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The use of plant extracts and bacteriophages as an alternative therapy approach in combatting bacterial infections: the study of lytic phages and *Stevia rebaudiana*

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Abstract

Introduction: In the light of the problem of antibiotic resistance, the use of combined alternative therapies in combatting bacteria-related disorders has gained popularity. Bacteriophages are one element implemented in new combination therapy. *Stevia rebaudiana* is known to have antimicrobial activity and regarded as potentially having a synergistic effect with bacteriophages. Therefore, possible interactions of lytic bacteriophages (MS2, T4 and Phi6) with acetone and methanol *S. rebaudiana* extracts (SRa and SRm) in the bacterial environment were examined. **Material and Methods:** The interactions were tested using a microdilution method, phage-extract co-incubation assay, static interaction (synography) and dynamic growth profile experiments in a bioreactor. **Results:** The interactions of the tested factors in a static environment differed from those in a dynamic environment. Dynamic conditions altered the effect of the extracts in a concentration-dependent manner. How different the effect of the SRa extract was to that of the SRm extract on bacterial growth in a dynamic environment depended on the species of the phage and bacterial host. The greatest differences were observed for *E. coli* strains and their phages, whereas *Pseudomonas syringae* and the Phi6 phage reacted very similarly to both extracts. Differences also emerged for the same extract in different *E. coli* strains and their phages. **Conclusion:** Every extract type should be tested on a case-by-case basis and experiment outcomes should not be generalised before gathering data. Moreover, many varied experiments should be performed, especially when examining such multifactorial mixtures. The tested mixtures could potentially be used in multidrug-resistant bacterial infection treatments.

Keywords: bacteriophage, *Stevia rebaudiana*, plant extract, co-application.

Introduction

Antibiotic resistance is a severe threat to health worldwide, with substantial repercussions for the health of humans and animals, as well as for food safety. Antibiotics are used excessively in human and animal treatment. Bacteria resistant to multiple antibiotics are a challenge in treating bacterial infections, and their transmission from animal to human and from human to human puts public health at risk. In the light of the emergence of resistant strains of microbes that cannot be effectively eradicated by antibiotics, exploring alternative therapy methods could be of great importance. Furthermore, antimicrobial resistance is still increasing worldwide (despite new treatments being employed) as the discovery rate of new antibiotics simultaneously decreases (3, 30). The beneficial effects of herbs and extracted compounds of plant origin gained through their antibacterial, antiviral or antioxidant properties have been shown in numerous studies (2, 3, 6, 10, 13). These substances, variously designated phytogenics, phytobiotics or botanical natural additives, have many advantages over commonly used antibiotics, foremost being that they may be a replacement for antibiotics in the treatment of resistant bacteria infections.

Stevia rebaudiana is a herbaceous perennial plant of the *Stevia* Cav. genus and *Asteraceae* family. The leaves of this plant produce diterpene glycosides (stevioside and rebaudiosides). They are a non-nutritive, non-toxic, high-potency sweetener and may serve as substitutes for sucrose as well as other synthetic sweeteners (44). The sweet compounds contained in *S. rebaudiana* do not chemically break down as they pass through the human digestive system. Therefore, stevia is considered safe for those whose blood sugar level must be controlled (14, 36). Moreover, stevia can be grown very easily like any other vegetable crop, which makes it simple to obtain (14) and it is also known not to be subject to insect predation. Therefore, insecticides are not required in its cultivation, which is of great benefit in producing an organic version of stevia (44). In addition to stevia's sweetening properties, it has medicinal values and uses. The literature points out other medical applications taking advantage of stevia's antihypertensive, antihyperglycaemic, antitumour, anti-inflammatory, antidiarrhoeal, diuretic, hepatoprotective and immunomodulatory effects (10, 44). Moreover, stevia leaves and callus have strong antioxidant activity and may be rich sources of antioxidants 37).

The particular importance of stevia in the light of the present research is attached to its antimicrobial activity, especially against certain bacteria. However, it is still unclear whether stevia extracts facilitate or impede bacterial growth (21, 29, 31). Furthermore, it has been shown that the antimicrobial potential of stevia depends on the type of extract used, different extraction methods having been applied and the activities of the resulting extracts having been dissimilar (33). The importance of all knowledge of the antibacterial action of stevia appears even greater in the light of recent studies showing that combinations of naturally antimicrobial plant extracts and other active agents with similar properties may be more effective than the substances used as individual therapies (26).

Bacteriophages (phages) could serve as alternative antimicrobial agents that could potentially be combined with plant extracts to create more effective preparations. Known for over a hundred years and regaining popularity today, phages are viruses that infect their specific bacterial hosts and can successfully supplement or in some cases even replace traditional methods of treatment and prevention of bacterial diseases. Their value is particularly high as a means of treating those diseases that are caused by antibiotic-resistant bacterial strains (1). The phage virion consists of molecules characterised by their biochemical complexity. Because of their varying sizes, levels of hydrophobicity and electric charges, bacteriophages interact with other materials and factors in various ways, often exerting stimulating or inhibiting effects. An example would be the alteration of a phage's lytic activity under synergistic or antagonistic influence (34, 45). The easy handling of bacteriophages and their ability to be cultivated in standard laboratory media are significant advantages that make bacteriophages easy to "produce". These benefits have been suggested to recommend phages as eukaryotic cell virus surrogates (11, 28, 41).

Therefore, in the present study, lytic bacteriophages and two different types of *S. rebaudiana* plant extract (methanol and acetone) were investigated for possible interactions in a bacterial environment, including any synergistic or antagonistic effects. To the best of our knowledge, this is the first work describing such phenomena.

Material and Methods

Microorganisms. Bacteriophages and their corresponding bacterial hosts were purchased from the German Collection of Microorganisms and Cell Cultures GmbH (Leibniz Institute DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen)). *Pseudomonas syringae* (DSM 21482) with phage Phi6 (DSM 21518), *Escherichia coli* (DSM 5695) with phage MS2 (DSM 13767) and *E. coli* (DSM 613) with phage T4 (DSM 4505) were selected as model microorganisms based on their different features, as presented below (Table 1).

