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Article

Variations of the Bacterial Community in Wheat (*Triticum aestivum*), Oats (*Avena sativa* L.), Barley (*Hordeum vulgare* L.) and Triticale (*Triticosecale*) from Three Regions of the Republic of Crimea

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Abstract: Russia is one of the largest cereal grain exporters in the world, churning out 34.3 million tons of export grain (wheat, barley and oats) in 2021. Plant infectious pathogens continue to be among the main factors in yield loss in the field and are a danger to the grain exporting industry's ability to expand internationally. This is primarily due to phytosanitary restrictions imposed by nations that monitor the presence and absence of certain phytopathogens in imported goods. Phytosanitary measures prevent the spread of plant pathogens, thus cutting the cost of dealing with them, once the pathogens invade new agricultural regions. This paper is devoted to the detection and identification of bacteria in samples of grain crops of three regions in The Republic of Crimea. The objects of the study were bacterial isolates from plant samples particularly wheat, oats, barley and triticale. The study was conducted in 2021. The identification of the isolates was carried out by sequencing a section 16–23S of the rRNA amplified by PCR with 8UA/519B, 27f/907r and PSf/PSr primers. Nucleotide sequences were deciphered using the Bio Edit program and compared with sequences placed in GenBank (<https://blast.ncbi.nlm.nih.gov>). The result of identification was considered an organism with maximum similarity. As a result, 38 samples of grain crops were collected, 95 bacterial colonies were isolated, of which 68 were identified to genus level and 22 were identified to species level. Some of the phytopathogens identified include: *Agrococcus jenensis*, *Pseudomonas* sp. and *Curtobacterium* sp. Some of the bacteria identified are beneficial like *Ochrobactrum* sp. *Erwinia* sp. and *Pantoea* sp. had a frequency of 28.95%, with *Pantoea agglomerans* having a frequency of 18.42%. *Ochrobactrum* sp. had a frequency of 10.53%. *Enterococcus mundtii* an frequency of 5.26%. Information about the species composition of bacteria on grain crops can be used to determine the spread of bacteria and their diagnosis and for bioinformatic analysis of genomes in search of species-specific genetic markers.

Keywords: Grain exports; primers; phyto-pathogens; identification; bacteria; wheat; barley; oats; Gene bank.

1. Introduction

2020 World bank data indicated that Russia's value-added agriculture expanded from \$45.9 billion in 2000 to \$66.2 billion in 2019 thus indicating a vast improvement. Grain production has increased significantly in the past years 2010 – 2019 [1]. This has allowed for improvement in the export value of grain from \$10.6 billion in 2019 to \$13 billion in 2021. Russia is currently one of the leading nations in grain export, trading about 34 million tons of grain (wheat, barley and maize) in 2021 alone, thus occupying a position of immense importance on the global market [2,3]. Grain export

generates a lot of money and in 2021 alone the nation made 8 962 670 000 USD [2]. To date the main importers of Russian grain are Egypt and Turkey [4]. China currently accounts for 15% of the flour (73 thousand tons in 2020 and in 2021) exported by Russia [2]. Exportation of grain might be limited and restricted by the presence of unwanted or quarantine pests and pathogens.

All grain exports have to meet certain criteria, specified by the importing country and these have to be highlighted in a phytosanitary certificate by the exporting nation [3]. Phytosanitary requirements have to be observed to prevent the spread of diseases across nations and continents. In grain crops there is regulation of *Pseudomonas syringae* and *Pseudomonas fuscovaginae*. Quarantine bacterial pathogens of wheat include members of the genera *Pseudomonas* and *Xanthomonas* for example *P. syringae* and *X. translucens* [5]. With these phytopathogens (*Xanthomonas translucens*, *Pseudomonas syringae* and *Pseudomonas fuscovaginae*) in mind and also given that the Republic of Crimea is a grain exporting region, from there grain samples were collected so as to detect and identify entire bacterial species within the plant samples, thus obtaining an estimation of their microbiome composition. Plant samples were collected, from them extracts were isolated and colonies were cultured so as to identify and detect various bacterial species within the samples and an estimate of the cultured bacterial composition of the plant samples was obtained. Bacteria have significant effects on plant growth, development, yield, health and nutrition thus an estimation of the microbiome composition of grain crops will help with the insight to all cultivated bacteria that can be found in grain crops [6]. The microbiome composition of crops varies depending on the crop thus the collection of different grain crops like barley and oats and not just wheat will help attain more comprehensive information on possible bacteria that make up a microbiome composition of grain crops. The species that can be present in the examined sample and from which the target species should be separated must all be known for bioinformatic prediction of a species-specific PCR target. PCR in conjunction with sequencing are widely used for identifying bacteria [7]. Prevention is better than cure and as such it is more important to prevent the spread of bacteria than having to try and control the damage caused to yield once they infest new areas. Hence, emphasis is stressed on the need to improve and develop efficient methods of bacterial detection [8]. The objective of this study were detection and identification of the cultivated bacteria in the samples collected from Belogorskij, Krasnogvardejskij and Simferopol'skij regions of the Republic of Crimea.

2. Materials and Methods

The objects of the study were bacterial isolates from samples of oats, wheat, triticale and barley plants. Sampling was carried out from the 1st to the 3rd of June 2021 from three regions (Belogorskij, Krasnogvardejskij and Simferopol'skij) of the Republic of Crimea. The plants upon sampling were in the initial phase of grain ripening known as the milky ripening stage. Analysis of the samples were conducted at the All-Russian Plant Quarantine Center. An analytical sample was prepared from each sample in accordance with the previously described technique [9] in the following way. To 5–10g of plant tissues chopped with sterilized scissors, 20 ml of phosphate-buffered saline was added (per 1 liter of distilled water 2.9g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.2g $\text{KH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 8g NaCl and 0.2g KCl; pH 7.0–7.2), left on the shaker for 1 hour at 200 rpm, then liquid part of the sample was passed through filters with a pore size of 3–5 μl and centrifuged for 10 min at 4°C, 10000g. The supernatant was removed and the precipitate was suspended in 1 ml of phosphate-buffered saline. Sample preparation was carried out within 1 week after sample collection. Before preparation samples were stored at 4°C in the dark. Bacterial isolates were isolated on Yeast extract-dextrose- CaCO_3 (YDC) [10] nutrient medium, for 5 to 7 days plated on three Petri dishes by the Drygalsky method, 20 μl of analytical samples. Using a sterile bacteriological loop, individual colonies were inoculated onto YDC nutrient medium. Some of them were not selected because they had morphological similarities to the colonies already selected. Different varieties of colony morphotypes grown on plates were selected. Small fragments of single colonies of the pure cultures thus obtained were collected using a sterile bacteriological loop and suspended in 200 μl of distilled water. The suspensions were used for DNA isolation. DNA of bacterial cultures was obtained using a commercial Proba-GS kit (ZAO Agro-Diagnostics, Russia) in accordance with the manufacturer's instructions.

