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Effects of Natural Antimicrobials on *Xanthomonas* Strains Growth

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Abstract: The aim of this work was to investigate the most promising natural antimicrobials effective for the growth suppression of *Xanthomonas* spp. bacteria. The research objects were *Xanthomonas* spp. strains isolated from tubers and stem of plants growing in Lithuania: *Xanthomonas translucens* NRCIB X6, *X. arboricola* NRCIB X7, NRCIB X8, NRCIB X9, and NRCIB X10; the supernatants of lactic acid bacteria *Lactococcus lactis* strains 140/2, 57, and 768/5, *Lactobacillus helveticus* strains 14, 148/3, R, and 3, *Lb. reuteri* 3 and 7, *Streptococcus thermophilus* 43, *Enterococcus faecium* 59-30 and 41-2; endophytic bacterial strains *Bacillus*, *Pseudomonas*, and *Paenibacillus* spp.; and essential oils of lavender (*Lavandula angustifolia*), grapefruit (*Citrus paradisi*), pine (*Pinus sylvestris*), thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*), peppermint (*Mentha piperita*), lemon (*Citrus limetta*), aqueous extracts of blueberries (*Vaccinium myrtillus*), and cranberries (*Vaccinium vitis-idaea*). The antimicrobial activity of tested substances was determined by agar diffusion method. Supernatants of *Lb. reuteri* strain 7 and *Lb. helveticus* strains 14, R, 3, and 148/3 were found to have a high antimicrobial activity against *Xanthomonas* spp. bacteria strains when compared to the positive control—1.0% copper sulfate (diameter of inhibition zones was 28.8 ± 0.7 mm). The diameter of inhibition zones of supernatants ranged from 23.3 ± 0.6 mm to 32.0 ± 0.1 mm. Thyme (2.0%) and lavender (2.0%) essential oils inhibited the growth of *Xanthomonas* spp. strains. The diameter of the inhibition zones was from 14.7 ± 0.8 mm to 22.8 ± 0.9 mm. The aqueous extracts of blueberries had a weak antimicrobial activity. The diameter of inhibition zones ranged from 11.0 ± 0.2 mm to 13.0 ± 0.2 mm.

Keywords: antimicrobial substances; inhibition; phytopathogenic bacteria; *Xanthomonas*

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1. Introduction

Bacterial diseases of plants cause devastating damage to crops and significant economic losses [1–3]. They cause about 12% of plant-origin food loss every year. Phytopathogenic bacteria have the ability to quick adaptation; therefore, eradicating the causes of disease is not an easy task. In addition, climate change scenarios predict that the adaption and migration of plant pathogenic bacteria will accelerate, as changing conditions will help pathogens to spread geographically from Western Europe to the North [4,5].

The bacterial genus *Xanthomonas*, belonging to the family *Xanthomonadaceae*, harbour some of the most devastating plant pathogens that continually cause food safety problems. Together, all of these taxa can infect many plants found in agriculture, forest, or natural ecosystems. As many as three species of *Xanthomonas* are among the ten economically and scientifically significant pathogenic bacteria compiled by scientists [2]. Particular attention has been paid to these species due to the very wide range of plants and the exceptional potential pathogenicity to their plant hosts—the main pathogens of rice and cassava, *X. oryzae* pv. *oryzae*, and *X. axonopodis* pv. *manihotis*, respectively, and *X. campestris* pathovars, which are causal agents of many crops worldwide [2].

The aforementioned bacteria causing plant diseases reduce the quantity and quality of the yield obtained. Although various methods of controlling pathogenic bacteria are used, microbiological safety remains a major concern for the agricultural industry. Billions of tons of pesticides are used to fight these plant diseases every year, and many of the antimicrobials currently available are highly toxic, non-biodegradable, and cause significant environmental pollution and rise human health risks [6]. At the present, various compounds, such as copper oxychloride, copper hydroxide, and copper oxide, are used in agriculture to prevent the spread of *Xanthomonas* spp. bacteria [7,8]. However, the widespread use of these agrochemicals increases the resistance of bacterial pathogens and causes significant damage to the environment [6,9].

For these reasons, researchers have focused on plant-derived natural bactericides and their possible applications in agriculture. The search for measures to control plant bacterial diseases are being intensified, as they have enormous potential, and modern agrochemical research is carried out to develop sustainable plant protection strategies and significantly reduce yield losses [10–14]. Naturally occurring and biologically active plant products, such as essential oils, organic extracts, or plant-associated microbiota, could be a source of alternative classes of natural biopesticides and a source of new and more effective compounds suitable for controlling plant pathogenic microorganisms [12,15–17]. In this work, new effective antimicrobials derived from natural sources were sought and therefore economically valuable. Such substances include essential oils, supernatants of lactic acid bacteria, compounds produced by endophytic bacteria, and berry extracts.

The main objective of this work was to assess and select the most effective antibacterial agents in vitro against the pathogenic *Xanthomonas* spp. strains.

2. Materials and Methods

2.1. Identification of Isolated Endophytic and Xanthomonas spp. Bacteria

In 2017–2018, plant pathogenic and endophytic bacterial strains from roots and nodules were isolated from the Fabaceae plants in the Laboratory of Plant Pathology at the Nature Research Centre (Vilnius, Lithuania). Phytopathogenic bacteria were isolated from plant material with disease symptoms [18,19]. The following *Xanthomonas* strains were used: *X. translucens* (Xt) NRCIB X6 and *X. arboricola* (Xa) strains NRCIB X7, NRCIB X8, NRCIB X9, and NRCIB X10.