Table 1. Characteristics of the tested microorganisms

Bacteria	Bacterial features (reference)	Phage	Family	Phage features
Pseudomonas syringae DSM 21482	Isolated from diseased plants, one of the best-studied plant pathogens (43)	Phi6 (Φ6)	*Cystoviridae*	Size ~ 80–100 nm; dsRNA; enveloped (lipid membrane), medium sized, no tail
Escherichia coli DSM 5695	Pathogenic strains can cause distinct disease syndromes, are common food contaminants (23)	MS2	*Leviviridae*	Size ~ 23–28 nm; ssRNA; non-enveloped, small size and genome, no tail
Escherichia coli DSM 613		T4	*Myoviridae*	Size ~ 120–200 nm / 86–90 nm; dsDNA; non-enveloped, relatively big, contractile tail

DSM – bacterium from the Leibniz Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen

Bacterium revival and phage propagation was conducted as described earlier (35). Briefly, the bacteria were revived from storage at −20°C in trypticase soy broth (BioMaxima, Lublin, Poland) with 10% (v/v) glycerol on Luria–Bertani (LB agar) (BioMaxima) by streaking glycerol stocks onto agar plates. The plates were then incubated for 24 h at 37°C for *E. coli* strains and for 48 h at 28°C for *P. syringae*. Phages were amplified by inoculating 50 mL of LB agar with colonies from bacterial stock plates and incubated (as described above) with agitation at 120 rpm in an orbital rotating shaker (ES-20 Shaker-Incubator; BioSan, Józefów, Poland) to reach optical density of 0.2 at 600 nm (OD_{600nm}). The optical density was measured using an Infinite 200 PRO NanoQuant microplate reader (Tecan, Männedorf, Switzerland). At this point the phages were added and samples were further incubated until lysis occurred. For MS2 and T4 lysate purification, samples were supplemented with 10% (v/v) chloroform, vortexed for 5 min and then centrifuged (model 5810 R; Eppendorf, Hamburg, Germany) at 5,000 rpm for 25 min at 4°C. The supernatant (phage lysate) was collected immediately and stored at 4°C for further use. For Phi6 lysate purification the sample was centrifuged at the first stage at 5,000 rpm for 15 min at 4°C and then sterilised through a 0.22 µm polyethersulfone (PES) filter. The lysate was collected and stored at 4°C for further use. The phages' activities and titres were tested by a double-overlay agar plaque assay (19).

Preparation of acetone and methanol extracts of *S. rebaudiana*. Preparation of *S. rebaudiana* extracts was carried out as described in our previous work (24) with some modifications. Fifty grams of dried stevia leaves (Flos, Mokrsko, Poland) were placed in glass bottles. The first bottle was intended for methanol extraction; therefore, 100 mL of 70% aqueous methanol (MeOH) was introduced. The second bottle intended for acetone extraction was supplemented with 100 mL of 70% aqueous acetone. Next, the samples were placed on a shaker (Ika, Staufen im Breisgau, Germany) and extracted at 150 rpm for 2 h at 70°C. The crude extracts were filtered through a Büchner funnel equipped with a cellulose filter. The extracts were then concentrated by evaporation at 50°C to obtain 10 mL of each aqueous solution. After the evaporation of methanol and acetone, the samples were diluted with 20 mL of water and sterilised through a 0.22 µm PES filter. The samples were then used for further experiments. At this stage the *S. rebaudiana* water solution of active compounds obtained by methanol extraction was marked "SRm", and the *S. rebaudiana* water solution of active substances obtained by acetone extraction was marked "SRa". Additionally, the dry mass of each extract was determined *via* moisture analyser (Radwag, Radom, Poland). For the next experiments, stock solutions of the extracts were serially diluted twofold in sterile, deionised water and kept at −20°C until further analysis.

The influence of *S. rebaudiana* extracts on bacterial cells. In order to test the influence of *S. rebaudiana* SRa and SRm extracts on bacterial cells, a 96-well microplate dilution experiment was performed. For the microplate method, modified minimum inhibitory concentration (MIC) determination was used (42). The colonies from stock bacterial plates were used to inoculate Falcon tubes with 30 mL of LB agar and the tubes were shaken at 120 rpm and incubated at 37°C for *E. coli* strains and 28°C for *P. syringae* on the orbital rotating shaker until the cultures reached an OD_{600nm} of 0.2. Polystyrene flat-bottomed plates with 96 wells were filled with 50 µL of twofold serial dilutions of SRa and SRm (50%–0.003%) and 50 µL of bacterial suspensions. Sterile deionised water added in place of extracts was used as the positive control (as a bacteria growth control). The plates were then placed in incubators for 24 h at temperatures appropriate for the tested bacteria. Optical density values were measured using the Infinite 200 PRO NanoQuant microplate reader (Tecan, Männedorf, Switzerland). The experiment was conducted in triplicate.

Co-incubation studies of phages and *S. rebaudiana* extracts. For direct phage–extract interaction testing, a co-incubation experiment was performed (34). The test was carried out in order to reveal the influence of extracts on bacteriophages' plaque-forming ability and titres. It was conducted in a static environment in order to detect basic interactions between the tested factors. Briefly, 12-well flat-bottomed polystyrene plates were initially filled with 1 mL of phage lysates (Phi6 and T4 at 10^8 plaque-forming units (PFU)/mL and MS2 at 10^9 PFU/mL). Then 1 mL of either SRa or SRm was added to reach final concentrations of 50.000–0.049% by twofold serial dilution. Extract-free deionised water with phage lysate was used as a positive control, *i.e.* as a phage control. The plates were then incubated at room temperature for 24 h in the dark. Later, samples were titrated in tris–magnesium sulphate buffer (50 mM tris-HCl, 10 mM $MgSO_4$ at pH 7.5) by spotting 3 µL of each tenfold dilution onto an LB agar plate precoated with a top agar layer (7%) mixed with overnight bacterial culture, implementing the double-layer agar technique. The experiment was conducted in triplicate.

Interaction stoichiometries of phages and *S. rebaudiana* extracts. Phage-extract synography (an optically based real-time microtiter plate readout combined with a matrix-like heat map of treatment potencies to measure phage and antibiotic synergy (PAS)) was performed as described elsewhere (15) with minor modifications. For test culture preparation, 5 mL of the overnight culture was diluted in LB agar in order to achieve OD_{600nm} of 1 (approximately 1×10^9 CFU/mL) and 100 µL of the suspension was transferred into each

well of the 96-well flat-bottomed plates. The plates had previously been inoculated with 50 µL per well of phage solution at one of a range of concentrations (10^2–10^8 PFU/mL) and 50 µL per well of extract (25%–0.049%), creating a checkerboard assay. The plates were incubated for 18 h at 37°C for *E. coli*, and at 28°C for *P. syringae*. Bacterial turbidity was determined by measuring OD_{600nm} values using the Infinite 200 PRO NanoQuant microplate reader (Tecan, Männedorf, Switzerland). Afterwards, a resazurin assay was carried out for bacteria metabolic activity testing. The dye was added to the samples in wells to reach a final 1 mg/mL concentration, and the plates were further incubated in the dark for 3 h when the bacteria were *E. coli* and for 4.5 h when the bacterium was *P. syringae*. Next, fluorescence measurements were performed using a fluorescent plate reader (Synergy HTX, BioTek Instruments, Winooski, VT, USA) at 540 nm excitation and 590 nm emission. The experiment was conducted in triplicate.

