ACTA SCIENTIARUM POLONORUM

Acta Sci. Pol. Technol. Aliment. 20(4) 2021, 473–484

www.food.actapol.net pISSN 1644-0730 eISSN 1898-9594 http://dx.doi.org/10.17306/J.AFS.2021.0968

ORIGINAL PAPER

Received: 26.05.2021
Accepted: 13.10.2021

THE USE OF BACTERIOPHAGES AGAINST SAPROPHYTIC MESOPHILIC BACTERIA IN MINIMALLY PROCESSED FOOD

Michał Wójcicki[1,2]✉, Aleksander Żuwalski[2], Olga Świder[2,3], Iwona Gientka[2], Dziyana Shymialevich[1], Stanisław Błażejak[2]

[1]Department of Microbiology, Prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology – State Research Institute Rakowiecka 36, 02-532 Warsaw, **Poland**
[2]Department of Food Biotechnology and Microbiology, Institute of Food Science, Warsaw University of Life Sciences (WULS – SGGW), Nowoursynowska 166, 02-787 Warsaw, **Poland**
[3]Department of Food Safety and Chemical Analysis, Prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology – State Research Institute, Rakowiecka 36, 02-532 Warsaw, **Poland**

ABSTRACT

Nowadays, food producers strive to meet the changing needs of consumers while maintaining the highest nutritional value of the products they supply. Physicochemical methods, which include modified atmosphere packaging, membrane techniques or ultrasounds, are the most frequently used to preserve food. Alternatively, biological methods can be applied, one of which is the use of bacteriophages (phages) to limit bacterial growth in the food environment. The purpose of our research was to verify the possibility of the use of bacteriophages as an antibacterial agent in minimally processed food environments of vegetable origin. The first stage of the research involved the isolation of phages against the dominant bacterial microflora in the analyzed products: broccoli sprouts, spinach leaves and freshly squeezed carrot-celery juice. Bacteriophages were isolated from municipal waste collected from sewage-treatment plants. Specific bacteriophages were isolated for twenty-nine out of thirty identified bacterial strains. The lytic activity of the phages was tested using a Bioscreen C automatic growth analyzer. Three methods for applying the phage cocktail were tested: direct addition of the cocktail, spraying it on, and placing the food product on a pad soaked with the phage mixture. The food products were packaged in a protective atmosphere and stored at 20°C. The total number of bacteria after adding the phage cocktail to the products was determined during the subsequent hours of incubation using classical microbial culturing. A significant decrease in the total number of bacteria was observed in the products containing phage suspensions. The obtained results suggest that application of the phage cocktail offers the possibility to extend the shelf life of the analyzed minimally processed food products by reducing the total number of saprophytic bacteria.

Keywords: bacteriophages (phages), saprophytic bacteria, food quality, Bioscreen C, minimally processed food

INTRODUCTION

The Western-style diet, a nutrition model that is widely used nowadays, can be linked to an increased risk of chronic diseases, including type 2 diabetes, hypertension, neoplasms and obesity among consumers. Constantly increasing consumer awareness has led to a growth in interest in high-quality nutritional products of natural origin in recent years (Ragaert et al., 2007; Tirpanalan et al., 2011). Aiming to meet consumer

✉michal.wojcicki@ibprs.pl, http://dx.doi.org/0000-0003-0573-3337

expectations, the food industry has introduced soft methods of food processing. This approach allows the bioactive ingredients of foods, which are naturally present in large amounts in products of plant origin, to be preserved (Tirpanalan et al., 2011).