Endophytic bacteria were isolated from legume roots and nodules. The plants were dug out and placed in sterile plastic bags, taken to the laboratory, and kept in the fridge at 4 °C until samples were prepared for further analysis. Soil was removed from roots and nodules carefully by gentle washing in running tap water; the surface was sterilized by 70% ethyl alcohol for 1 min. and followed by a solution of 6% sodium hypochlorite for 10 min. Samples were rinsed with sterile dH_2O three times to remove surface sterilization agents. Each root sample was cut into approximately 1-cm fragments and ground in 20 µL of sterile water, and 3–5 µL of each root suspension was spread on King's B agar plates and incubated at 27 °C for 3–5 days till the colonies of endophytic bacteria developed; then, bacterial colonies were transferred onto the same agar and purified [18,19]. Endophytic bacterial strains belonging to *Bacillus*, *Paenibacillus*, and *Pseudomonas* genera were used: Bacillus sp. strains NRCIB B1, NRCIB B2, NRCIB B3, NRCIB B4, NRCIB B5, NRCIB B6, *Pseudomonas* sp. strains NRCIB P1, NRCIB P2, NRCIB P3, and *Paenibacillus* sp. NRCIB PB1.

Strains were identified by Gram staining [19]. All gram-negative bacteria were tested for hypersensitive reaction (HR) on tobacco (*Nicotiana tabacum* L. 'Samsun') [20] and tomato (*Solanum lycopersici*) plants [18]. A milky suspension (10^8 cells/mL) was infiltrated in the mesophyll between the two epidermis of tobacco and tomato leaves using a small sterile syringe. Plants were evaluated after 24 h, and if the tissues became necrotic, the reaction was considered as positive [20,21]. The ability to cause rot on potato and pectolytic activity were investigated; 2-day old bacterial isolates grown on King's B medium at 28 °C were inoculated in a center of 2.5-cm diameter sterile potato slice placed in a sterile Petri dish on

moist, sterile filter paper. Pectinolytic properties were evaluated after 24 h of incubation at 28 °C [18,22].

All bacterial isolates were selected for further molecular studies: bacterial genomic DNA was extracted using CTAB protocol [23] and Aljanabi and Martinez [24] protocol with slight modifications described by Kałużna et al. [22]. Yellowish *Xanthomonas*-like bacteria were screened by PCR using genus-specific primers X1 and X2 [25]. PCR reaction mix (15 µL) contained 1.5 µL 10× PCR buffer (Green Buffer DreamTaq, ThermoFisher, Vilnius, Lithuania), 1.3 µL dNTPs (2 mM), 1 µL of each primer (10 pmol), 0.08 µL (2 U) Taq DNA polymerase, and 2 µL of bacterial DNA (15 ng). PCR was carried out in a thermal cycler (MJ Mini, Biorad, Hercules, CA, USA). Cycling conditions were initial denaturation for 2 min at 95 °C and 29 cycles of 45 s at 95 °C, 45 s at 37 °C, and 1.5 min at 62 °C; the final extension step took 10 min at 62 °C. The PCR products were visualized on UV by running in a Tris-acetate agarose gel (1.5%) electrophoresis.

Then, all pathogenic and endophytic bacterial isolates were identified by sequenced bacterial 16S rDNA. PCR reaction mix with universal primers 27F and 1492R and amplification conditions according to Lane et al. [26] were used: initial denaturation for 5 min at 94 °C and 32 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1.5 min at 72 °C; the final extension step took 10 min at 72 °C. The PCR products were visualized on UV by running in a Tris-acetate agarose gel (1.5%) electrophoresis. Then, DNA sequence of each strain was subjected to the analysis using DNA LaserGene package National Center for Biotechnology Information database [27].

2.2. Microorganisms and Growth Media

This study focuses on twelve lactic acid bacteria (LAB) strains [28] obtained from the Kaunas University of Technology (KTU) Food Institute LAB collection: *Enterococcus faecium* 59–30, *E. faecium* 41-2B 2v, *Lactobacillus helveticus* 14, *Lb. helveticus* 3, *Lb. helveticus* 148/3, *Lb. helveticus* R, *Lb. reuteri* 3, *Lb. reuteri* 7, *Lactococcus lactis* 140/2, *L. lactis* 57, *L. lactis* 768/5, and *Streptococcus thermophilus* 43. Cultures of each bacterial strain were stored at minus 80 °C in 15% glycerol. Active bacterial strains for experimental use were prepared by transferring a loopful of cells from stock cultures onto yeast extract calcium carbonate dextrose agar (YDA) (HiMedia, Mumbai, India) at 27 °C for 24–72 h (in the case of *Xanthomonas* spp. and endophytic bacteria) and MRS broth (Oxoid Ltd., Basingstoke, UK) at 30 °C or 37 °C for 48 h (in the case of LAB strains).

2.3. Determination of Antibacterial Activity

The agar well diffusion method was used to determine the antibacterial activity of substances of natural origin: supernatants of lactic acid bacteria (LAB), essential oils, blueberry and cranberry extracts, and endophytic bacteria obtained from the roots and nodules of *Fabaceae* plants.

Briefly, the *Xanthomonas* spp. strains were pre-cultivated on YDA slants for 48 h at 27 ± 1 °C. The grown-up bacterial cultures were washed off from the agar with sterile phosphate-buffered saline (PBS) solution (Na_2HPO_4 0.27%, NaH_2PO_4 0.04%, NaCl 0.8%, pH 7.2 in distilled water), and the density of cell suspension of each culture was adjusted according to McFarland standard No 0.5. One milliliter of the prepared suspension was added to the YDA medium, melted before, cooled to 45 °C, and was mixed thoroughly. The prepared mixture of bacteria cell suspension and the medium was poured into 90-mm Petri dishes, 12 mL each. After the medium had solidified, and agar surface had dried, wells of 8-mm diameter were made in the plates, which were filled with 50 µL of the examined solution. Antimicrobial effect against the bacterial pathogens was evaluated after 48 h of growth at 27 ± 1 °C according to the diameter (in mm) of inhibition zones around the wells. If no inhibition zones formed around the wells, the test solution had no antibacterial effect on the tested bacteria culture. The zones of inhibition (ZOI) were evaluated as follows: 8–9 mm, no effect; 10–15 mm, weak effect; 16–22 mm, medium effect; and 23–32 mm, strong effect.