Phage infection and lysis profile experiments. Based on results from the static synography experiment without mixing conditions, combinations in which interesting phenomena were detected, particularly increased bacterium activity in the resazurin assay with simultaneous OD values showing a reduction in bacteria biomass were chosen. These combinations were selected in order to analyse the influence of combined treatments of phage extracts on bacterial host growth rate in real time in a dynamic environment, *i.e.* one with mixing conditions. Appropriate controls for result comparison (phage + extract maximal dose, extract maximal dose, and bacterial growth control) were also applied (Table 2).

In order to keep the experimental assumptions of the synography test, overnight bacterial host cultures were diluted in LB agar to achieve an OD_{600nm} of 1, and 5 mL of the suspensions were then transferred to 50 mL Falcon tubes. Afterwards, 2.5 mL of phage lysate was added to give a final titre of 10^8 PFU/mL, along with 2.5 mL of SRa or SRm extract concentrations, in order to obtain the chosen final concentrations, *e.g.* 50% extract was added to obtain a final concentration of 12.5%. For extract-only treatments, 2.5 mL of sterile deionised water was added instead of phage. For the host bacterium growth control, 5 mL of sterile deionised water was added to 5 mL of the bacterial suspension. Samples were then shaken at 150 rpm and incubated for 16 h, *E. coli* at 37°C and *P. syringae* at 28°C, and real time OD_{850nm} values were measured using BioSan bioreactors (BS-010160-A04, BioSan, Riga, Latvia).

Statistical analysis. One-way (plant extract antimicrobial studies) and two-way (phage-extract co-incubation assay) analyses of variance were used to statistically analyse the results, along with Dunnett's multiple comparisons test. Differences were considered significant at $P \leq 0.05$. All statistical analyses were carried out using GraphPad Prism 8.01 (GraphPad Software, San Diego, CA, USA).

Table 2. Chosen treatment combinations for real time assessment of bacterial growth

Bacterial host	Phage	Phage titre	Extract	Extract (%)	Treatment type
P. syringae DSM 21482	Phi6	10^8	SRa	25	Phage + extract
P. syringae DSM 21482	-	-	SRa	25	Extract
P. syringae DSM 21482	Phi6	10^8	SRa	0.049	P + E + B combination *
P. syringae DSM 21482	Phi6	10^8	-	-	Phage infection control
P. syringae DSM 21482	-	-	-	-	Growth control
E. coli DSM 5695	MS2	10^8	SRa	25	Phage + extract
E. coli DSM 5695	-	-	SRa	25	Extract
E. coli DSM 5695	MS2	10^8	SRa	0.19	P + E + B combination *
E. coli DSM 5695	MS2	10^8	-	-	Phage infection control
E. coli DSM 5695	-	-	-	-	Growth control
E. coli DSM 613	T4	10^8	SRa	25	Phage + extract
E. coli DSM 613	-	-	SRa	25	Extract
E. coli DSM 613	T4	10^4	SRa	0.049	P + E + B combination *
E. coli DSM 613	T4	10^8	-	-	Phage infection control
E. coli DSM 613	-	-	-	-	Growth control
P. syringae DSM 21482	Phi6	10^8	SRm	25	Phage + extract
P. syringae DSM 21482	-	-	SRm	25	Extract
P. syringae DSM 21482	Phi6	10^8	SRm	0.049	P + E + B combination *
P. syringae DSM 21482	Phi6	10^8	-	-	Phage infection control
P. syringae DSM 21482	-	-	-	-	Growth control
E. coli DSM 5695	MS2	10^8	SRm	25	Phage + extract
E. coli DSM 5695	-	-	SRm	25	Extract
E. coli DSM 5695	MS2	10^8	SRm	0.19	P + E + B combination *
E. coli DSM 5695	MS2	10^8	-	-	Phage infection control
E. coli DSM 5695	-	-	-	-	Growth control
E. coli DSM 613	T4	10^8	SRm	25	Phage + extract
E. coli DSM 613	-	-	SRm	25	Extract
E. coli DSM 613	T4	10^8	SRm	0.19	P + E + B combination *
E. coli DSM 613	T4	10^8	-	-	Phage infection control
E. coli DSM 613	-	-	-	-	Growth control

DSM – bacterium from the Leibniz Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen; SRa – *S. rebaudiana* acetone extract; SRm – *S. rebaudiana* methanol extract

* P + E + B combination – phage, extract and bacteria combination in which interesting phenomena were detected

Results

***S. rebaudiana* extract antimicrobial studies.** The tested plant extracts had dry masses of 32.8433% (328.433 g/L) in the case of SRa and 21.4921% (214.921 g/L) in the case of SRm. In order to test the effects of the extracts on bacterial cells, a microdilution assay was performed. The mixtures of plant extracts and bacterial cultures cultivated in static conditions produced varied effects that were strictly dose dependent (Fig. 1). The SRa extract resulted in a decreased bacterial biomass of *E. coli* DSM 613 and of *E. coli* DSM 5695, except when mixed at three concentrations: 6.25% and 3.125% raised the bacterial counts and 1.56% kept bacteria biomass at the control level of 0%.