All DNA samples were tested by conventional PCR. Amplification was carried out on a T100 thermal cycler (Bio-Rad, United States) using oligonucleotides synthesized at CJSC Evrogen (Russia) and ready-made mixtures for PCR 5½ MasDDTaqMIX-2025 (CJSC "Dialat", Russia). The first test was performed with primers 8UA/519B (8UA: 5'-AGAGTTTGATCMTGGCTCAG-3', 519B: 5'-GTATTACCGCGGCKGCTG-3') for the 16–23S rRNA region [11]. The PCR mixture per reaction contained 14 µl of water, 5 µl of 5x MasDDTaqMIX-2025, 2 µl of each primer at a concentration of 10 µmol, and 2 µl of DNA. Amplification: 96 °C - 10 min; then 35 cycles: 95°C - 15 s, 55°C - 30 s, 72°C - 30 s; 72 °C -10 min. The presence of the PCR product was checked using horizontal electrophoresis in 1.5% agarose gel. Amplicon residues not used for electrophoresis were subjected to purification and sequencing as described above. DNA samples for which PCR products with primers 8UA/519B were of low concentration for sequencing or those whose results in Blasts did not give exact identification were subjected to PCR with primers 27F/907R (27F: 5'-AGAGTTTGATYMTGGCTCAG-3', 907R: 5'-CCGTCAATTCMTTGGAGTTT-3') for the 16–23S rRNA region [12]. PCR mixture per reaction contained 16 µl water, 5 µl of 5x MasDDTaqMIX-2025, 1 µl of each primer at a concentration of 10 µmol and 2 µl of DNA. Amplification program: 95 °C - 5 min; then 35 cycles: 95 °C - 15 s, 58 °C - 30 s, 72 °C - 60 s; then 72 °C - 5 minutes. In the absence of 880 bp PCR product further PCR was done using primers PSF/PSR (PSF: 5'-AGCCGTAGGGGAACCTGCGG-3', PSR: 5'-TGACTGCCAAGGCATCCACC-3') [13]. Several copies of the 610 bp sequence, amplifiable with the indicated primers are located in tRNA in bacteria of the genus *Pseudomonas*. PCR mixture per reaction contained 16 µl water, 5 µl of 5x MasDDTaqMIX-2025, 1 µl of each primer at a concentration of 10 µmol and 2 µl of DNA. Amplification program: 95 °C - 10 min; then 25 cycles: 95 °C - 20 s, 64 °C - 15 s, 72 °C - 15 s; then 72 °C - 2 minutes. The presence of the PCR product was checked using a gel-documenting system (Bio-Rad, USA) after electrophoretic separation of PCR products in 1.5% agarose gel. Thus, members of the genus *Pseudomonas* were identified amongst the isolates. The amplicons remaining in the tube were purified using the kit Gene JET PCR Purification Kit (Thermo Fisher Scientific, USA) and used for Sanger sequencing using the Big Dye Kit, BigDye®XTerminator™ Purification Kit (Thermo Fisher Scientific, USA) on genetic analyzer AB-3500 (Applied Biosystems, USA) according to an adapted method [14]. The presence of the PCR product was checked using horizontal electrophoresis in 1.5% agarose gel. Amplicon residues not used for electrophoresis were subjected to purification and sequencing as described above.

The sequencing results were processed using the BioEdit program (<https://bioedit.software.informer.com/>). The decoded nucleotide sequences were compared using the BLAST service with the sequences available in GenBank (<https://blast.ncbi.nlm.nih.gov>). The result of identification was considered the organism with the maximum similarity. (Max score), automatically calculated by the BLAST service based on the calculation of the Query coverage and Percent identity indicators. If several such organisms were found in a taxon, the oldest taxon was considered as the result of the identification process. For each identified species and genus, the frequency of occurrence (A) was calculated using the formula [15]: $A = B/C * 100\%$, where B is the number of samples on which a bacterium with a certain species was found, C is the total number of analyzed samples. When calculating the frequency of occurrence of bacterial genera, both isolates identified to the species level and those identified only at the genus level were taken into account.

3. Results

In three regions (Belogorskij, Krasnogvardejskij and Simferopol'skij) of the Republic of Crimea, 38 samples of grain crops were collected including winter barley, winter wheat, oats, triticale and cereal legume mix as shown in Table 1. The sampling period for winter grain crops was in the milky ripening phase. There were no symptoms of bacterial diseases during sampling of winter crops on plants. A total of 38 samples of grain crops were taken (Table 1).

Table 1. The results of samples collected of grain crops from Belogorskij, Krasnogvardejskij and Simferopol'skij of the Republic of Crimea.

Crop	Cultivar	Number of samples
Winter Wheat	Aksiniya	3
	Gubernator Dona	6
	Asket	7
	Anka	1
	Karavan	1
	Korona	1
Winter Barley	Onega	5
	Vosxod	3
	Rubezh	2
	Espada	1
	Master	1
	Toma	1
Oats	Verny`j	1
	Skakun	1
	Podgorny`j	1
	-	1
Cereal mix (Triticale; wheat and barley)	-	1
Cereal-legume mix	-	1

Note. -means the cultivar is unknown. Amongst the samples collected 13 samples of Winter barley, 19 samples of Winter Wheat, 4 samples of Oats, 1 sample of Cereal mix (Triticale; wheat and barley) and 1 sample of Cereal-legume mix.

The three regions (Belogorskij, Krasnogvardejskij and Simferopol'skij) of the Republic of Crimea, 38 samples of grain crops were collected including winter barley, winter wheat, oats, triticale and cereal legume shown in the maps below:

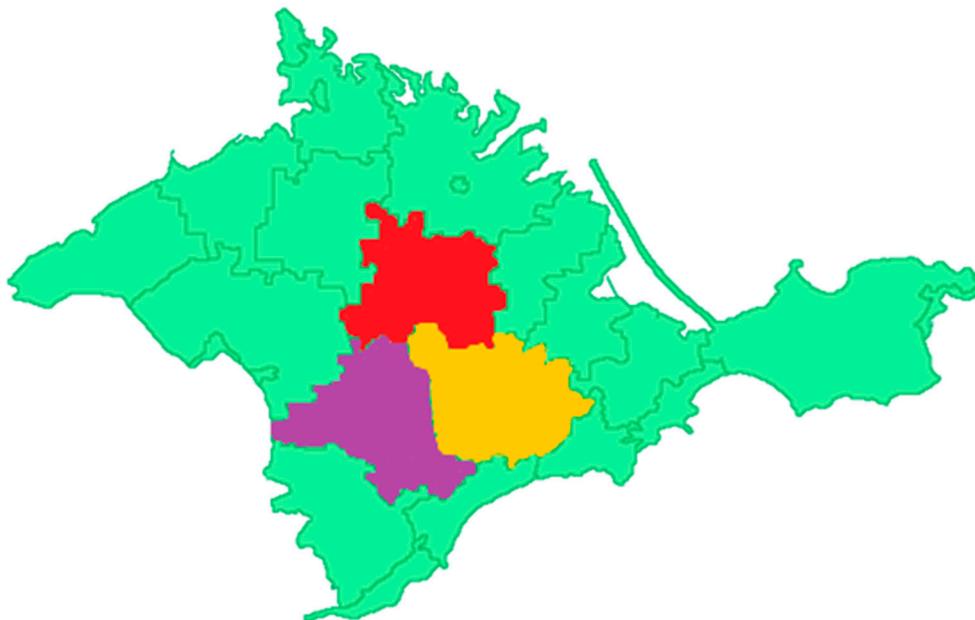


Figure 1. A map of The Republic of Crimea, in orange- Belogorskij from which samples C1 -C19 were collected, in red- Krasnogvardejskij from which samples C20, C21, C24, C26, C28, C33, C37, C38, C41, C42, C45, C46, C48, C51 and C53 were collected and in purple- Simferopol'skij from which samples C54, C58, C59 and C60 where C refers to Crimea and the sample number collected.

3.1. Bacteria identification

3 primer pairs used in identification of the bacteria, 8UA/519B, 27F/907R and PSF/PSR.

As a result of PCR with 8UA/519B primers, the 500 bp amplicon was obtained for 92 tested samples of DNA from bacterial cultures (Figure 2).

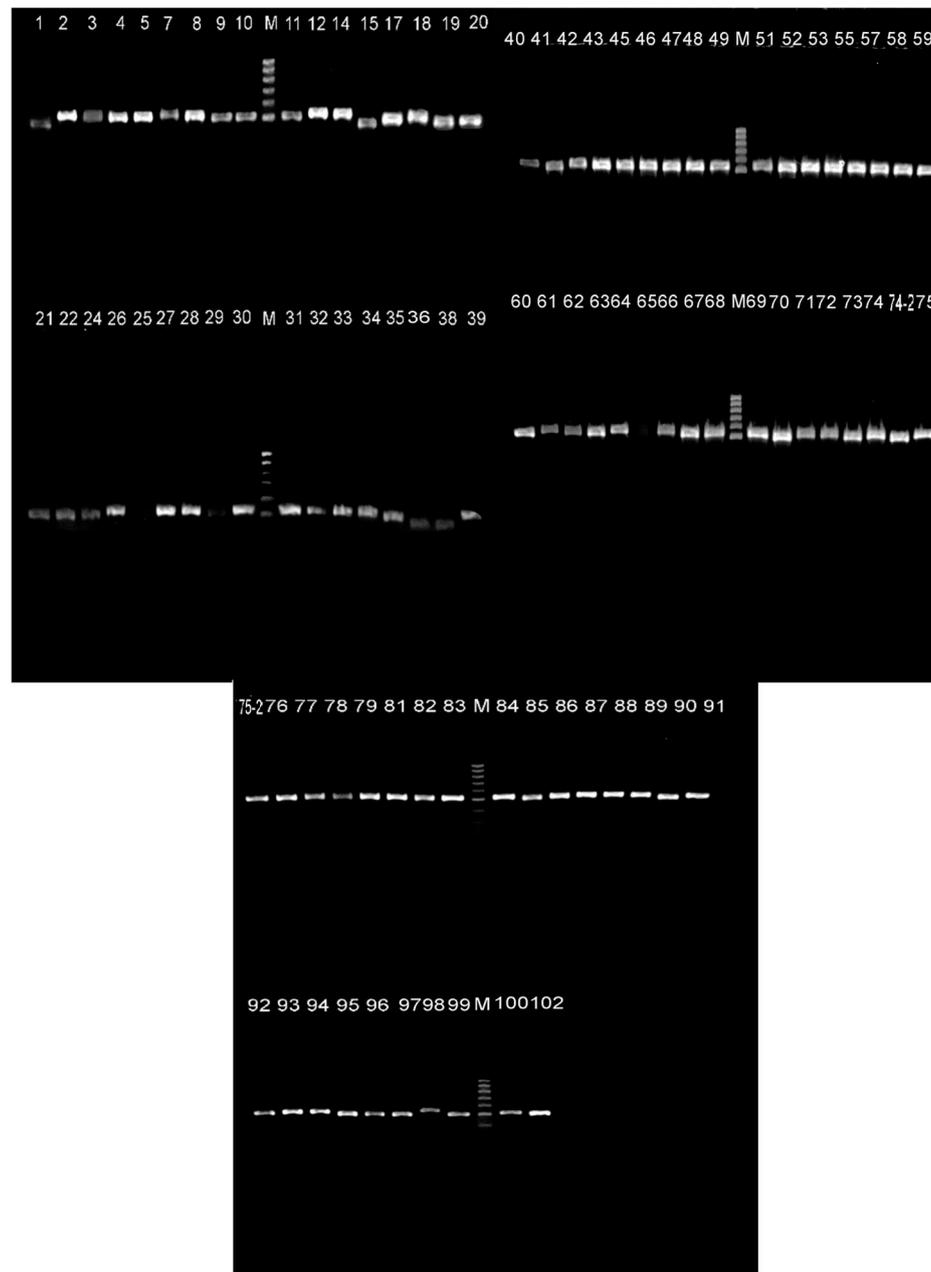


Figure 2. PCR products with primers 8UA/519B (500 bp) obtained for DNA samples of bacterial isolates from cereal varieties: 1-5 7-9 Winter Barley Onega, 10-12 Winter wheat Aksiniya, 14-15, 17 Winter wheat Asket, 18-22, 24 Winter barley Vosxod, 25-30 Winter wheat Gubernator Dona, 31-35 Winter barley Vosxod, 36, 38, 39 Winter wheat Asket, 41-42 Winter barley Onega, 43, 45-53, 55, 57-60 Winter wheat Gubernator Dona, 61-64 Winter wheat Asket, 66-75-1 Winter barley Onega, 75-2-79, 81-82 Winter wheat Asket, 83-85 Winter barley Rubezh, 86 Winter barley Espada, 87 Winter wheat Anka, 88 Winter wheat Karavan, 89, 90 Oats Verny`j, 91, 92 Oats Skakun, 93, 94 Oats Podgorny`j, 95-96 Winter barley Master, 97 Winter barley Rubezh, 98.1, 98.2 Winter barley Toma, 99 Triticale, 100 Cereal legume, and 102 Winter wheat Korona; M Molecular weight marker GeneRuler 100 bp Plus (100-1000 bp), ("Thermo Fisher Scientific, USA) (Belogorskij, Krasnogvardejskij and Simferopol`skij of the Republic of Crimea 2021).