2.4. Cell-Free Supernatants of Lactic Acid Bacteria

For determining antibacterial activity, 1 mL of LAB (in sterile milk) was added to 10 mL of MRS broth (Oxoid, UK) and incubated for 48 h under the optimal temperature (30 °C or 37 °C). Cell-free supernatants (CFS) were obtained, removing cells by centrifugation at 6000 rpm for 15 min. Then supernatant was filtered through the 0.2-µm pore filter.

2.5. Essential Oils

Ten essential oils were obtained from commercial sources (UAB "Mėta", Lithuania). Essential oils of thyme (*Thymus vulgaris*), grapefruit (*Citrus paradisi*), lavender (*Lavandula angustifolia*), rosemary (*Rosmarinus officinalis*), lemon (*Citrus limon*), peppermint (*Mentha piperita*), pine (*Pinus sylvestris*), tea tree (*Melaleuca alternifolia*), juniper (*Juniperus communis*), and silver fir (*Abies sibirica*) in concentrations of 1.0% (*v/v*) and 2.0% (*v/v)* were used.

2.6. Blueberry and Cranberry Aqueous Extracts

Blueberries (*Vaccinium myrtillus*) were collected from three different location sites in four countries (Lithuania, Latvia, Finland, and Norway), and cranberries (*Vaccinium vitis-idaea*) were collected from two different location sites in Lithuania. The berries were harvested during the ripening period (July–September and November) and stored in the freezer at minus 80 °C. For extraction, 5 g of frozen berries were crushed in a mortar and shaken for 30 min in 90 mL of deionized water at 40 °C. Then, the extracts were filtered through Whatman No.1 filter paper. The obtained extracts were transferred into vials and used for further analysis.

2.7. Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of antimicrobial essential oils (thyme, grapefruit, lavender) was determined using the broth microdilution method. The test samples of essential oils were dissolved in methanol and added to LB medium (Liofilchem diagnostici, Roseto degli Abruzzi, Italy,) to get a final concentration of 1000 µg/mL, which were further serially diluted to 500, 250, 125, 62.5, 31.25, 15.62, 7.81, and 3.9 µg/mL, respectively. The final concentration of methanol in the culture medium was maintained at 0.5% (*v/v*). A total of 50 µL of test organism suspension (1.5×10^8 CFU/mL approximately) was transferred into each tube. All tubes, including the control, containing the bacterial suspension in LB medium were incubated at 27 ± 1 °C for 48 h. The lowest concentrations of the test samples which, after macroscopic evaluation, did not show any growth of the test organisms, were determined as MICs and were expressed in µg/mL [29]. All tests were performed in triplicate.

2.8. Statistical Analysis

Statistical analysis of test findings was carried out using SPSS 16 package for statistical data processing. Tukey's HSD test was used in order to determine the significant difference ($p < 0.05$) between the obtained research results among inhibition zone sizes against different microorganisms. The tests were repeated 4–5 times.

3. Results

3.1. Antibacterial Activity of Endophytic Bacterial Strains

During 2017–2018, plant material from different species of *Fabaceae* growing in Lithuania was collected. In this study, 295 bacterial isolates were obtained and investigated. All gram-negative bacteria were tested for hypersensitive reaction (HR) on tobacco (*Nicotiana tabacum* L. 'Samsun') [20], and *Xanthomonas*-like isolates were tested on tomato (*Solanum lycopersici*) plants (Figure 1) [18]. *Xanthomonas*-like isolates showing pathogenicity to tomato plants and having pectolytic activity were identified by 480-bp band produced in PCR using genus-specific primers X1 and X2 [25]. The approximately 1300-bp PCR product of the 16S DNA fragment [26] was then sequenced. Our studies revealed that only five isolates belong to the genus *Xanthomonas*: one strain was classified as *X. translucens* (Xt)

(NRCIB X6) and four as *X. arboricola* (Xa) (NRCIB X7, NRCIB X8, NRCIB X9, and NRCIB X10) (accession numbers in GenBank: OL504772-OL504776).

(**a**) (**b**)

Figure 1. The yellowish bacteria *X. arboricola* NRCIB X8 (**a**) and hypersensitive reaction test on tomato (**b**).

Endophytic bacteria can protect the plant from pathogens. Thus, 10 out of 73 endophytic non-pathogenic isolates without pectolytic activity were selected as the most effective isolates against pathogenic *Xanthomonas* strains (Table 1). These bacteria were identified by sequenced 16S rDNA (accession numbers in GenBank: OL505113-OL505122).

Table 1. Antibacterial activity of different endophytic bacterial strains against pathogenic *Xanthomonas* spp. strains.

Antagonistic Bacteria Strain	Average Zone of Inhibition (ZOI), mm				
	Xanthomonas spp. Strain				
	***X. translucens* NRCIB X6**	***X. arboricola* NRCIB X7**	***X. arboricola* NRCIB X8**	***X. arboricola* NRCIB X9**	***X. arboricola* NRCIB X10**
Pseudomonas sp. NRCIB P2	33.0 ± 0.0 d	33.0 ± 0.0 d	25.9 ± 0.5 b,f	24.1 ± 0.4 a,f,g	23.0 ± 0.5 b,d,g
Pseudomonas sp. NRCIB P1	32.7 ± 0.8 d	31.2 ± 0.7 c	23.2 ± 0.3 a,e,i	24.0 ± 1.5 a,e,g	23.7 ± 0.3 b,d,f,g
Pseudomonas sp. NRCIB P3	27.2 ± 0.4 e	25.5 ± 0.6 b	25.1 ± 0.8 a,b,g	22.5 ± 0.6 d,g	24.8 ± 1.4 c,g
Bacillus sp. NRCIB B5	24.6 ± 0.0 c	25.2 ± 0.3 b	22.7 ± 0.3 c,d,e,g	22.9 ± 0.1 c,d,e,f	22.2 ± 0.2 d,i
Bacillus sp. NRCIB B4	21.8 ± 0.5 a,g	21.5 ± 0.6 a	25.1 ± 0.7 a,b	26.2 ± 1.1 a	24.5 ± 1.4 b,c
Bacillus sp. NRCIB B1	22.2 ± 0.1 a	21.0 ± 0.1 a	23.5 ± 0.2 a,c,f	25.1 ± 0.6 a,c	25.2 ± 0.2 a,c,f
Bacillus sp. NRCIB B2	-	21.4 ± 0.2 a	25.3 ± 0.2 a,b	30.3 ± 0.5 b	27.0 ± 0.4 a
Bacillus sp. NRCIB B3	21.2 ± 0.9 a,g	20.6 ± 0.4 a	26.8 ± 2.2 b	25.4 ± 1.3 a	23.0 ± 0.0 b,d,g
Bacillus sp. NRCIB B6	22.8 ± 0.8 a	20.6 ± 0.2 a	20.6 ± 0.1 d	21.6 ± 0.3 d	22.4 ± 0.2 d,e,i
Paenibacillus sp. NRCIB PB1	30.3 ± 0.2 f	20.7 ± 0.3 a	16.5 ± 0.2 h	17.0 ± 0.1 h	16.4 ± 0.6 h
K *	20.3 ± 1.2 g	21.8 ± 0.8 a	21.0 ± 0.9 d,i	20.8 ± 0.6 d,i	20.5 ± 0.6 i