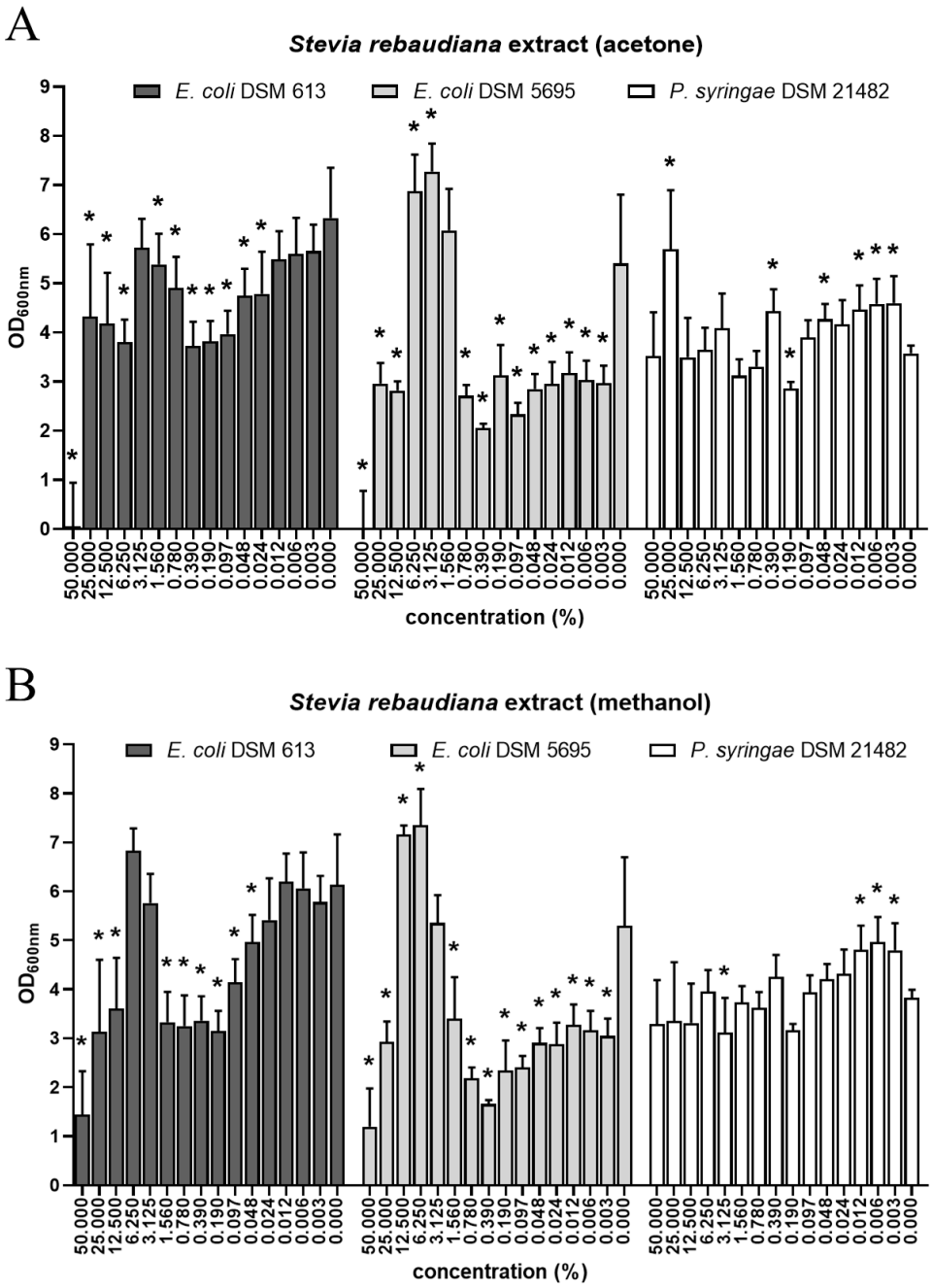

Fig. 1. Activity of the *Stevia rebaudiana* extracts on cells of *E. coli* and *Pseudomonas syringae* expressed in optical density (OD) changes of biomass. Acetone extract activity assessed by microdilution method (A) and methanol extract activity assessed by microdilution method (B). DSM – bacterium from the Leibniz Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen. Error bars represent standard deviation between samples. Means sharing the asterisk are significantly different from the control (extract concentration 0%) at $P \leq 0.05$

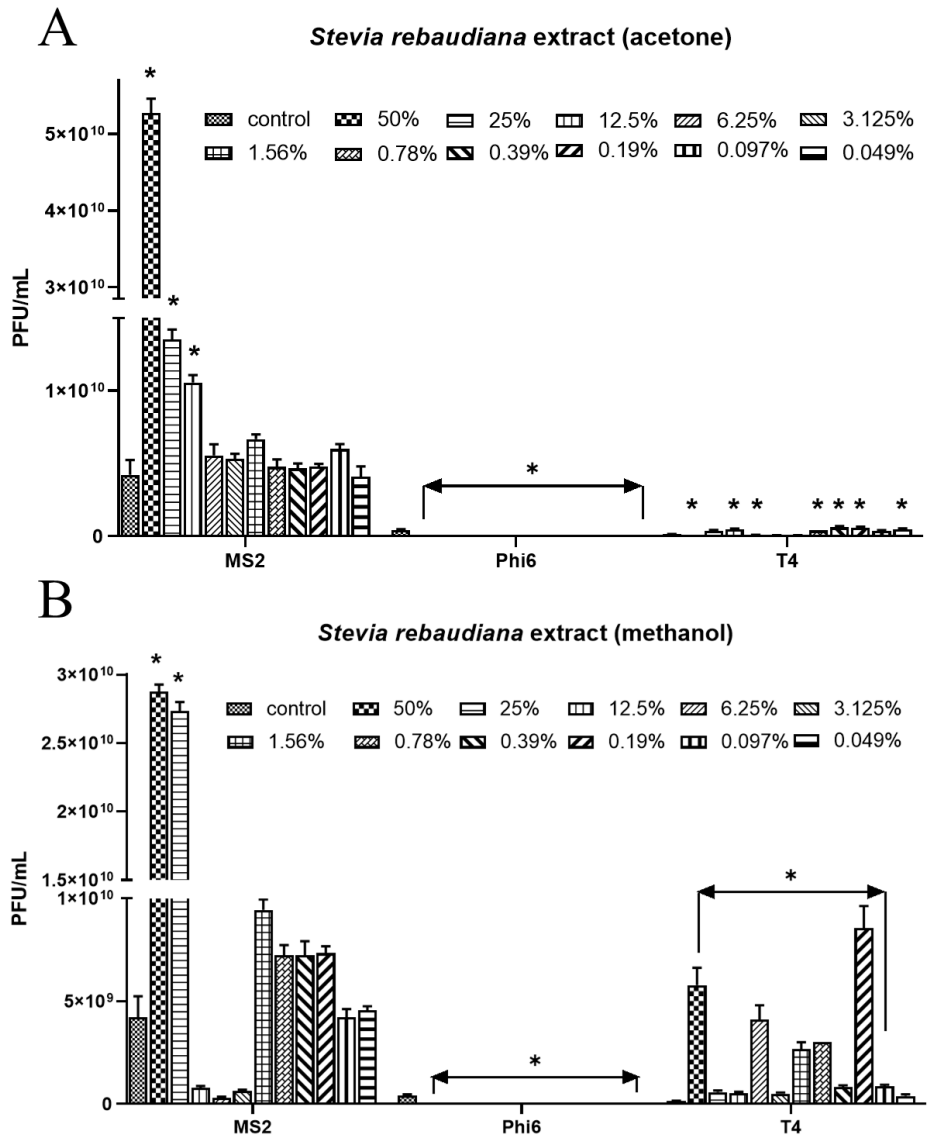

Fig. 2. Phage titres (MS2, Phi6 and T4) after 24 h exposure to different concentrations of *S. rebaudiana* extracts. Phage counts after co-incubation of phages with acetone extract (A) and after co-incubation of phages with methanol extract **(**B). Error bars represent standard deviation between samples. Means sharing the asterisk are significantly different from the control at $P \leq 0.05$. PFU – plaque-forming unit

The SRa extract addition caused a reduction in *P. syringae* DSM 21482 cell density when used at 0.19%, whereas the biomass was kept at the control level for most concentrations, specifically 50%, 12.5%, 6.25%, 3.125%, 1.56%, 0.78%, 0.097% and 0.024%, or was increased, this occurring at 25%, 0.39%, 0.048%, 0.012%, 0.006% and 0.003% (Fig. 1A). The SRm extract also showed an inhibitory effect on *E. coli* DSM 613 cells at the majority of the tested concentrations, except for 6.25%, 3.125%, 0.024%, 0.012%, 0.006% and 0.003%, at which SRm did not influence bacterial growth. A similar effect was observed for the SRm extract on *E. coli* DSM 5695, where the majority of the tested concentrations decreased the bacteria biomass, except for 12.5% and 6.25%, which propagated the bacteria's growth, and 3.125%, which did not alter bacteria multiplication. The results were very similar to those obtained with the SRa extract. The addition of the SRm extract to *P. syringae* DSM 21482 cells at most concentrations induced no effect on the biomass of the bacilli, four concentrations being exceptions: 3.125% inhibiting and 0.012%, 0.006% and 0.003% stimulating bacteria growth (Fig. 1B).