As a result of PCR with 27F/907R primers, the 880 bp amplicon was obtained for 61 tested samples of DNA from bacterial cultures (Figure 2)

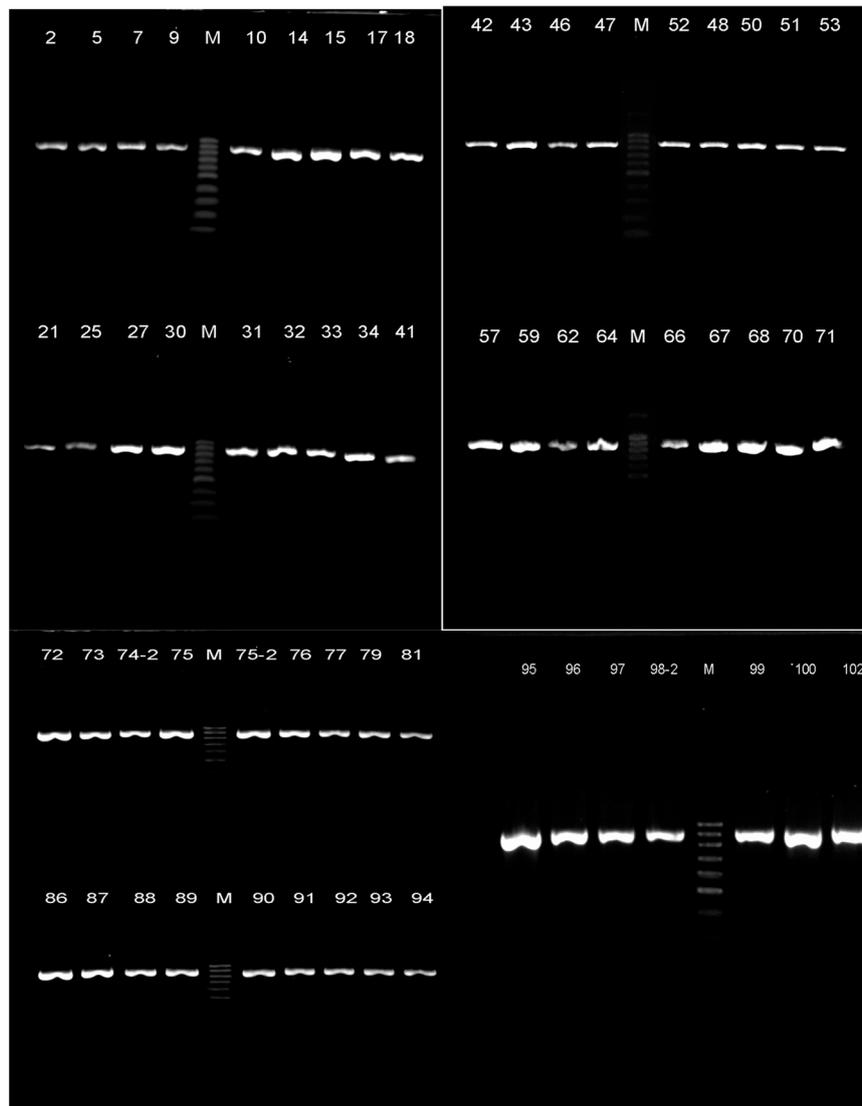


Figure 3. PCR products with primers 27F/907R (880 bp) obtained for DNA samples of bacterial isolates from cereal varieties: 2 5 7 9 Winter Barley Onega, 10 Winter wheat Aksiniya, 14,15, 17 Winter wheat Asket, 18 25 Winter barley Vosxod, 27, 30 Winter wheat Gubernator Dona, 31-34 Winter barley Vosxod, 41 42 Winter barley Onega, 43, 46-48, 50, 51, 53, 57,59 Winter wheat Gubernator Dona, 62, 64 Winter wheat Asket, 66-68, 70-73, 74-2, 75-1 Winter barley Onega, 75-2, 76,77, 79, 81 Winter wheat Asket, 86 Winter barley Espada, 87 Winter wheat Anka, 88 Winter wheat Karavan, 89, 90 Oats Verny`j, 91, 92 Oats Skakun, 93, 94 Oats Podgorny`j, 95-96 Winter barley Master, 97 Winter barley Rubezh, 98.2 Winter barley Toma, 99 Triticale, 100 Cereal legume, and 102 Winter wheat Korona; M Molecular weight marker GeneRuler 100 bp Plus (100-1000 bp), ("Thermo Fisher Scientific, USA) (Belogorskij, Krasnogvardejskij and Simferopol`skij of the Republic of Crimea 2021).

As a result of PCR with PSF/PSR primers, the 610 bp amplicon was obtained for 4 tested samples of DNA from bacterial cultures (Figure 4).

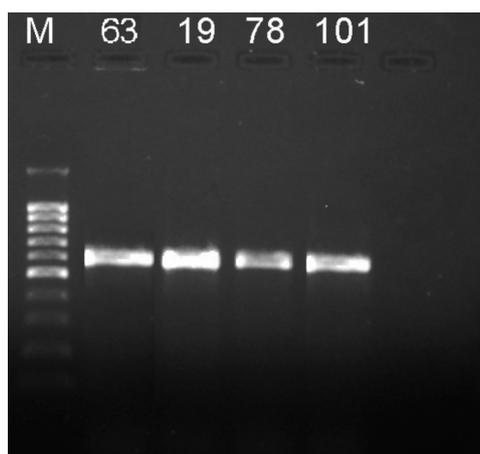


Figure 4. PCR products with primers PSF/PSR (610 bp) obtained for DNA samples of bacterial isolates from cereal varieties: 19 Winter barley Vosxod, 63 Winter wheat Asket, 78 Winter wheat Asket, 101 Oat (no name); M Molecular weight marker GeneRuler 100 bp Plus (100-1000 bp), ("Thermo Fisher Scientific, USA) (Belogorskij, Krasnogvardejskij and Simferopol'skij of the Republic of Crimea 2021).

Purification, sequencing and processing, using the BioEdit program made it possible to extract nucleotide sequences for each of the selected isolates and align these sequences in the BLAST service (<https://blast.ncbi.nlm.nih.gov>). These methods also made it possible for the identification of *Pantoea ananatis* in a winter wheat sample which is well known for forming brownish lesions with clear margins and yellow halos on wheat leaves causing bacterial disease [16]. Table 2 provides examples of the results achieved.