*—control solution (1% $CuSO_4$). Note: The values with different letters in a column are significantly different at $p < 0.05$ (1% $CuSO_4$).

All endophytic bacteria tested belong to the genera *Pseudomonas*, *Bacillus*, and *Paenibacillus*. However, *Pseudomonas* spp. bacteria, especially *Pseudomonas* sp. strain NRCIB P2 (average ZOI ranged from 23.00 ± 0.47 mm to 33.00 ± 0 mm), had a stronger inhibitory effect on pathogenic *Xanthomonas* spp. than other endophytic bacteria. The efficacy of some bacterial strains has been found to depend on the species of pathogenic microorganism. *Paenibacillus* sp. NRCIB PB1 showed significantly greater inhibition against *X. translucens* NRCIB X6 (average ZOI 30.28 ± 0.25 mm) compared to *X. arboricola* strains (average ZOI ranged from 16.43 ± 0.58 mm to 20.68 ± 0.33 mm), but *Bacillus* sp. NRCIB B2 was not effective against *X. translucens* NRCIB X6 and inhibited colony growth of *X. arboricola* strains (average ZOI ranged from 21.33 ± 0.24 mm to 30.3 ± 0.46 mm).

3.2. Lactic Acid Bacteria Strains

In our study, the tested LAB strains that exposed antibacterial activity against *Xanthomonas* spp. strains were chosen for further analysis (Table 2). The data in Table 2 showed that LAB strains and their supernatants were antimicrobially active against *Xanthomonas* spp. strains' growth. Comparing the antibacterial activity among LAB strains, supernatants of *Lb. helveticus* 148/3, 3, and 14 demonstrated a strong inhibitory effect against

Xanthomonas spp. strains (average ZOI ranged from 23.3 ± 1.0 mm to 32.0 ± 0.2 mm) (Figure 2).

Table 2. Antibacterial activity of different LAB strains against *Xanthomonas* spp. strains.

Lactic acid Bacteria Strain	Average Zone of Inhibition (ZOI), mm				
	Xanthomonas spp. Strain				
	***X. translucens* NRCIB X6**	***X. arboricola* NRCIB X7**	***X. arboricola* NRCIB X8**	***X. arboricola* NRCIB X9**	***X. arboricola* NRCIB X10**
L. lactis 140/2	20.1 ± 0.6 a	20.9 ± 1.0 a	20.6 ± 0.8 a	22.0 ± 1.0 b,e	22.5 ± 1.0 a
L. lactis 57	19.1 ± 0.9 a	20.0 ± 0.2 a,b	19.8 ± 1.0 a	20.0 ± 1.0 a,b	22.0 ± 1.0 a
L. lactis 768/5	18.5 ± 0.6 a	18.3 ± 0.5 b	19.3 ± 0.7 a	18.5 ± 0.6 a	20.8 ± 1.0 a
E. faecium 59/30	17.9 ± 0.3 a	19.8 ± 0.5 a,b	19.0 ± 0.8 a	19.0 ± 0.8 a	20.5 ± 0.6 a
E. faecium 41/2	19.3 ± 0.5 a	20.3 ± 1.0 a,b	19.8 ± 0.5 a	19.5 ± 0.6 a	20.8 ± 0.8 a
Lb. helveticus 14	23.3 ± 1.0 c	29.0 ± 0.8 c,g	25.3 ± 1.0 c	28.8 ± 1.2 c	26.5 ± 1.0 c
Lb. helveticus 3	29.5 ± 1.0 b,d	28.5 ± 1.0 d,e	28.3 ± 1.0 c,d	28.5 ± 1.0 c,d	30.0 ± 0.8 b,d,e
Lb. helveticus 148/3	32.0 ± 0.8 b	29.3 ± 1.0 c,g	32.0 ± 0.2 b	28.8 ± 1.0 c,d	30.0 ± 0.8 b,d,e
Lb. helveticus R	27.3 ± 1.0 d,f	27.0 ± 1.0 c,d	27.3 ± 1.0 c,d	27.0 ± 1.0 d	31.0 ± 1.0 b
S. thermophilus 43	19.8 ± 0.5 a	18.8 ± 0.5 b,f	20.0 ± 0.8 a	18.9 ± 1.0 a	21.5 ± 1.0 a
Lb. reuteri 3	27.1 ± 1.0 d,e,f	24.5 ± 0.6 e	27.3 ± 1.2 c,d	24.8 ± 1.0 e	26.0 ± 1.0 c,d
Lb. reuteri 7	26.0 ± 1.0 f	25.3 ± 1.0 d,e	27.0 ± 1.0 c,d	28.0 ± 1.2 c,d	26.8 ± 1.0 c,e
K *	28.3 ± 1.0 d,f,g	29.8 ± 0.5 g	29.0 ± 1.0 b,d	28.8 ± 1.0 c,d	28.5 ± 1.0 b,c

*—control solution (1 % $CuSO_4$). Note: The values with different letters in a column are significantly different at $p < 0.05$.