Co-incubation assay. Co-incubation showed varied results (Fig. 2). The addition of the SRa extract did not significantly influence MS2 phage counts, the 50%, 25% and 12.5% concentrations exerting a phage stimulating effect, however. When the SRa extract was added to the Phi6 phage lysate, the extract caused a phagicidal effect, where no phage plaques were present in any tested concentrations. The combination of this extract with the T4 phage resulted in mixed effects. Four SRa concentrations, which were 25%, 3.125%, 1.56% and 0.097%, did not alter phage counts

significantly; two, namely 50% and 6.25%, decreased phage PFU/mL; and five stimulated the counts, these being 12.5%, 0.78%, 0.39%, 0.19% and 0.049% (Fig. 2A). Similarly to the SRa extract, the SRm extract did not significantly influence MS2 phage counts, with the exception of two concentrations, 50% and 25%, which raised the number of phage plaques. In contrast, the addition of the SRm extract to the Phi6 phage caused a significant phagicidal effect at all of the tested concentrations, which was also the case for the SRa extract. When the SRm extract was incubated with the T4 phage, only 0.049%, the lowest concentration, did not significantly alter the phage counts, whereas all higher concentrations caused a stimulating effect (Fig. 2B).

Stoichiometries of the phage–extract interactions. For most of the tested samples, the dark colour of the concentrated extract darkened the sample. This was taken into account when analysing the results – the extracts' optical density backgrounds were removed from the data for clear results interpretation.

The synograms revealed complex relationships between the tested extracts and bacteriophages shaping their effect on their bacterial hosts (Fig. 3). Interestingly, in most of the cases the highest tested extract concentration (25%) caused increased growth of bacteria biomass; however, after 24 h of incubation in the stationary environment the cells were no longer active (Fig. 3A-A'–F-F'). This may indicate increased cell proliferation in the initial growth stage and earlier achievement of the stationary phase due to the presence of the extracts.

Specific interactions between the tested elements were also observed. The 0.78%, 0.39% and 0.19% concentrations of the SRa extract increased *P. syringae* cell activity, even though the OD values of the bacteria biomass were found to decline concomitantly with the decreasing concentration of the extract. Peak cell activity was detected in a mixture of 0.19% SRa and no phage. Interestingly, at an SRa concentration of 0.049% and maximum dose of the Phi6 phage at 10^8 PFU/mL, the highest biomass reduction was detected, while cell activity grew by 44% compared to the control (Fig. 3A-A'). In general, the SRa extract influenced *E. coli* DSM 5695 by decreasing biomass proliferation, lowering the OD in parallel with the decreasing concentration of the extract, while at the same time cell activity rose almost to positive control levels. Here, an interesting phenomenon was also detected, when the highest biomass reduction was discovered with the SRa concentration at 0.19% and the MS2 level at 10^8 PFU/mL, but the cell activity level remained at 70% of that of the control (Fig. 3B-B'). The SRa extract combined with T4 phage showed a similar influence on *E. coli* DSM 613 cells as on *E. coli* DSM 5695; however, more scattered phenomena regarding cell activity were detected. To summarise, bacterial activity in some cases was extract-concentration and phage-concentration independent, and this mainly applied to peaks in cell activity. However, in one case, with the SRa concentration at 0.049% and the T4 level at 10^4 PFU/mL, the highest biomass reduction was seen, along with a notable decrease in cell activity level to only 20% of that of the control (Fig. 3C-C').

When SRm extract was used on *P. syringae* cells, the results obtained were very similar to those of adding the SRa extract: the 1.56%, 0.78%, 0.39% and 0.19% concentrations increased bacteria cell activity, with OD values of the biomass dropping simultaneously and proportionally to the decreasing concentration of the extract. As with the SRa extract, also for the SRm extract peak cell activity was detected in the mixture of 0.19% and no phage. A further parallel with the findings for the SRa extract was that at an SRm concentration of 0.049% and maximum dose of phage, the highest biomass reduction was detected, while cell activity rose by 62% compared to the control (Fig. 3D-D'). A similar trend was also maintained for the results of the SRm extract and *E. coli* DSM 5695, in that they were comparable with those of the SRa extract under the same conditions. However, several cases of increased cell activity compared to the control were present. An interesting phenomenon and one similar to that of the SRa experiment was also observed when the SRm extract was investigated: the highest biomass reduction was discovered at an SRm concentration of 0.19% and MS2 phage concentration of 10^8 PFU/mL, while the detected cell activity remained at 98% of that of the control (Fig. 3E-E'). Similar tendencies were also manifested by the SRm extract and *E. coli* DSM 613 to those presented by the SRa extract and this bacterium – scattered phenomena in the case of cell activity were also detected. However, the most interesting phenomenon observed concerned SRm at 0.019% and T4 at 10^8 PFU/mL, which was the highest biomass reduction along with a decrease in the cell activity level to only 16% of that of the control (Fig. 3F-F').

Lytic performance of phages in the presence of plant extracts. To test the course and lysis ability of the phages in real time, phage performance against bacteria was tested in the presence of the extracts at different concentrations, measured by changes in the OD of the samples that were cultivated in the bioreactors with agitation at 150 rpm (Fig. 4). The extract concentrations were chosen on the basis of results gained from the synography experiment and they were the concentrations in combinations in which the described phenomena were present with maximal treatment doses as controls. The experiment time was also shortened to 16 h because the synography experiment duration of >18 h revealed inactive cells. For most of the tested samples, the sample darkened because of the dark colour of the concentrated extract. This was taken into account when analysing the data – the extracts' optical density backgrounds were removed from the curves for clear results interpretation.