Table 2. The results of alignment of nucleotide sequences of bacterial isolates obtained from Wheat (*Triticum aestivum*) by Sanger sequencing (BLAST service, <https://blast.ncbi.nlm.nih.gov>; Belogorskij, Krasnogvardejskij and Simferopol'skij of the Republic of Crimea 2021).

Crop	Cultivar	Isolate №	Primers	Result of identification
Winter wheat	Aksiniya	10	27f-907r	<i>Frigoribacterium</i> sp.
		11	8UA-519B	<i>Pantoea</i> sp.
		12	8UA-519B	<i>Pantoea</i> sp.
Winter wheat	Aksiniya	14	27f-907r	<i>Ochrobactrum</i> sp.
		15	27f-907r	<i>Frigoribacterium</i> sp.
Winter wheat	Aksiniya	17	27f-907r	<i>Pantoea agglomerans</i>
		25	27f-907r	<i>Arthrobacter</i> sp.
Winter wheat	Gubernator Dona	26	8UA-519B	<i>Pantoea</i> sp.
		27	27f-907r	<i>Erwinia</i> sp.
Winter wheat	Gubernator Dona	28	27f-907r	<i>Clavibacter michiganensis</i>
		29	8UA-519B	<i>Pantoea agglomerans</i>
		30	27f-907r	<i>Erwinia aphidicola</i>
Winter wheat	Asket	36	27f-907r	<i>Pantoea</i> sp.
		37	27f-907r	<i>Erwinia</i> sp.
		38	8UA-519B	<i>Ochrobactrum</i> sp.
Winter wheat	Gubernator Dona	39	8UA-519B	<i>Pantoea agglomerans</i>
		43	27f-907r	<i>Rosenbergiella</i> sp.
		45	27f-907r	<i>Pantoea ananatis</i>
Winter wheat	Gubernator Dona	46	27f-907r	<i>Erwinia</i> sp.
		47	27f-907r	<i>Erwinia</i> sp.
		48	27f-907r	<i>Rosenbergiella</i> sp.
		49	8UA-519B	<i>Pantoea agglomerans</i>
		50	27f-907r	<i>Stenotrophomonas</i> sp.
		51	27f-907r	<i>Erwinia</i> sp.

Winter wheat	Gubernator Dona	52	27f-907r	<i>Rosenbergiella</i> sp.
		53	27f-907r	<i>Erwinia</i> sp.
Winter wheat	Gubernator Dona	55	8UA-519B	<i>Rosenbergiella</i> sp.
		57	27f-907r	<i>Erwinia</i> sp.
		58	8UA-519B	<i>Pantoea</i> sp.
		59	27f-907r	<i>Pantoea</i> sp.
		60	8UA-519B	<i>Pantoea</i> sp.
		61	8UA-519B	Uncultured bacterium
Winter wheat	Asket	62	27f-907r	<i>Erwinia</i> sp.
		63	PSf- PSr	<i>Pseudomonas</i> sp.
		64	27f-907r	<i>Exiguobacterium</i> sp.
Winter wheat	Asket	75-2	27f-907r	<i>Pantoea</i> sp.
Winter wheat	Asket	76	27f-907r	<i>Pantoea</i> sp.
Winter wheat	Asket	77	8UA-519B	<i>Pantoea</i> sp.
		78	PSf- PSr	<i>Pseudomonas</i> sp.
Winter wheat	Asket	79	27f-907r	<i>Pantoea agglomerans</i>
Winter wheat	Asket	81	27f-907r	<i>Plantibacter</i> sp.
		82	27f-907r	Bacteria
Winter wheat	Anka	87	27f-907	<i>Rosenbergiella</i> sp.
Winter wheat	Karavan	88	27f-907	<i>Rosenbergiella</i> sp.
Winter wheat	Korona	102	27f-907	<i>Pantoea agglomerans</i>

19 samples of Winter wheat were collected from which 45 colonies were isolated and identified with the use of 3 pairs of primers.

One sample of grain crops contained either a particular kind of detected bacterium, or a few detected bacteria (Table 3). *Erwinia rhapontici* was amongst some of the detected and identified bacterium in the samples of barley. *E. rhapontici* is known to cause premature death in infected plants [17]. Table 3 shows the list of the bacteria identified from the barley samples collected from The Republic of Crimea.

Table 3. The results of alignment of nucleotide sequences of bacterial isolates obtained from Barley (*Hordeum vulgare*) by Sanger sequencing (BLAST service, <https://blast.ncbi.nlm.nih.gov>; Belogorskij, Krasnogvardejskij and Simferopol'skij of the Republic of Crimea 2021).

Crop	Cultivar	Isolate №	Primers	Result of identification
Winter barley	Onega	1	8UA-519B	<i>Ochrobactrum</i> sp.
		2	27f-907r	<i>Erwinia aphidicola</i>
		3	27f-907r	<i>Rathayibacter festucae</i>
		4	27f-907r	<i>Arthrobacter</i> sp.
		5	27f-907r	<i>Arthrobacter</i> sp.
		7	27f-907r	<i>Stenotrophomonas maltophilia</i>
		8	27f-907r	<i>Erwinia</i> sp.
		9	27f-907r	Bacteria
		Winter barley	Vosxod	18
19	PSf- PSr			<i>Pseudomonas poae</i>
20	8UA-519B			<i>Plantibacter flavus</i>
21	8UA-519B			<i>Frigoribacterium</i> sp.
22	8UA-519B			<i>Erwinia rhapontici</i>
24	8UA-519B			<i>Erwinia rhapontici</i>
Winter barley	Vosxod	31	8UA-519B	<i>Erwinia</i> sp.
		32	27f-907r	<i>Pantoea</i> sp.
Winter barley	Vosxod	33	27f-907r	<i>Erwinia</i> sp.
		34	27f-907r	<i>Curtobacterium</i> sp.
		35	27f-907r	<i>Ochrobactrum</i> sp.
Winter barley	Onega	41	8UA-519B	<i>Enterobacter</i> sp.
		42	27f-907r	Uncultured bacterium

Winter barley	Onega	66	27f-907r	<i>Exiguobacterium</i> sp.
		67	27f-907r	<i>Stenotrophomonas</i> sp.
		68	27f-907r	<i>Stenotrophomonas</i> sp.
Winter barley	Onega	69	8UA-519B	<i>Pseudomonas</i> sp.
		70	27f-907r	<i>Plantibacter</i> sp.
		71	27f-907r	<i>Stenotrophomonas</i> sp.
		72	27f-907r	<i>Stenotrophomonas</i> sp.
		73	27f-907r	<i>Agrococcus jenensis</i>
Winter barley	Onega	74-1	8UA-519B	<i>Pantoea vagans</i>
		74-2	27f-907r	<i>Frigoribacterium</i> sp.
		75-1	27f-907r	<i>Rosenbergiella</i> sp.
Winter barley	Rubezh	83	27f-907r	<i>Pantoea pleuroti</i>
		84	27f-907	<i>Pantoea</i> sp.
		85	8UA-519B	<i>Microbacterium</i> sp.
Winter barley	Espada	86	27f-907	Uncultured bacterium
Winter barley	Master	95	27f-907	<i>Curtobacterium</i> sp.
		96	27f-907	<i>Clavibacter michiganensis</i>
Winter barley	Rubezh	97	27f-907	<i>Microbacterium</i> sp.
Winter barley	Toma	98	8UA-519B	<i>Enterococcus mundtii</i>
		98-2	27f-907	<i>Frigoribacterium</i> sp.