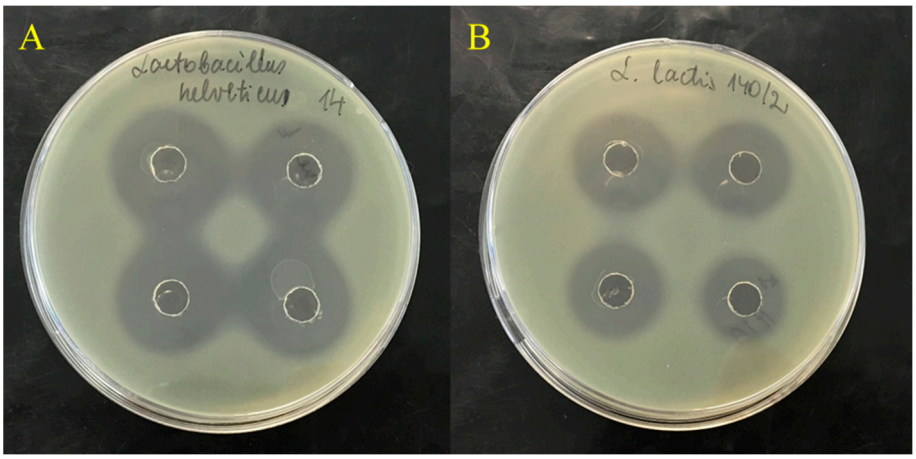


Figure 2. Antibacterial activity of *Lb. helveticus* 14 (**A**) and *L. lactis* 140/2 (**B**) strains against *X. arboricola* NRCIB X7.

Lb. reuteri 3 supernatant also showed the strong activity against the growth of *X. translucens* NRCIB X6 (ZOI was 27.3 ± 1.2 mm) and *X. arboricola* NRCIB X8 (ZOI was 27.3 ± 1.2 mm). Supernatant of *Lb. reuteri* 7 inhibited the growth of *X. arboricola* NRCIB X9 strongly (ZOI was 28.0 ± 1.2 mm).

The scientific literature suggests that the size of the zone of inhibition depends on the type of LAB, the test method, and the concentration of LAB [30]. *L. lactis*, *E. faecium* and *S. thermophilus* strains showed a moderate antibacterial effect (ZOI ranged from 17.9 ± 0.3 mm to 22.0 ± 1.0 mm). The results demonstrated the influence of genus and species on the potential in select of LAB or endophytic strains with enhanced antibacterial activities against *Xanthomonas* spp. strains.

3.3. Essential Oils and Aqueous Extracts of Berries

The antimicrobial activity of plant oils and extracts has been recognized for many years [12,16,17,31]. In our study, we tested different essential oils and obtained that the tested essential oils exposed antibacterial activity against *Xanthomonas* spp. strains' growth (Table 3).

Table 3. Antibacterial effect of essential oils on *Xanthomonas* spp. strains.

Essential Oil	Concentration, %	Average Zone of Inhibition, mm				
		Xanthomonas spp. Strain				
		***X. translucens* NRCIB X6**	***X. arboricola* NRCIB X7**	***X. arboricola* NRCIB X8**	***X. arboricola* NRCIB X9**	***X. arboricola* NRCIB X10**
Rosemary (*Rosmarinus officinalis*)	1	0.0 ± 0.0 b	0.0 ± 0.0 c	9.0 ± 0.1 i	0.0 ± 0.0 b	0.0 ± 0.0 c
	2	0.0 ± 0.0 b	0.0 ± 0.0 c	10.0 ± 0.1 e	11.0 ± 0.0 g,h,i	9.0 ± 0.0 h
Lemon (*Citrus limon*)	1	0.0 ± 0.0 b	0.0 ± 0.0 c	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 c
	2	0.0 ± 0.0 b	0.0 ± 0.0 c	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 c
Thyme (*Thymus vulgaris*)	1	16.0 ± 0.0 c	11.0 ± 0.0 a	16.5 ± 0.1c	14.0 ± 0.0 c	17.0 ± 0.1 d
	2	22.0 ± 0.1 d	18.0 ± 0.1 d	20.0 ± 0.1 d	28.0 ± 0.2 d	26.0 ± 0.2 e
Grapefruit (*Citrus paradisi*)	1	11.0 ± 0.1 e	10.0 ± 0.1 e	10.0 ± 0.1 e	10.5 ± 0.2 e,h	10.0 ± 0.0 a
	2	14.5 ± 0.5 f	11.0 ± 0.1 a	11.0 ± 0.1 f	12.3 ± 0.2 a	12.0 ± 0.1 f
Peppermint (*Mentha piperita*)	1	0.0 ± 0.0 b	0.0 ± 0.0 c	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 c
	2	11.0 ± 0.1 e,g	10.0 ± 0.0 e	11.0 ± 0.0 f,h	10.0 ± 0.0 e	11.0 ± 0.0 b
Pine (*Pinus sylvestris*)	1	0.0 ± 0.0 b	0.0 ± 0.0 c	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 c
	2	0.0 ± 0.0 b	0.0 ± 0.0 c	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 c
Lavender (*Lavandula angustifolia*)	1	11.5 ± 0.1 a,e	11.5 ± 0.0 f	11.5 ± 0.0 a,f	11.5 ± 0.1 a,i	10.5 ± 0.0 a,b
	2	15.0 ± 0.0 f	15.0 ± 0.1 g	14.5 ± 0.0 g	15.5 ± 0.1 f	13.5 ± 0.0 g
Tea tree (*Melaleuca alterfolia*)	1	11.5 ± 0.0 a,e	11.2 ± 0.1 a,f	11.3 ± 0.2 a,f	11.5 ± 0.0 a,g	10.0 ± 0.1 a
	2	12.0 ± 0.1 a	12.0 ± 0.1 b	12.0 ± 0.1 a	12.0 ± 0.1 a	11.0 ± 0.2 b
Silver fir (*Abies sibirica*)	1	0.0 ± 0.0 b	0.0 ± 0.0 c	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 c
	2	0.0 ± 0.0 b	0.0 ± 0.0 c	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 c
Juniper (*Juniperus communis*)	1	0.0 ± 0.0 b	0.0 ± 0.0 c	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 c
	2	0.0 ± 0.0 b	0.0 ± 0.0 c	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 c