Minimal processing technology (MPT) enables food producers to supply consumers with fresh products which are rich in health-promoting nutrients. The food industry uses numerous physical methods to preserve food. These methods include high hydrostatic pressure (HHP), radiation, ultrasound sonification and pulsed electric field (PEF). Applied methods also include sous-vide and cook-chill packaging, use of protective edible coating, modified atmosphere packaging and preservation using protective microorganisms (Tirpanalan et al., 2011). However, use of the above methods can negatively affect both the sensory features and nutritional value of food. Furthermore, not all methods are effective in decreasing microbiological contamination of products. HHP is bactericidal for vegetative cells, although the spores are not totally inactivated. Gram-positive bacteria in a steady state of growth are more resistant to high pressures than Gram-negative bacteria or the cells in a logarithmic phase of growth (Aymerich et al., 2008; Garriga et al., 2004). Ionizing radiation is effective in limiting the growth of pathogenic bacteria, but even small doses (0.5 kGy) negatively affect the quality of leafy vegetables. The FDA recommends using significant doses of radiation in order to preserve an iceberg lettuce (Tirpanalan et al., 2011). Ultrasound sonification is an inadequate method for preservation of foods of vegetable origin because it causes leaking of cellular contents out to the environment. PEF enables the inactivation of microorganisms simultaneously with the protection of desired sensory and physical features of food products. However, PEF induces conformational changes in proteins, and thus can affect the activity of the native enzymes of products (Ohshima et al., 2007; Tirpanalan et al., 2011; Zhao et al., 2010). One of the major factors affecting the quality of products of vegetable origin is the storage atmosphere. Research by Lopez-Galvez et al. (1996) confirmed the positive influence of controlled atmosphere packaging on the sensory quality of food, especially with respect to red spotting and leaf-edge browning. However, the application of physical methods in the food industry does not always guarantee that a food product with satisfactory sensory and microbiological characteristics will be obtained.

Application of natural "enemies" of bacteria represents a relevant approach in food production. Biological alternatives to physicochemical methods are applicable particularly in the preservation of minimally processed food with the biggest microbiological risk, such as freshly squeezed juices, sprouts or mixed-leaf salads. The use of bacteriophages prolongs the shelf life of the product without altering its physical properties (Wójcicki et al., 2019). Bacteriophages are highly specific bacterial viruses which can usually infect only one species or one type of bacteria, and, unlike antibiotics, they do not destroy the natural commensal microbiota in the gastrointestinal tract of humans (Gientka et al., 2021). Research shows that phages are invulnerable to many stressful conditions during food processing (wide temperature range, pH). Their pH stability expands at lower temperatures. Phages can protect products from contamination during distribution (Wójcicki et al., 2019).

Phages are applied in three sectors in the food industry: primary production (mainly used to prevent the formation of biofilms on the surface of equipment), bio-sanitization (mainly used in production facilities), and bio-preservation (used to extend the shelf life of products by combatting pathogenic bacteria that spoil food). In the case of minimally processed food, the reduction of product quality due to the growth of accompanying saprophytic microflora poses a significant problem (e.g., spoilage of products, rotting, deterioration of color and taste; Gientka et al., 2021).

There are multiple companies which offer commercial bacteriophage preparations intended for the food industry. Many of these preparations have been approved by the FDA (preparations made by PhageGuard (e.g., Listex™ P100), Secure Shield E1, EcoShield™, ListShield™, ShigaShield™, SalmoFresh™) and USDA (PhageGuard, Finalyse®) (Moye et al., 2018; Svircev et al., 2018). Most of the phage preparations are effective in bacteria eradication in the food environment. Ten commercial phage preparations have received a temporary GRAS status (Kahn et al., 2019). In 2016, the EFSA issued a report concerning the evaluation of safety and efficacy in eradicating pathogenic bacteria with the Listex™ P100 developed by Micros Food Safety. Its effectiveness has been confirmed in

scientific research (Guenther et al., 2009; Lewis et al., 2019). However, not all studies have clearly proved the efficacy of commercial bacteriophage preparations. In a study conducted by Arthur et al. (2016), the use of a bacteriophage preparation caused no significant reduction in the number of *Escherichia coli* O157:H7 compared to the control samples. In turn, Oladunjoye et al. (2017) showed an increased efficacy of the coupled use of a bacteriophage preparation with sucrose monolaurate, i.e., a compound exhibiting antibacterial activity.

Commercial bacteriophage preparations are targeted at pathogenic bacteria which prevail in the food environment. However, saprophytic bacteria can also pose a risk of food spoilage, deteriorating food quality, and thereby shorten its shelf life. Therefore, future studies should aim at developing an antibacterial agent with a wide spectrum of activity targeted against saprophytes of minimally processed food (Wójcicki et al., 2019).

The aim of this study was to assess the possibility of the use of bacteriophages in limiting the growth of natural, dominant, saprophytic bacterial strains as well as choosing the most effective method of phage suspension application aimed at protection of the analyzed minimally processed food matrices.

MATERIALS AND METHODS

Biological material and research material

The subject of our study was bacteriophages directed against autochthonous bacterial microflora of the analyzed minimally processed products. The phages were isolated from municipal sewage which was collected from a wastewater treatment plant in Izabelin "Mokre Łąki" (near Warsaw, Poland).

