	4%	4%	-	-	-	-	-	-	-	18%
Pseudomonas	-	-	-	23.5%	-	-	-	-	-	-	20%	-	-	-	5%	-	-	3%
Pediococcus	-	-	-	-	-	6%	-	-	21%	30%	-	-	-	-	-	-	-	1%
Aeromonas	-	-	-	-	-	-	-	-	-	5%	-	-	-	-	4.5%	-	1.5%	3%
Clostridium sensu stricto 13	-	-	-	-	-	-	-	-	-	-	6.5%	-	-	-	-	-	-	1.5%
Acinetobacter	-	-	-	-	-	-	-	-	-	-	-	6%	-	-	-	-	38%	-
Streptomyces	-	-	-	-	-	-	-	-	-	-	-	-	1%	-	13.5%	-	-	-
Afipia	-	-	-	-	-	-	-	-	-	-	-	-	-	100%	-	-	-	-

4. Discussion

Twenty commercially available bioformulations used in agriculture as biofertilizers and biopesticides were analyzed in our study. Although the market of bioformulations is growing rapidly [33–35], such studies have not been conducted to date. However, there are similar studies of complex commercially available mixtures of bacteria. A comparative analysis of classical and molecular methods was conducted to assess the composition of the microbiological inoculants used for wastewater treatment and soil reclamation [36]. There is research on the application of high-throughput sequencing for the study of commercial bacterial biologics in the food industry. Studies of the microbiological composition of commercial starter cultures of bacteria and probiotics with high-throughput sequencing have also been conducted [37,38].

Two identification methods were used in the analysis of bioformulations in our study: the classical microbiological method and high-throughput sequencing. It was revealed that the results of identifying target groups of bacteria with these two methods coincided for 80% of the bioformulations. The main limitation of the classical microbiological method is the identification of only a specific bacterial taxon through the use of selective media. It is impossible to obtain a complete picture of the bacterial composition with the classical microbiological method. At the same time, high-throughput sequencing makes it possible to detect most of the bacterial taxa present in the sample. The disadvantage of the high-throughput sequencing method is its inability to identify the bacterium at the species level. However, it is worth noting that, in most cases, it is enough to determine the generic affiliation of the bacteria to draw a conclusion about the quality of the bioformulations and the presence of foreign bacteria. The analysis of DNA sequences of both living and dead bacterial cells is another limitation of high-throughput sequencing, since the method does not allow the assessment of the viability of cells.

We are the first to show that the real composition of commercial bioformulations may differ from the declared composition. The most common microbial contaminants were *Enterococcus* and *Lactobacillaceae* (in 40% of the bioformulations), *Klebsiella* and *Escherichia-Shigella* (in 35% of the bioformulations), and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (in 30% of the bioformulations). This can be dangerous for both plants and

humans. For example, some species of the bacterial genera *Klebsiella* and *Escherichia-Shigella* we identified are pathogenic to humans [39,40], and some members of the genus *Pseudomonas* cause diseases in and the death of cultivated plants [41–43]. This fact especially points to the need to introduce more detailed quality control of commercial bioformulations for their active use in the agricultural sector. The differences in the compositions are expressed both in the presence of additional bacterial components and in the absence of the bacterial taxa declared by the manufacturer. In some cases, the composition of the bioformulations differed from the declared one in two parameters at once.

It is interesting to note that, among the identified extraneous bacteria, there were microorganisms neutral for plants: *Lactobacillus* and *Acinetobacter* [44,45]. *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* is a symbiotic soil microorganism that promotes nitrogen absorption and stimulates the growth of the plant organism due to the formation of rhizospheres [46]. We have previously shown that these bacteria are typical contaminants of bioformulations and, probably, they can lead to spoilage of products due to their properties [47,48]. More detailed studies are required for species and strain identification of contaminants.

The above-mentioned bacteria identified by us were typical contaminants of bioformulations. Based on the data we obtained, further experiments can be aimed at studying the effect of these bacteria on the growth and development of agricultural plants.

5. Conclusions

As a result of our comprehensive study of commercially available bioformulations, data were obtained on the actual composition of these products. The classical microbiological method made it possible to confirm or refute the presence of certain bacteria declared in the bioformulations' composition by their manufacturers. For example, 20% of the samples did not grow any of the expected types of microorganisms. Analysis based on high-throughput sequencing made it possible not only to confirm the composition indicated on the packaging of the bioformulations, but also to reveal the presence of taxa not declared by the manufacturers in the samples. In 40% of the bioformulations, *Enterococcus* and *Lactobacillaceae*, uncharacteristic for their supposed composition, were identified. Only 10% of bioformulations fully corresponded to the composition. Typical contaminants of bioformulations were *Enterococcus*, *Lactobacillaceae*, *Klebsiella*, *Escherichia-Shigella*, and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*.

A comparative analysis of the results of the classical microbiological method and the high-throughput sequencing method showed similar results for 80% of the studied bioformulations. However, unlike the classical microbiological methods, high-throughput sequencing made it possible to assess the full bacterial composition of the bioformulations. We showed that high-throughput sequencing can be an effective tool for bioformulations' quality and safety control.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr10112243/s1, Table S1: Raw sequencing data; Table S2: Composition of microbiological media used for analysis.

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