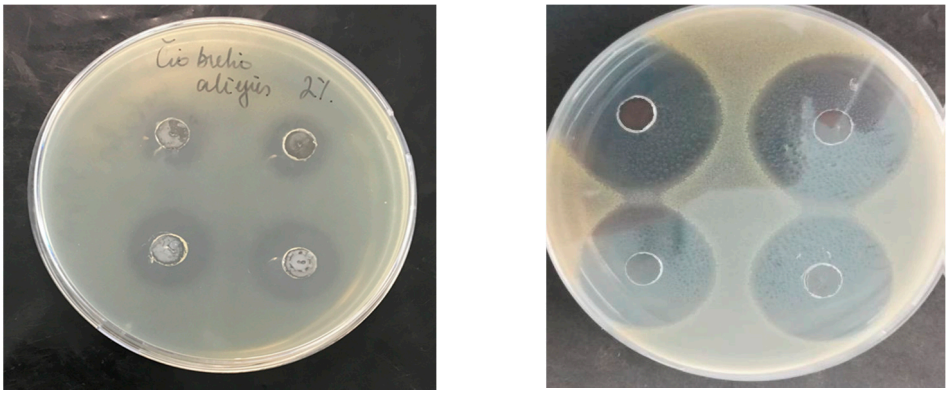
Note: The values with different letters in a column are significantly different at $p < 0.05$.

The results showed that the tested essential oils had a different antibacterial activity against *Xanthomonas* spp. strains. The 1% (*v/v*) essential oils of lemon, pine, silver fir, juniper, rosemary, and peppermint had no antibacterial activity. Weak antibacterial activity was found in 2% (*v/v*) essential oils of grapefruit, lavender, tea tree, and peppermint. The 2% (*v/v*) thyme essential oils showed a medium to strong antibacterial activity (ZOI ranged from 18.0 ± 0.1 mm to 28.0 ± 0.2 mm) (Figure 3). Our finding that tested emulsions of essential oils possessed an antibacterial activity against *Xanthomonas* spp. is in close agreement with the previously reported results [16,29,32].

The efficiently of essential oils (thyme, lavender, and grapefruit) on investigated *Xanthomonas* strains was assessed by determining the minimum inhibitory concentration.

Essential oils of thyme, lavender, and grapefruit exhibited a strong antibacterial activity against *Xanthomonas* spp. The minimum inhibitory concentration values were 7.81–31.25 µg/mL. All *Xanthomonas* spp. strains were found to be the most sensitive organisms to thyme essential oil (MIC value 7.81 µg/mL).

Aqueous extracts of blueberries and cranberries showed a weak and moderate inhibitory effect in vitro against *Xanthomonas* spp. strains (Table 4). Based on the results obtained, the antibacterial efficacy of blueberry extracts does not depend on the region (country) or their habitat. Inhibition of blueberry extracts resulted in zones of inhibition whose diameters were ranging from 10.0 ± 0.1 mm to 13.0 ± 0.0 mm. That indicates a weak antibacterial effect against *Xanthomonas* spp. bacterial strains. Aqueous cranberry extracts showed sufficiently moderate antibacterial activity—the average of inhibition zones ranged from 17.5 ± 0.5 mm to 18.1 ± 0.2 mm (location site No. 1).



(**a**) (**b**)

Figure 3. Antibacterial activity of thyme (*Thymus vulgaris*) essential oils concentration 1% (**a**) and 2% (**b**) against *X. arboricola* NRCIB X7.

Table 4. Antibacterial activity of blueberry and cranberry aqueous extracts against *Xanthomonas* spp. strains.

Berry Extract (Country)	Berry Location Sites Number	Average Zone of Inhibition, mm				
		Xanthomonas spp. Strains				
		***X. translucens* NRCIB X6**	***X. arboricola* NRCIB X7**	***X. arboricola* NRCIB X8**	***X. arboricola* NRCIB X9**	***X. arboricola* NRCIB X10**
Blueberry (Lithuania)	1	11.0 ± 0.0 a	11.0 ± 0.0 a	11.0 ± 0.0 a	11.5 ± 0.0 a,c,e,f,g,h,j,k	12.0 ± 0.0 a
	2	11.0 ± 0.0 a	11.0 ± 0.0 a	11.0 ± 0.0 a	11.0 ± 0.0 a,b	12.0 ± 0.0 a
	3	11.0 ± 0.0 a	10.0 ± 0.0 b	10.0 ± 0.1 b	12.0 ± 0.0 c	13.0 ± 0.0 b
Blueberry (Finland)	1	10.0 ± 0.0 b	10.0 ± 0.0 b	10.0 ± 0.0 b	11.0 ± 0.0 b,j	11.0 ± 0.0 c
	2	10.0 ± 0.0 b	10.0 ± 0.0 b	10.0 ± 0.0 b	11.0 ± 0.0 b,k	11.0 ± 0.0 c
	3	10.0 ± 0.0 b	10.0 ± 0.0 b	10.0 ± 0.0 b	12.0 ± 0.0 c,l	12.0 ± 0.0 a
Blueberry (Latvia)	1	11.0 ± 0.0 a	11.0 ± 0.0 a	11.0 ± 0.0 a	12.0 ± 0.0 c,d	12.0 ± 0.0 a
	2	11.0 ± 0.0 a	11.0 ± 0.0 a	11.0 ± 0.0 a	11.0 ± 0.0 b,e	11.0 ± 0.0 c
	3	11.0 ± 0.1 a	11.0 ± 0.0 a	11.0 ± 0.0 a	11.2 ± 0.0 b,f	11.0 ± 0.0 c
Blueberry (Norway)	1	10.0 ± 0.0 b	10.0 ± 0.0 b	10.0 ± 0.0 b	11.0 ± 0.0 b,g	11.0 ± 0.0 c
	2	10.0 ± 0.0 b	10.0 ± 0.0 b	10.0 ± 0.0 b	11.0 ± 0.0 b,h	11.0 ± 0.0 c
	3	11.0 ± 0.0 a	11.0 ± 0.0 a	11.0 ± 0.0 a	12.0 ± 0.0 c,i	12.0 ± 0.0 a
Cranberry (Lithuania)	1	17.8 ± 0.3 c	18.1 ± 0.2 c	17.8 ± 0.4 c	18.1 ± 0.2 m	17.5 ± 0.5 d
	2	17.5 ± 0.1 c	17.8 ± 0.3 c	17.1 ± 0.2 c	17.8 ± 0.5 m	16.8 ± 0.1 e