13 samples of Winter barley were collected from which 41 colonies were isolated and identified with the use of 3 primers.

The bacteria identified in the study were either beneficial or harmful to grain plants and in some cases both. *Pantoea agglomerans* (Table 3) is known to cause bacterial blight in grain crops and yet some of its strains are beneficial to the rhizosphere of some plants [18–20]. The results of bacteria identified from oats, Triticale and cereal legume mix are shown in Table 3.

Table 3. The results of alignment of nucleotide sequences of bacterial isolates obtained from Oats (*Avena sativa*) and 1 sample of Triticale and 1 sample of Cereal-legume by Sanger sequencing (BLAST service, <https://blast.ncbi.nlm.nih.gov>; Belogorskij, Krasnogvardejskij and Simferopol'skij of the Republic of Crimea 2021).

Crop	Cultivar	Isolate №	Primers	Result of identification
Oats	Verny`j	89	27f-907	<i>Pantoea</i> sp.
		90	27f-907	<i>Curtobacterium</i> sp.
Oats	Skakun	91	27f-907	<i>Rosenbergiella</i> sp
		92	27f-907	<i>Pseudomonas</i> sp.
Oats	Podgorny`j	93	27f-907	<i>Pantoea agglomerans</i>
		94	27f-907	<i>Pantoea</i> sp.
Oats	–	101	PSf- PSr	<i>Pseudomonas</i> sp.
Triticale, wheat, barley	–	99	27f-907	<i>Arthrobacter</i> sp.
Cereal-legume mix	–	100	27f-907	<i>Frigoribacterium</i> sp.

4 samples of Winter Oats were collected from which 7 colonies were isolated and identified with the use of 2 primers and 1 sample of Triticale, and 1 sample of Cereal-legume were collected from which 2 colonies were isolated and identified with the use of 1 primer set (27f-907).

From the 38 samples collected in The Republic of Crimea, 95 bacterial colonies were isolated whose DNA was extracted, of these 68 were identified to genus level and 22 to species level.

The methods applied in the study made it possible to determine the frequency of occurrence of various bacteria at genera and a species level. Frequency of occurrence of the *Pantoea* and *Erwinia* genera was the highest at 28.95% (Table 4). The diversity of the bacteria is shown with the most common being *Pantoea agglomerans*, *Pantoea ananatis*, *Pantoea vagans* and *Pantoea pleuroti* with various

frequency of occurrence. Table 4 shows the frequency of occurrence of all the detected and identified bacteria.

Table 4. The frequency of occurrence of species and genera of bacteria in samples. of grain crops from Belogorskij, Krasnogvardejskij and Simferopol'skij of the Republic of Crimea 2021.

Genus	Frequency %	Species	Frequency %
<i>Pantoea</i>	28.95	<i>Pantoea agglomerans</i>	18.42
		<i>Pantoea ananatis</i>	2.63
		<i>Pantoea vagans</i>	2.63
		<i>Pantoea pleuroti</i>	2.63
<i>Erwinia</i>	28.95	<i>Erwinia rhapontici</i>	2.63
		<i>Erwinia aphidicola</i>	5.26
<i>Rosenbergiella</i>	18.42	-	-
<i>Frigoribacterium</i>	15.79	-	-
<i>Stenotrophomonas</i>	7.89	<i>Stenotrophomonas maltophilia</i>	2.63
<i>Pseudomonas</i>	13.16	<i>Pseudomonas poae</i>	2.63
<i>Ochrobactrum</i>	10.53	-	-
<i>Arthrobacter</i>	10.53	-	-
Uncultured bacterium	7.89	Uncultured bacterium -	7.89
<i>Exiguobacterium</i>	5.26	-	-
<i>Curtobacterium</i>	7.89	-	-
<i>Microbacterium</i>	5.26	-	-
<i>Clavibacter</i>	5.26	<i>Clavibacter michiganensis</i>	5.26
Bacteria	5.26	Bacteria	5.26
<i>Rathayibacter</i>	2.63	<i>Rathayibacter festucae</i>	2.63
<i>Enterococcus</i>	5.26	<i>Enterococcus mundtii</i>	5.26
<i>Agrococcus</i>	2.63	<i>Agrococcus jenensis</i>	2.63
<i>Plantibacter</i>	5.26	<i>Plantibacter flavus</i>	2.63
<i>Enterobacter</i>	2.63	-	-

Note – means species were not identified within the given genus frequency of the various bacteria identified. For each identified species and genus, the frequency of occurrence (A) was calculated using the formula [13]: $A = B/C * 100\%$, where B is the number of samples on which a bacterium with a certain species was found, C is the total number of analyzed samples.

4. Discussion

From the 38 plant samples, 95 colonies were collected and 22 were identified to species level and other 68 were identified to genus level from which DNA was extracted and PCR was run and as a result the bacteria shown in Tables 2–4. Colony morphology, color shape and elevation we studied [21].

Pantoea sp., bacteria are mostly gram negative and form yellow, dark brown lesions on wheat [22]. This genus encompasses phytopathogenic bacteria and beneficial bacteria. *Pantoea ananatis* (strain TZ39) is a phytopathogen in rice where it causes bacterial blight [23]. Some *Pantoea* sp species have some strains that are beneficial towards plants particularly in the rhizosphere [24]. *P.*

agglomerans (BSL 2) strains are a good example. The strains of this bacterium, in particular strains C9-1 and E325 are used as biocontrol agents commercially [9]. *Pantoea* sp. TW18 has been reviewed and is highly recommended for industrial use that is in remediation of radionuclides in environmental cleanup [24,25].

Pantoea agglomerans is a plant bacterium that exists both as an endophyte and epiphyte [26]. Some *P. agglomerans* strain causes disease like bacterial blight in grain crops including maize; wheat; cotton onion and some ornamental plants [18,19]. *Pantoea agglomerans* has some strains that are beneficial to plants as it contributes to the wholesomeness of the rhizosphere and is beneficial towards the host plants [20].