Three kinds of minimally processed plant products characterized by high microbiological instability were tested – broccoli sprouts, spinach leaves and freshly squeezed carrot-celery juice.

Isolation and taxonomic identification of the bacterial strains from food products

Saprophytic mesophilic bacterial strains were obtained by isolating their colonies from a PCA medium (during the Total Number of Bacteria (TNB) determination). To ensure pure bacterial cultures, single colonies were repeatedly picked and streaked on nutrient agar (PCA, BTL, Poland), and cultured at 37°C. Then, strains were cultured on selectively differentiating media: Endo, VRBL and XLD (all microbiological media: BTL, Poland). The biochemical features of the strains were determined using the API-20E test (bio-Mérieux, Poland). The taxonomic identification of the isolated bacteria was determined by analysis of the protein profile using Matrix Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) analyzer. All strains were maintained at –80°C in Luria Bertani (LB) broth (bactotryptone 10.0 g/L, NaCl 10.0 g/L, yeast extract 5.0 g/L, pH 7.2 ±0.2) supplemented with 20% glycerol.

Isolation and propagation of bacteriophages from municipal sewage

The sample of municipal sewage collected for analysis was centrifuged with the aim of separating the organic and mineral parts from the microflora. In order to separate bacteria from the potentially present bacteriophages, the supernatant was filtered using syringe filters with a 0.22 μm membrane. In the next step, 20 mL of phage supernatant was added to 20 mL of T-Broth's double-concentrated liquid medium (nutrient broth 8.0 g/L, peptone 5.0 g/L, sodium chloride 5.0 g/L and glucose 1.0 g/L). The medium was inoculated with an overnight bacterial culture and held for 24 h at 37°C. Subsequently, the medium was filtered by a syringe filter with a 0.45 μm membrane. Concentration of the phages (the phage titer, i.e., the number of phages in 1 mL lysate, expressed in PFU/mL) in the obtained lysates was determined using the method of two-layer plates on a substrate with a strain of bacteria cultured. To this end, a series of phage lysate dilutions were prepared. 100 μL of fresh bacterial suspension in a TSB medium (BTL, Poland) and 500 μL phage lysate from the appropriate dilution were introduced into the sterile tube. The mixture was vortexed and left at 20°C for 15 min in order to let the phages adsorb to the surface of the host cells. Then, 3 mL of top-agar medium liquefied and cooled to 45°C (nutrient broth 25.0 g/L, agar-agar 7.5 g/L) was added to the tube, vortexed and poured onto the surface of the plate with nutrient agar. The dishes were held upside down overnight at 37°C (according to Alves et al., 2019). The plaques were counted after incubation. The phage titer was determined according to the following formula:

$$\text{Phage titer} = \frac{\text{number of plaques – forming units, PFU}}{\text{volume of phage suspension, mL} \times \text{dilution of the phage lysate}}$$

Then, phage plaques were cut with a scalpel and purified three times in SM buffer according to the method proposed by Mirzaei and Nilsson (2015).

Verification of the activity of lytic phages

The lytic activity of the phages was verified using a Bioscreen C automatic growth analyzer (according to Islam et al., 2020). To this end, the all-night bacterial culture carried out in LB broth was diluted 1:100 in a fresh medium with the addition of magnesium sulphate and calcium chloride with a final concentration of 0.01 M. 190 µL of culture was applied to the wells in the multi-area plates and incubated in the apparatus at 37°C until the optical density increased $\Delta OD_{600} \sim 0.1$. The wells were infected by adding 10 µL of a phage lysate with an index of multiplicity of infection (MOI) of 1.0 and 0.1, respectively. Multiplicity of infection is the ratio of the number of infectious virus particles to a known number of bacterial cells in the medium. The plates were left for 15 min at room temperature to let the phages adsorb to the surface of host cells. Then, in the Bioscreen C growth analyzer, a 24-hour culture was carried out at 37°C. The apparatus measured the optical density of the culture every 15 min. Each variant was tested ten times. Based on the obtained data, bacterial cell growth curves were plotted. For each of the strains, the coefficients of specific growth rate – µ were determined according to the formula:

$$\mu = \frac{\ln OD_{max} - \ln OD_{min}}{t}$$

where:

$\ln OD_{max}$ – natural logarithm from the maximum optical density value of the culture during exponential growth,

$\ln OD_{min}$ – natural logarithm from the maximum optical density value of the culture during exponential growth,

t – duration of the exponential growth phase of the culture, h.