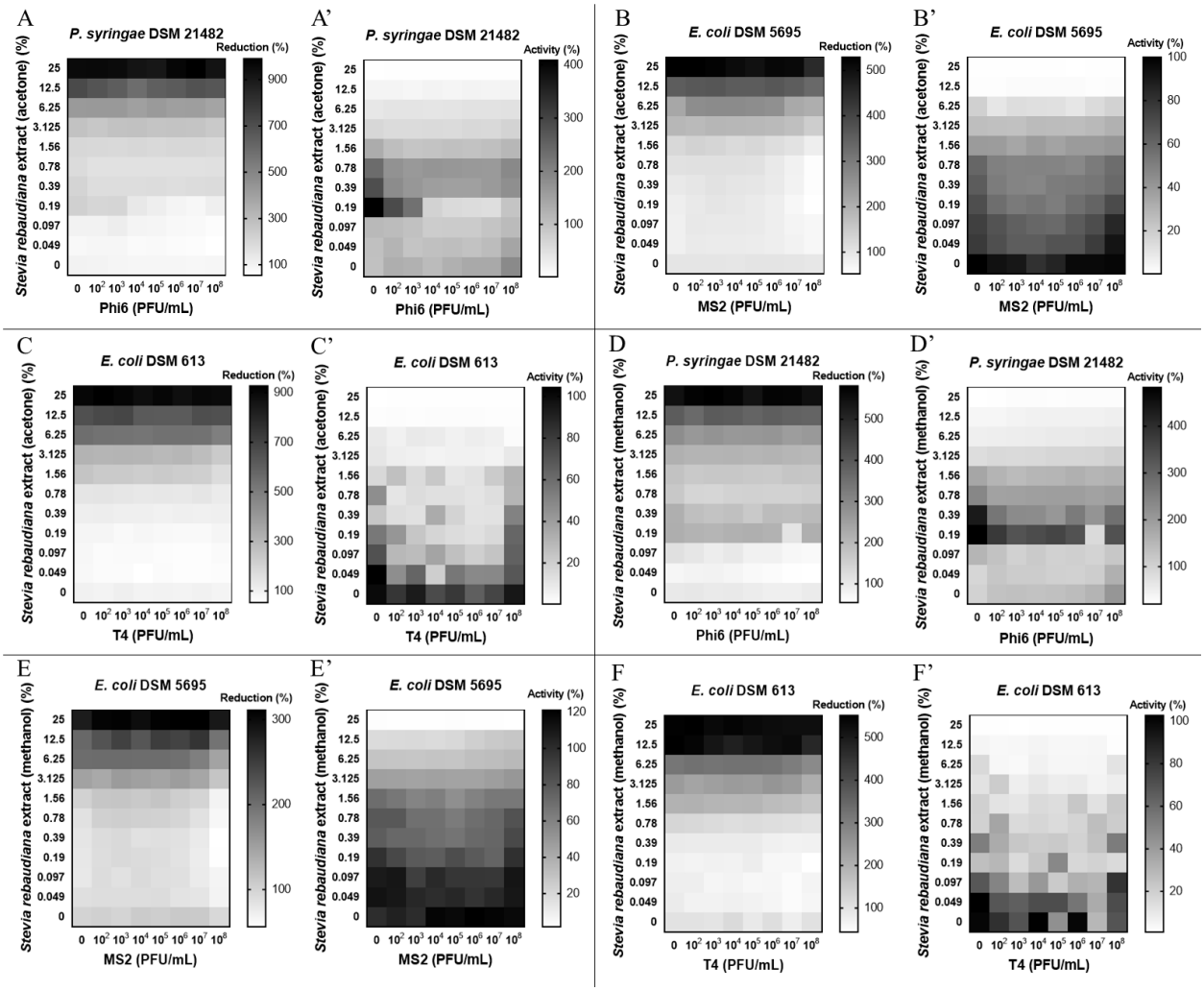

Fig. 3. Effects of phage–extract combinations on host bacteria. Paired heat-maps represent optical density measurements at 600 nm (A–F) and corresponding fluorescence measurements (A'–F'). Combined treatment of *Stevia rebaudiana* acetone extract with phage Phi6 (A–A'), phage MS2 (B–B') and phage T4 (C–C'). Combined treatment of *S. rebaudiana* methanol extract with phage Phi6 (D–D'), phage MS2 (E–E') and phage T4 (F–F'). Synograms (t = 24 h) represent the mean reduction (% of the control) or activity (% of the control) percentage of each treatment from three replicates. DSM – bacterium from the Leibniz Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen; PFU – plaque-forming unit

The tested extracts influenced bacterial cell and phage activity in a varying manner; however, some dependencies were noted (Fig. 4). In general, high concentrations (25%) of the tested extracts resulted in decreased phage lytic efficiency in most cases, either through a stimulating effect on bacterial cells cancelling the lytic effect of the phages or through a deactivating action on the phage particles themselves. The combination of *P. syringae* and the SRa extract at a concentration of 25% resulted in a decrease in OD with or without phage Phi6, flatteining the growth curves – phage addition did not decrease the OD further. In addition, the curves were still in a rising stage, therefore bacterial cells were still multiplying. However, when the SRa concentration was 0.049%, this led to the Phi6 and control lysis curves being the same (Fig. 4A). The mixture of *E. coli* DSM 5695 and 25% SRa extract caused a clear, gradual decrease in ODs, showing the extract's toxicity to bacteria. On the other hand, introducing the MS2 phage into this mixture resulted in a lytic effect lasting for up to 5 h of the incubation. Afterwards, bacterial growth rebounded, climbing rapidly. The reduction in SRa concentration to 0.19% led to the bacterial lysis curve being almost identical to the control lysis curve; however, lysis began a little later and was noted after 2.5 h *vs* after 2 h in the controls (Fig. 4A'). When the SRa extract at 25% was added to the *E. coli* DSM 613 cells, this visibly reduced the OD, flattening growth curves regardless of whether the T4 phage was used or not, which was similar in the case of *P. syringae*. However, in this case the curves were in the gradual reduction stage. When an SRa concentration of 0.049% was used to challenge the *E. coli* DSM 613 cells, it led to a slightly negative cell growth curve, and a contrasting one to the growth control curve (Fig. 4A"). The application of the SRm extract at 25% to the *P. syringae* suspension offered almost the same result as when the SRa extract was used. Regardless of the presence of the Phi6 phage, both growth curves were clearly flattened. However, after 12 h of incubation, the OD values started to fall gradually.