Pantoea ananatis is a gram-negative, facultatively anaerobic, rod shaped, aerobic bacterium. *P. ananatis* forms brownish lesions with clear margins and yellow halos on wheat leaves causing bacterial disease. Cereal leaf beetle (CLB, *Oulema melanopus*, Coleoptera, Chrysomelidae) is a vector of *Pantoea ananatis* and feeds also on, oat and barley. The pathogen is transmitted via oral secretion by insects [16]. Other vectors of *P. ananatis* are *Diabrotica virgifera*, tobacco thrips, onion thrips, cotton fleahoppers (*Pseudatomoscelis seriatus*), mulberry pyralid (*Glyphodes pyloalis*), ticks, lice, and fleas [27–31]. This phytopathogen was noted for causing mulberry bacterial wilt [32]. It is also noted for increasing crop yield of host plants as a result of promoting plant growth. [33].

Pantoea vagans is a hydrogen-oxidase positive, non-spore forming gram negative short-roded and oxidase negative bacterium [34]. This bacterium's colonies are convex yellow-beige, smoothed edged colonies [35]. Previously known as a strain of *Pantoea agglomerans* it is a beneficial bacterium. It controls *Erwinia amylovora* which causes fire blight in apple and pear trees [36].

Pantoea pleuroti is a short-roded, non-spore forming, motile non-capsulated gram-negative bacterium that causes bacterial blight disease in *Pleurotus eryngii* an edible mushroom [37]. It grows well on tryptone glucose soy agar, forming yellow, convex, rough, round with entire margins. The bacteria grow well between 20- 37 °C and also at 40 °C and does not grow at 4, 10 and 44 °C [37].

Erwinia sp., bacteria are short rodged, non-spore forming facultative anaerobic gram-negative bacteria [38]. Some of the strains like *Erwinia* sp. (PR6) are actually beneficial as they promote plant growth [39]. The colonies are usually translucent, light beige circular colonies with soft margins. *Erwinia* sp is reported to have been found in wheat aphids in Russia [40]. Whilst some strains are beneficial some are harmful to plants. Its species mainly affect grain crops, vegetables and fruit [41].

Erwinia rhapontici was identified in this paper which is responsible for soft rot and pink seed [42]. In infected seeds it mainly causes premature plant death and poor plant emergence [17].

Erwinia aphidicola is a fermentative, oxidase negative bacterium [43]. When it infects seed poor germination is a common trait [40] It also causes bacterial leaf spot in common bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) [44].

Stenotrophomonas sp. is a gram-negative bacterium. It is a soil borne pathogen and also affects humans. Its hosts are both plants and soils. Usually located in either internal tissue like the root and stem vascular tissues and the external areas like the rhizosphere Bacterium pathogens belonging to this group are up to ten, including *Stenotrophomonas koreensis*, *Stenotrophomonas rhizophila*, *Stenotrophomonas maltophilia*, *Stenotrophomonas acidaminiphila*, *Stenotrophomonas humi*, *Stenotrophomonas nitritireducens*, *Stenotrophomonas terrae* and *Stenotrophomonas chelatiphaga* [45].

Stenotrophomonas. maltophilia is a beneficial bacterium to the ecosystem and to the health of the plant, thus making it of economic value. This species plays a vital role in plant and soil cycles thus making them very valuable to the soil ecosystem. Further laboratory work has seen this bacterium being used in the production of biomolecules, bioremediation and biocontrol. Some of its strains however have been noted to be pathogenic to humans with a compromised or weak immune system, this bacterium has been noted to be resistant to drugs. *S. maltophilia* is found either inside the plant that is vascular tissues of the stem and the roots, or outside the plant that is in the rhizosphere of the plants as it is involved in sulphur and nitrogen cycles. This bacterium has not only been noted to assist with plant growth but also assisting in the mitigation of abiotic stress within the plant and also in control of some pathogens [46] This bacterium is usually found in association with plants like oilseed, wheat, potatoes, various weeds and maize amongst others [45,47].

Pseudomonas sp. is a genus that has more than 150 species whose bacteria belong to the family Pseudomonadaceae and are rod shaped, facultative aerobes and gram negative [48]. The species of this genus are of concern as it occupies 20% of the bacteria identified in the grain excluding oats, triticale and barley. *Pseudomonas* spp comprise of phytopathogens like *Pseudomonas putida* and *Pseudomonas syringae* [49,50]. *Pseudomonas syringae* pv. *atrofaciens* causing a basal glume rot [51]. Not all species within this genus are harmful some of them are actually beneficial, to plant growth particularly *Pseudomonas gramilis*, *Pseudomonas fluorescens*, *Pseudomonas migulae* and *Pseudomonas lini* [52]

Pseudomonas poae is a beneficial microbe in the rhizosphere of plants. One study showed that in the rhizosphere of *P. heterophylla*, the presence of *F. oxysporum* promotes the growth of *P. poae* and also is responsible for attracting it [53]. This bacterium has been noticed to be beneficial in the conversion of phosphorous to make it available for plant uptake and in some cases also for nitrogen [54]. In enabling the uptake of nutrients by breaking them down this bacterium is known to enhance plant height [55].

Clavibacter michiganensis it is a gram-positive bacterium [56]. this bacterium affects mainly the xylem vessels on the plants [57]. It causes systematic vascular bacterial wilt of plants and leaf blight [58]. In wheat the bacterium is known to cause mosaic disease symptoms. It mainly affects tomatoes by causing bacterial canker. This bacterium transmitted via seed and bacteria infested plant residues [59]

Plantibacter flavus promotes plant growth. Two strains of the bacterial pathogen M251 and M259 were found to increase the length root-length and total biomass and promote shoot and root growth respectively. These two bacterial strains were found to have genes that are beneficial to plant pathways namely auxin and cytokinin biosynthesis and 1-aminocyclopropane-1-carboxylate deaminase production due to the 70 actinobacterial putative plant-associated genes [60].

Microbacteriaceae bacterium are mainly aerobic, rod shaped, motile, gram positive, mesophilic and a non-spore forming bacteria [60]. They are widely dispersed in diverse terrestrial and aquatic settings and frequently live inside of plants as pathogens or endophytes [61]. In plants afflicted by various nematodes, numerous putative novel species of multiple genera in this family were discovered alongside representatives of recognized species [62]. The leaves of various herbaceous and woody plants infected with nematodes, leaf-mining insects, and plant parasitic mites were found to include *Microbacteriaceae* strains in both affected and unaffected areas [63].