Application of the phage cocktail to the analyzed food products

The entire study was based on proprietary methodology. The phage cocktail was a mixture of 29 isolated bacteriophages with known titer lysates. Before infecting the products, the phage mixture was filtered through a cellulose syringe filter with a membrane pore diameter of 0.45 µm. The titer of the prepared phage cocktail equaled $2.6 \cdot 10^9$ PFU/mL and that was the average number of all phages in the prepared application mixture.

Solid products (broccoli sprouts and spinach leaves) were infected in two ways. Firstly, a cellulose absorbent pad with a capacity of 2500 mL/m^2 impregnated with a phage suspension was placed on a styrofoam tray. Then, 50 g of the product was applied and packed in a protective atmosphere. In the second case, 50 g of product was applied to the styrofoam tray and direct spraying (5 mL) of the phage cocktail was carried out. The prepared samples were also packed in a protective atmosphere.

In the case of a liquid product (freshly squeezed carrot-celery juice), a phage suspension (5% of the volume of juice) was applied directly to the glass bottle. The bottles were protected with a layer of parafilm.

All samples were stored at 20°C. Measurements of changes in the total number of mesophilic bacteria were carried out in triplicate at the 0th, 6th, 24th, and 48th hours of experiment on nutrient agar (PCA, BTL, Poland). Simultaneously, control samples (without the addition of phage mixtures) were performed.

Statistical analyses

In the analysis of the practical application of the phage cocktail, a two-way analysis of variance with a confidence interval of 0.95 was performed. Statistical tests and calculations were performed using the Statistica 13.1 software suite.

RESULTS AND DISCUSSION

The high-quality of minimally processed food can be maintained not only thanks to the eradication of pathogenic bacteria, but generally by preventing the growth of any microorganisms, including saprophytic ones. In previous studies, we determined the microbiological quality of minimally processed food during storage.

In accordance with the legal requirements, the presence of *Salmonella* spp. and *Listeria monocytogenes* was examined in the products. The presence of these pathogens was not found in any of the tested batches. Contamination of the products with bacilli strains was relatively low. The conducted microbiological quality tests of minimally processed plant products (unpublished studies) drew our attention to the high contamination of these products with bacteria from the Enterobacteriaceae family. We hypothesized that the reduction of bacteria belonging to the Enterobacteriaceae family would significantly reduce microbial contamination of food products.

During the research, pure bacterial cultures were isolated. Based on morphological (according to Bergey's, i.e., Gram staining result, cell shape, oxygen tolerance, and spore formation) and biochemical properties (API 20E test), 30 isolated strains belonged to the Enterobacteriaceae family. The analysis of the protein spectra (using MALDI-TOF MS) of the isolated strains confirmed their taxonomic affiliation (Table 1). The pure bacterial cultures then served as hosts for the bacteriophages. Host-specific strains of phages were found against 29 of the isolated strains of bacteria. A phage titer was determined for each virus (Table 1).

There are many studies available that assess the microbiological quality of minimally processed plant products. An assessment of microbiological quality of sprouts carried out by Gientka et al. (2021) showed that the most microbiologically contaminated were alfalfa sprouts (TNB = 8.14 log CFU/g). In other studies (Berthold-Pluta et al., 2017), the total count of aerobic mesophilic bacteria in samples of lettuces, sprouts and non-pasteurized fruit, vegetable and fruit-vegetable juices was in the range of 5.6–7.6, 6.7–8.4 and 2.9–7.7 log CFU/g, respectively. A study of the

Table 1. Identification of isolated bacterial strains and concentration of viruses in lysates (phage titer)

Bacterial strain code	Classification according to Bergey's				Bacteria identification according to API 20E test	Bacteria identification according to MALDI-TOF MS	Phage titer (M_F) in the lysate PFU·mL^{-1}
	Gram staining result	cell shape	oxygen tolerance	spore formation			
1	2	3	4	5	6	7	8
BRS-02	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Escherichia coli*	*Escherichia coli*	$3.6 \cdot 10^4$
BRS-03	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Escherichia coli*	*Escherichia coli*	$3.1 \cdot 10^8$
BRS-06	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Citrobacter youngae*	*Citrobacter* sp.	$3.9 \cdot 10^9$
BRS-10	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Serratia fonticola*	*Serratia liquefaciens*	$9.3 \cdot 10^9$
BRS-14	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Escherichia coli*	*Escherichia coli*	$7.5 \cdot 10^9$
BRS-18	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	$6.4 \cdot 10^6$
BRS-19	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Pantoea* spp.	*Pantoea agglomerans*	$1.4 \cdot 10^9$
BRS-24	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Escherichia coli*	*Escherichia coli*	$2.0 \cdot 10^7$
BRS-25	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Enterobacter cancerogenus*	*Enterobacter* sp.	$3.5 \cdot 10^5$
BRS-28	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Escherichia coli*	*Escherichia coli*	$4.0 \cdot 10^6$

Table 1 – cont.