Note: The values with different letters in a column are significantly different at $p < 0.05$.

4. Discussion

Bacterial pathogens belonging to *Xanthomonas* genus are the causal agents of very devastating diseases of economically important crops worldwide [1,2]. However, in recent decades, the excessive use of pesticides to control plant diseases has caused environmental pollution, harmful effects on organisms, including human health [6]. Thus, biological control promises an alternative sustainable management of plant diseases caused by the genus *Xanthomonas* [11,13]. The most commonly reported genera with antimicrobial activity against phytopathogens are *Actinobacteria*, *Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Serratia*, where *Bacillus* and *Pseudomonas* are among the most commonly described genera that can inhibit bacterial plant pathogens [13,15,33]. Our studies also found that these two bacterial genera had the greatest antagonistic effect against *Xanthomonas* spp. strains. The same effectiveness was found with LAB strains, supernatants of *Lb. helveticus* 148/3, 3, and 14, where average ZOI of endophytic and LAB strains ranged from 23.3 ± 1.0 mm to 33.0 ± 0 mm. Unfortunately, to date, insufficient attention has been paid to finding appropriate biological control measures against *X. arboricola* and *X. translucens*. *X. oryzae* pv. *oryzae*, *X. campestris*, and *X. axonopodis* pathovars are among the most economically harmful pathogens [2], and therefore, the greatest amount of existing information is on the use of microorganisms to control these pathogens [34,35]. However, several studies have been published on antagonist bacteria suitable for biocontrol against *X. arboricola* and *X. translucens*. For example, the sweet osmanthus (*Osmanthus fragrans*) endophyte *Bacillus*

sp. is a promising biocontrol candidate for *Xanthomonas arboricola* pv. *juglandis*, the causal agent of walnut blight [36].

LAB are found in food-related environments and also in many plants as endophytic microorganisms [37]. The antagonistic activity of LAB has been described in detail in many studies and now known as producers of various antagonistic compounds, including organic acids, diacetyl, hydrogen peroxide, ethanol, acetaldehyde, acetoine, carbon dioxide, bacteriocins (reuterin and reutericyclin), bactericidal proteins, etc. [38]. Twelve unique LAB strains previously characterized as highly antagonistic were chosen in this study to assess antimicrobial activity against *Xanthomonas* spp. species. These LAB strains appeared to produce and secrete natural antimicrobial compounds, such as lactic (6.04–19.90 g/L), citric (0.30–3.30 g/L), benzoic (0.2–1.80 mg/L), and sorbic (0.1–1.20 mg/L) acids, ethanol (0.30–0.87%), and hydrogen peroxide (0.006–0.009 g/L) [39].

Antagonistic properties of bacteria mostly depend on the ability to produce a wide range of antimicrobials including small bioactive secondary metabolites (bacteriocins, antibiotics, toxins), hydrolytic enzymes, volatile organic compounds, etc. [17,33,40–42]. The genus *Lactobacillus* is the largest group among LABs, and the most studied and widely described species suitable for biocontrol of plant pathogens is *Lb. plantarum* [41,43]. Therefore, in our study, we attempted to evaluate *Lactobacillus*, *Streptococcus*, and *Enterococcus* spp. as antagonists that could be used to control pathogenic *Xanthomonas* spp. bacteria. Our studies showed that all LAB strains tested had antibacterial activity against *Xanthomonas* spp. The 43 *S. thermophilus* strains analysed here did not have high antibacterial activity against gram-negative bacteria (18.8–21.5 mm). The highest activity against *Xanthomonas* spp. strains was demonstrated by *Lb. helveticus* strains 148/3 and 3. Their activity ranged from 23.3 to 32.0 mm, and they were the best producers of benzoic acid [39]. The most prospective LAB strains tested were *Lb. helveticus* 3, 148/3, and R and *Lb. reuteri* 3 and 7. In our previous study, *Lb. reuteri* strains 3 and 7 showed antibacterial activity due to high amount of ethanol production. *Lb. helveticus* and *Lb. reuteri* strains produced the most of all antimicrobials [39]. Our finding that LAB showed good antibacterial activity against *Xanthomonas* spp. strongly agrees with previously reported results.

Although many authors describe microorganisms suitable for controlling pathogenic *Xanthomonas* spp. bacteria [17,33,40–43], the knowledge about the antibacterial activity of bacteria, such as endophytic or LAB strains, is still insufficient. Antagonistic bacteria are a good alternative to manage plant diseases because they do not show toxicity to living organisms and environment and thus are more sustainable compared to chemical pesticides. Bacteria producing a complex of antimicrobial compounds could have great potential for commercial application.