Curtobacterium sp., has been identified not just an endophyte in plants but also a plant disease causative agent in grain plants including rice and sorghum and in citrus like grapevine and strawberries [64]. This genus includes the species like *Curtobacterium flaccumfaciens* this is a seed borne, motile, aerobic, gram positive bacterium whose motility is aided by flagella whose colonies are usually shiny, round, yellow and smooth in some cases even convex on Potato Dextrose Agar (PDA) [65–67]. This pathogen is of economic significance as it infects both juvenile and aged plants by causing wilting because it affects the vascular system of plants [68]. *Meloidogyne incognita* is a nematode that facilitates this pathogen in entering the plant via wounds. It is responsible for diseases mainly in beans like bean bacterial wilt [69].

Rathayibacter festucae mainly affects wheat and cause yellow bacterial slime in wheat kernels on the leaves and stems leading to gumming diseases. This bacterium produces rose orange colonies [70]. The bacteria are known to cause yellow bacterial slime on host plants' leaves, seedheads and stems therefore causing gumming diseases [71]. Some bacterial pathogens like *Rathayibacter* sp are transmitted by nematode and upon infection they cause decay and more openings for more bacteria to infect the plants [70].

Agrococcus jenensis is a yellow pigment, non-spore forming, yellow pigment, gram positive and non-motile bacterium [72]. It grows between 15 to 37°C with optimal growth at 30°C at 42°C no growth is noted and a pH of 5 to 11 with an optimal pH of 7.5 [72]. In its genome this bacterium has alkanesulfonate assimilation genes. These are commonly used in paints and thus it is also classified as a beneficial pathogen[73].

Enterococcus mundtii is a bacterium that can be isolated from plant surfaces. It is a catalase negative, gram-positive, facultative anaerobic bacterium that can survive in extreme conditions of temperatures in the range 5-65 °C and pH 4.5-10 and also in other hostile environments [74]. This bacterium is also known as a lactic acid bacteria (LAB). LAB species aid in silage fermentation and as such influence silage quality [75]. LAB species produce bacteriocins these can be used in food preservation and most importantly in the control of *Listeria monocytogenes* which are known to infect vegetables via the roots and are a hazard to both human and animal health. Application of *E. mundtii* strains (WFE3, WFE20 and WFE31) to soil has reduced levels of *Listeria monocytogenes* in soil thus aiding in the improvement of nitrate nitrogen (NO₃-N) levels of soil [76].

Rosenbergiella sp. are oxidase negative, gram-negative, facultatively anaerobic, motile, rod-shaped bacteria that will not grow beyond 36 °C with optimum growth at 28–30 °C. It forms yellow-orange-pigmented colonies on nutrient media [77]. They are known to produce high levels of IAA (indole acetic acid) and thus improving plant germination and the growth rate of plants [78].

Frigoribacterium sp., are aerobic, short rod-shaped, non-motile gram positive bacteria that produce white colonies. Optimum conditions for growth are between 8 pH level and 25 - 30 °C, though growth has been noted even at 10 to 45 °C and pH levels of 5 - 10 [79,80].

Ochrobactrum sp. are salt tolerant, ammonia oxidizing bacteria. They are beneficial bacteria at a commercial level where they are used to amend soils. These bacteria are also cold resistant thus making it very useful in ammonia degradation for nitrogen fixation [81].

Arthrobacter sp. are aerobic, gram-stain-positive, non-spore-forming bacteria that is soil borne. Bacteria strains within this genome aid plant growth by assisting in phosphate solubilization [82]. They only grow within a pH range of 5 to 11 and a temperature of 10 °C to 37 °C, however growth optimums for temperature and pH are 25 °C and 7 respectively. On nutrient media it produces cream circular convex colonies [83].

Exiguobacterium sp. are bacteria that can survive in a wide range of temperatures that has proven to be vital in biodegradation of plastic [84]. These bacteria considered beneficial as they help improve soil nitrogen content by harboring nitrogen fixing traits [85]. Some of its bacterial strains (*Exiguobacterium* sp. WS1-12) were also noted to have a positive effect on root hair formation thus a positive effect on plant growth including grain (wheat) [82].

Plantibacter sp. are gram-positive bacteria of varying sizes that is rod shaped and produces yellow pigments [86].

Enterobacter sp. are motile, gram-negative, non-spore forming facultatively anaerobic bacteria [87,88].

5. Conclusion

38 samples of grain crops were collected from three regions of Crimea from which we isolated 95 bacterial colonies were isolated, 68 were identified to genus level, 22 were identified to species level using the molecular genetic diagnostic methods described in this paper. Among them, phytopathogens include *Agrococcus jenensis*, *Pseudomonas* sp. and *Curtobacterium* sp., *Pantoea ananatis* and *Clavibacter michiganensis*. *Erwinia rhapontici* is included in the A1 list of the Euro-Mediterranean Plant Protection Organization (EPPO) meaning it is a missing or undetected quarantine organism in Brazil and thus regulated by phytosanitary requirements of Brazil, Sudan, Colombia and Mali (<https://fsvps.gov.ru/ru>). *Pantoea ananatis* is included in the alarm list of the North American Plant Protection Organization (NAPPO). Whilst *Pseudomonas* sp. is not mentioned as quarantine pathogen some of the genera within this species are considered to be Quarantine pathogens like *Pseudomonas syringae* in Mexico, Taiwan and Colombia. *P. syringae* is currently under regulation but not considered a Quarantine pathogen in countries like Great Britain, Egypt and Zimbabwe. *Clavibacter michiganensis* is currently on the alarm list of Guinea (<https://fsvps.gov.ru/ru>, <https://gd.eppo.int/>). Bacteria with economically useful properties were also isolated and identified: *Pantoea agglomerans*, *Arthrobacter* sp., *Exiguobacterium* sp., *Ochrobactrum* sp., *Rosenbergiella* sp., *Enterococcus mundtii*, *Agrococcus jenensis*, *Plantibacter flavus*, *Pantoea vagans*, *Pseudomonas poae* and *Stenotrophomonas maltophilia*. According to the literature, *Frigoribacterium* sp., *Plantibacter* sp. and *Enterobacter* sp. neither have pronounced

harmful nor beneficial properties. The highest frequency of occurrence, 28.9%, was noted in species belonging to the genus *Pantoea* and *Erwinia*. Representatives of the genera *Rosenbergiella* (18.42%), *Frigoribacterium* (15.79%), *Pseudomonas* (13.16%), *Ochrobactrum* and *Arthrobacter* (10.53%) are also characterized by a high frequency of occurrence. The obtained experimental data on the species composition of bacteria on grain crops can be used in the analysis of the spread of bacterioses in the territory of the Russian Federation and their diagnostics, as well as for the bioinformatic analysis of bacterial genomes in the search for species specific.

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