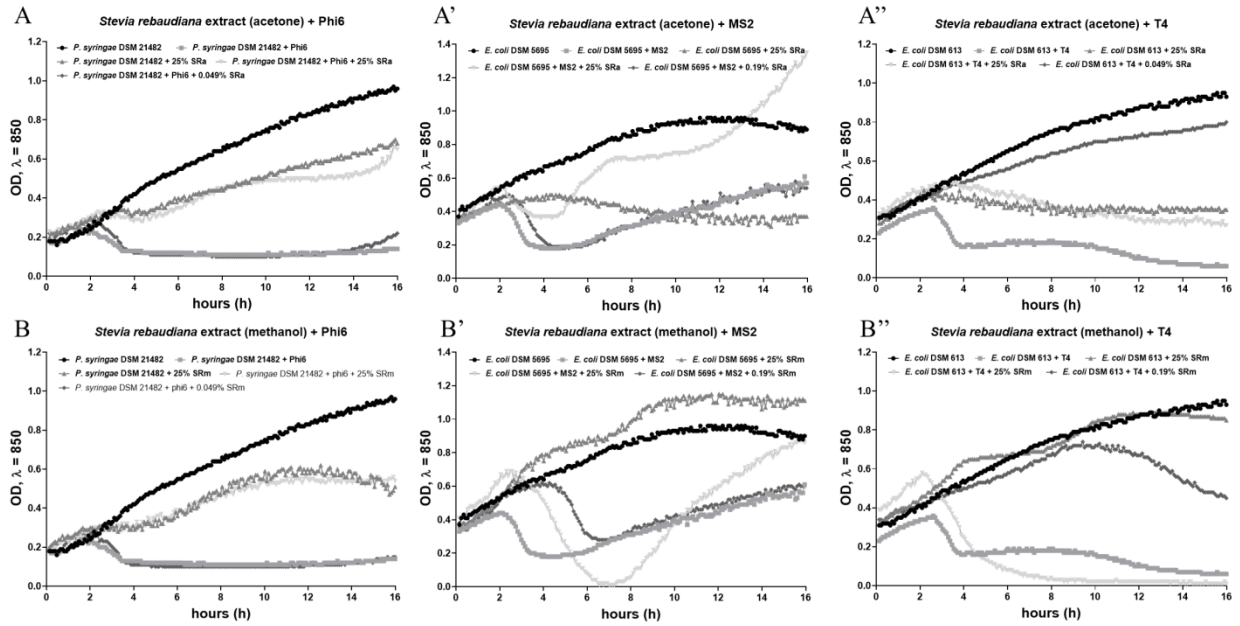

Fig. 4. Bacteria growth curves treated by lytic Phi6, MS2 and T4 phages and different extract concentrations chosen in the synograms assay. Combined treatment of phages with of *Stevia rebaudiana* acetone extract (Phi6 – A; MS2 – A'; T4 – A") and combined treatment of phages with *Stevia rebaudiana* methanol extract (Phi6 – B; MS2 – B'; T4 – B''). OD – optical density; DSM – bacterium from the Leibniz Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen

When the SRm concentration of 0.049% was applied, it also led to the Phi6 phage lysis curve being the same as the control lysis curve, as was also the case when SRa was the tested extract (Fig. 4B). A 25% concentration of SRm extract addition to the *E. coli* DSM 5695 caused a visible increased proliferation of the bacteria, stimulating the cells. When the MS2 phage was added into this mixture, it led to a very strong lytic effect from the 2.5 h point to the 7 h point of incubation and this was stronger and longer lasting than the lysis in the lysis control. However, strong bacterial proliferation was observed later. Lowering the SRm concentration to 0.19% delayed but elongated phage lysis compared to the control curve. Lysis was noted in the control from 2 h until 3.5 h, *i.e.* for 1.5 h; lysis induced by the tested sample was evident from 4.5 h until 6.5 h, *i.e.* for 2 h (Fig. 4B'). The addition of SRm at the concentration of 25% to the *E. coli* DSM 613 cells did not generate any specific effect on the bacteria, and the growth curve was almost identical to the bacterial control growth curve. When bacteriophage T4 was also added, what it mediated was similar to what the MS2 phage induced in *E. coli* DSM 5695: a marked strong lytic effect was observed. The effect was also longer and stronger than in the control – lytic activity was detected from the 2 h point of incubation, and no bacterial growth rebound was observed. Lowering the SRm concentration to 0.19% caused the curve to be highly comparable to the control growth curve until 9 h of incubation had passed. After that time, the curve began to fall, indicating the death of the bacterial cells (Fig. 4B").

Discussion

Stevia is mainly known as a natural sweetener; however, it is also gaining interest because of its many beneficial effects not only for humans (12, 25, 32), but also for animals, where the herb can serve as a feed supplement or alternative feed source (4, 5, 12, 27). It has also been shown that phages can be used as feed additives to reduce bacteria in animals preslaughter without negatively impacting normal gut microbial communities (40). Mindful of this, we tested possible interactions between *S. rebaudiana* acetone and methanol extracts and lytic phages as antibacterial agents in an environment of bacteria related to fauna and flora diseases.

The extracts of *S. rebaudiana* in acetone and methanol revealed diverse and multidimensional interactions with the mixtures of phages and their bacterial hosts. In order to explain these interactions, it is necessary to draw general conclusions from the experiments performed and then propose hypotheses explaining the results.

The lysis profile experiment of *P. syringae* and SRa at a concentration of 25% resulted in OD reductions with or without phage Phi6, and when the SRa concentration was 0.049%, this led to the Phi6 and control lysis curves being the same. These concentrations stimulated the cells in the modified MIC test, but proved to be phagicidal in the tests in static conditions with no mixing. It is possible that the introduction of dynamic conditions (mixing) neutralised the extracts' stimulating effect on bacteria and the phagicidal effect in a concentration-dependent manner. When *E. coli* DSM 5695 was challenged, the

SRa extract at 25% concentration caused a gradual decrease in OD, while after the MS2 phage was added, a lytic effect was observed for up to 5 h of the incubation, after which bacterial growth rebounded. The acetone extract at 0.19% produced almost identical lysis and control curves. These concentrations were toxic to cells in the MIC test, and SRa 25% potentiated the action of the MS2 phage. In this case, we also hypothesise that dynamic conditions diminished the extracts' influence on the bacteria and their phage-inducing effect, albeit to a lesser extent. When SRa 25% was added to the *E. coli* DSM 613 cells, it resulted in a similar outcome to that in the case of *P. syringae* – a visible decrease in OD was noted, regardless of whether the T4 phage was used. An SRa concentration of 0.049% led to only a slight reduction in the cell growth curve's values compared to those of the growth control curve. These concentrations were toxic to cells in the modified MIC test, and SRa 0.049% stimulated the phage. Here, we also would argue that to a lesser extent, dynamic conditions weakened the extracts' effect on bacteria in a concentration-dependent manner. Moreover, the phage-inducing effect was not visible because of the small amount of phage particles (10^4 PFU/mL). The application of the SRm extract at 25% concentration to *P. syringae* cells gave almost the same result as when the similarly concentrated SRa extract was used – regardless of the presence of the Phi6 phage, both growth curves were clearly flattened. At the concentration of 0.049%, SRm with the addition of the Phi6 phage caused the lysis curve to be identical to the control lysis curve, the effect being the same as when SRa was used. These concentrations did not affect cells in the modified MIC test, but were phagicidal. In this case, dynamic conditions could also be the explanation for those results. They caused the extract to be slightly cell-growth inhibiting at 25%, and also in a concentration-dependent manner neutralised the phagicidal effect. The 25% concentration of SRm extract added to *E. coli* DSM 5695 caused increased bacterial proliferation. With the addition of the MS2 phage, a lytic effect was observed that was overall stronger and longer than that evident in the lysis control. However, bacterial growth rebound was detected. Lowering the SRm concentration to 0.19% delayed but also elongated phage lysis compared to the control curve. These concentrations effectively intoxicated the cells in the modified MIC test, and 25% SRm was phage stimulating. Mixing caused the extract to lose its toxicity to cells, the effect even changing to one of cell stimulation, but the effect on the MS2 phage remained an inducing one at 25% SRm. In the challenge to *E. coli* DSM 613, 25% SRm did not generate any specific effect on the bacteria, but when phage T4 was combined with it, a stronger and longer lytic effect was noted than that in the lysis control, which was similar to what occurred in the case of the MS2 phage and *E. coli* DSM 5695, but with no bacterial growth rebound. Lowering the SRm concentration to 0.19% caused the curve to be highly similar to the control growth curve, but after 9 h it began to fall. These concentrations inhibited cell growth in the modified MIC test and stimulated the MS2 phage. Therefore, the results presented here are also consequences of the extracts losing their toxic activity on *E. coli*, but an inductive effect on the T4 phage remained in a concentration-dependent manner.