Higher plants are sources of active compounds that are natural pesticides, which can be used against various plant pathogenic bacteria [16,44]. They are more attractive than chemical pesticides because are not toxic to the environment and to non-target organisms, including humans, and are easily biodegradable and therefore more sustainable. Essential oils are rich in various groups of chemical compounds that affect the permeability of the bacterial cell membranes and other structures, thus reducing the spread of plant diseases caused by bacterial pathogens [10,45,46].

In the present study, the in-vitro antibacterial activities of essential oils and aqueous extracts of blueberries and cranberries were assessed by the presence or absence of inhibition zones. The results of this in-vitro study showed that 2% (*v/v*) essential oils of thyme (*Thymus vulgaris*), grapefruit (*Citrus paradisi*), and lavender (*Lavandula angustifolia*) and aqueous cranberry (*Vaccinium vitis-idaea*) extracts (Lithuania) exerted potential antibacterial effect against *Xanthomonas* spp. It is sufficiently well known that the compounds present in these plants have antimicrobial activity against many gram-positive and gram-negative pathogenic bacteria, including bacteria of the genus *Xanthomonas* [16,32,44,47]. For example, the main components of *T. vulgaris* are thymol, geraniol, and carvacrol, and they are very effective against gram-negative plant pathogenic bacteria [48]. Our finding that tested essential oils possessed an antibacterial activity against *Xanthomonas* spp. agrees with

others previously reported. The 1% (*v/v*) essential oils of lemon, pine, silver fir, juniper, rosemary, and peppermint had no antibacterial activity. Some of these findings about *Xanthomonas* species were confirmed by previous studies, for example, juniper had no antibacterial activity for *Xanthomonas campestris* pv. *campestris* (Xcc), but some results were an opposite when Xcc were highly sensitive to rosemary; very sensitive to peppermint, silver fir, and lemon; and sensitive to pine [49] and had no effect. Thus, the results only confirm once again that the activity of the compounds against pathogens is highly dependent on the species of pathogenic microorganism tested and (or) the mode of action of the active compounds of the plant used.

Various publications mention plant extracts or essential oils that may be effective against bacterial microorganisms, and they be used to inhibit the growth of plant pathogenic bacteria in an agricultural system [10,12,16,17,31,32,44,47]. Interest in plant pathogenic bacteria of the genus *Xanthomonas* is still relevant, as it still causes significant yield losses, and effective and safe measures are being sought to control them. In this context, plant essential oils or their extracts can have promising uses because they are more environmentally friendly and sustainable, and trends in their use seem promising. Thus, many of the secondary metabolites present in plants as active compounds can be used for plant protection and food safety to ensure pathogenic *Xanthomonas* control [10,12,44]. However, much research still needs to be done to increase the reliability and effectiveness of these products. In this sense, the recent developments in biotechnology and analytical chemistry can help advance studies involving the biological control *Xanthomonas*.

5. Conclusions

Pseudomonas sp. strain NRCIB P2 (average ZOI ranged from 23.00 ± 0.47 mm to 33.00 ± 0 mm) had a stronger inhibitory effect on pathogenic *Xanthomonas* spp. compared to other endophytic bacteria as *Bacillus* or *Paenibacillus* spp. However, *Paenibacillus* sp. NRCIB PB1 had significantly greater inhibition against *X. translucens* NRCIB X6 (average zone of inhibition 30.28 ± 0.25 mm) compared to *X. arboricola* strains (average zone of inhibition ranged from 16.43 ± 0.58 mm to 20.68 ± 0.33 mm).

The supernatants of *Lactobacillus helveticus* strains 3, 148/3, and R and *Lb. reuteri* strains 3 and 7 and *Pseudomonas* sp. NRCIB P2 demonstrated a strong/potent antibacterial effect against the growth of *Xanthomonas* spp. strains. The diameter of the inhibition zones under their influence ranged from 24.5 ± 0.6 mm to 32.0 ± 0.8 mm, whereas the diameter of inhibition zones of the control sample (1% copper sulfate) ranged from 28.3 ± 1.0 mm to 29.8 ± 0.5 mm.

The antibacterial properties of 2% (*v/v*) thyme (*Thymus vulgaris*) essential oils showed a moderate and strong antibacterial activity against *Xanthomonas* spp. strains' growth. The thyme essential oil formed inhibition zones 18 ± 0.1−28 ± 0.2 mm in diameter. Grapefruit (*Citrus paradisi*) and lavender (*Lavandula angustifolia*) 2% (*v/v*) essential oils showed a moderate antibacterial activity against *Xanthomonas* spp. strains' growth (the diameter of inhibition zones were 11.0 ± 0.1–14.5 ± 0.5 mm and 13.5 ± 0.0–15.5 ± 0.1 mm, respectively). The minimum inhibitory concentration of thyme essential oil was 7.8 µg/mL and for lavender and grapefruit 31.25 µg/mL. The 2% (*v/v*) essential oils of lemon (*Citrus limon*), pine (*Pinus sylvestris*), silver fir (*Abies sibirica*), and juniper (*Juniperus communis*) did not show antimicrobial activity on *Xanthomonas* spp. strains' growth.

The inhibitory effect of aqueous blueberry extracts against *Xanthomonas* spp. strains was not dependent on region and location. Aqueous cranberry extracts (Lithuania) showed a sufficiently moderate antibacterial activity (the diameter of the inhibition zones ranged from 16.8 ± 0.0 mm to 18.1 ± 0.0 mm), while aqueous blueberry extracts (Lithuania, Latvia, Norway, and Finland) showed a weak antibacterial activity (the diameter of the inhibition zones ranged from 10.0 ± 0.0 mm to 13 ± 0.0 mm).

This information will allow the development of new biopesticides in the future based on targeted scientific researches. These results showed that natural antimicrobials (the supernatants of *Lb. helveticus* 3, 148/3, and R; *Lb. reuteri* 3 and 7 strains; the essential oils

of thyme, lavender, and grapefruit; and aqueous extracts of *Vaccinium vitis-idaea*) could be used in the development of new biopesticides to control plant bacterial diseases caused by pathogenic *Xanthomonas* species.

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