In general, the literature shows effects of stevia on bacterial cells varying from inhibitory to stimulating in the case of human microbiota (10, 31, 37). Inconsistencies have also been found in research concerning standard laboratory bacterial strains, mainly caused by differences in extract type. *S. rebaudiana* water, methanol, ethyl acetate and hexane extracts that were examined against a low number of selected microorganisms revealed that the water extract was only active against *Bacillus subtilis* and *Staphylococcus aureus* and the methanol extract was the most active against *Pseudomonas aeruginosa*. *Escherichia coli* proved to be most susceptible to hexane extract, less so to ethyl acetate and methanol extracts, and unaffected when water extract was used (38). In research by other authors, where water, ethanol, petroleum ether, cyclohexane, acetone and chloroform extracts were tested, a petroleum ether extract was found to inhibit the growth of *E. coli* completely. The highest antibacterial index was also obtained for petroleum ether extract against all pathogens, with the highest activity against *S. aureus*, *Enterococcus faecalis* and *P. aeruginosa*. This research also found *E. coli*, *Proteus mirabilis* and *B. subtilis* to be the most susceptible to water, ethanol and acetone extracts (13). When stevia extracts were obtained through the Ayurvedic Pharmacopeial method (water and alcohol), the Soxhlet method and column extraction, the highest rate of susceptibility was exhibited by *Enterobacter aerogenes* to all the extracts. The alcohol extract showed the highest activity against all tested bacteria. Visible inhibition of *E. coli* was achieved by the alcohol and Soxhlet extracts, the column extract was active to a lesser extent, but the water extract was not active at all (22). Other experiments revealed that an acetone extract had most effective antibacterial potential, followed by an ethyl acetate extract. The acetone extract showed greater activity against Gram-positive than against Gram-negative organisms. *E. coli* was equally susceptible to ethyl acetate and acetone extracts, but insusceptible to the water extract (17). In contrast to those results, it was also shown in another experiment that a methanolic extract was the most effective against all the tested bacteria. The susceptibility of *E. coli* to chloroform and methanol extracts was similar, and no effect on the bacterium was observed when the water extract was applied. However, it is worth noting that the chloroform and methanol extract exhibited concentration-dependent antibacterial inhibition, and that when extracts were diluted, inhibition was greater in some cases (8).

Difficulty in the interpretation of results and their comparison is even greater when another variable is taken into consideration. It has been shown that even the methods by which *S. rebaudiana* is dried can affect its quality and antimicrobial activity, the optimal method providing the herb with longer-lasting inhibitive effect on bacteria (20). Unfortunately, all of the data referred to were collected by simple diffusion methods; therefore, it is also possible that other authors could obtain different outcomes when using microdilution or real-time growth methods. Even if there are no papers with precedents describing the influence of stevia on bacteriophages, the antiviral activity of the plant was confirmed – hot water extracts of stevia showed anti-human rotavirus (HRV) activity. Stevia inhibited the replication of all four serotypes of HRV *in vitro*. It was indicated that the inhibitory mechanism is a blockade of virus binding and that the inhibitory components were heterogeneous anionic polysaccharides with different ion charges (10). These findings support our research with regards to SRa and SRm activity, as well as tend to sustain the dynamic environment thesis that charged particles could behave differently in mixing conditions rather than in static ones.

Currently, there is limited information in the literature regarding the effects of extracts on phages or the effects of the simultaneous application of both. Even fewer works describe these interactions in the environment of bacterial hosts. Previous scientific publications have mainly described the plaque-forming ability of the phages after their contact with plant extracts (6, 7, 9, 18). However, there are some findings that describe more complex interactions. One of these shows the influence of *Phoenix dactylifera* L. acetone extract on a phage which lyses *Pseudomonas* and described the extract's inhibition of phage infectivity and complete prevention of bacterial lysis; however, it presented no information about the extract's influence on bacteria (16). Some plant extracts have also shown concentration-dependent antiviral activity and a reduction of phage yield (2). The importance of performing multiple tests in order to understand such complex interactions, which can change in different environments, was noted in another study. It was found that black cumin extract increased phage plaque size; however, this effect was not reflected in phage titres in a liquid medium. In general, in liquid media experiments, no synergistic effects were detected. Furthermore, the observed interactions were more closely related to antagonism, something we also noted in our present work (39). Moreover, plant extracts and lytic phages can significantly reduce bacterial concentrations compared to untreated and extract-treated controls, but these reductions were not sustained over time (26).

We hypothesise that changing environmental conditions by mixing could be responsible for some of the observed effects of the stevia extracts. It is worth pointing out that in our previous article, we also observed plant extracts' stimulation of bacteria after testing the mixtures in the dynamic environment of a bioreactor (24). This phenomenon could be explained by the heavier physical contact of the mixtures' molecules due to mixing, which in consequence enhances the interactions between them. However, a thorough understanding of this phenomenon may require further research.

The effects of *S. rebaudiana* acetone (SRa) and methanol (SRm) extracts on the course of phage lysis and activity in the dynamic environment depended on the species of the phage and bacterial host. The greatest differences between the effectiveness levels of the extracts were noted for *E. coli* strains and their phages, whereas *P. syringae* and the Phi6 phage reacted similarly to the SRa and SRm extracts. Differences also emerged for a single extract, whether SRa or SRm, within *E. coli* strains and their phages – therefore each extract type should be tested on a case-by-case basis and no generalisations should be made.

Dynamic conditions can also alter the effect of extracts on bacterial cells and phages in a concentration-dependent manner. Because phage–extract interactions against bacteria in a static environment are often different to those in a dynamic environment, many varied experiments should be performed, especially when examining multifactorial mixtures. Further studies are needed to understand the basics of the interactions between phages and plant extracts for the possible future use of phage–extract combinations as antibacterial mixtures. An appropriate concentration of the selected herb extract may have antibacterial properties and may also increase the activity of phages. The activity of phages combined with the selected extract being more powerful than that of the phages alone, these mixtures could be used in multi-drug resistant bacterial infection treatments.

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