1	2	3	4	5	6	7	8
BRS-38	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Enterobacter cloacae*	*Enterobacter* sp.	$4.0 \cdot 10^4$
BRS-39	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Cronobacter sakazakii* (formerly *Enterobacter sakazakii*)	no identification	$3.0 \cdot 10^7$
BRS-45	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Enterobacter ludwigii*	*Enterobacter cloacae*	$5.4 \cdot 10^7$
SPL-07	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Serratia fonticola*	*Serratia fonticola*	$2.0 \cdot 10^9$
SPL-08	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Escherichia coli*	*Escherichia coli*	$1.3 \cdot 10^9$
SPL-20	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Citrobacter koseri / farmeri*	*Citrobacter farmeri*	$1.7 \cdot 10^8$
SPL-23	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Cronobacter sakazakii* (formerly *Enterobacter sakazakii*)	*Enterobacter cloacae*	$2.2 \cdot 10^9$
SPL-27	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Escherichia coli*	*Escherichia coli*	phages were not isolated
SPL-30	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Escherichia coli*	*Escherichia coli*	$1.1 \cdot 10^{10}$
SPL-33	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Shigella sonnei*	*Shigella sonei*	$5.2 \cdot 10^9$
SPL-36	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Serratia ficaria*	*Serratia fonticola*	$2.6 \cdot 10^8$
SPL-37	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Cronobacter sakazakii* (formerly *Enterobacter sakazakii*)	no identification	$8.0 \cdot 10^8$
SPL-40	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Enterobacter asburiae*	no identification	$5.7 \cdot 10^7$
CCJ-04	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Escherichia coli*	*Escherichia coli*	$9.6 \cdot 10^9$
CCJ-16	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Shigella* spp.	*Shigella dysenteriae*	$1.1 \cdot 10^{10}$
CCJ-21	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Pantoea* spp.	no identification	$1.3 \cdot 10^9$
CCJ-26	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Rahnella aquatilis*	no identification	$3.6 \cdot 10^9$
CCJ-27	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Citrobacter freundii*	*Citrobacter* sp.	$2.7 \cdot 10^9$
CCJ-29	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Enterobacter ludwigii*	*Enterobacter* sp.	$2.7 \cdot 10^8$
CCJ-35	Gram-negative	bacilli rod-shaped	facultative anaerobes	non-spore-forming	*Cronobacter sakazakii* (formerly *Enterobacter sakazakii*)	*Enterobacter cloacae*	$2.9 \cdot 10^9$

microbiological quality of mung bean sprouts (Iacumin and Comi, 2019) showed that in all tested samples, the microbial load was less than 6 log CFU/g. In addition, the results demonstrated that no pathogenic microorganisms were present. Similarly, in studies by Baenas et al. (2017), pathogenic microorganisms were absent during the shelf life of sprouts. Nevertheless, the slight growth of bacteria from the Enterobacteriaceae family, aerobic mesophilic and psychrophilic bacteria, was observed.

No identification means that results obtained show conflict, e.g., more than one bacterial species identified or the base used does not contain protein spectra characteristic for the tested bacteria.

The phages' ability to lyse bacterial host cells was examined using an automatic Bioscreen C growth analyzer. Tables 2–4 show changes in the specific growth rate of bacterial cultures after adding the appropriate phage lysate. Measurement of the optical density using the Bioscreen C automatic growth analyzer allowed us to determine the beginning and duration of the log phase of growth of the tested strains that were deliberately infected with complementary phages with MOI of 1.0 and 0.1 against the control culture.

The changes in the coefficient of specific growth rate of bacteria cultures isolated from broccoli sprouts (BRS) after adding a phage lysate are presented in Table 2. Thirteen competent phages were isolated from the broccoli sprout samples. Significantly lower (compared to the control samples) coefficients of specific growth rate in the phage infected samples indicate a significant reduction in cell division during the log phase of the growth of the tested strains. The use of MOI = 1.0 in most cases had a stronger effect on inhibiting the cell division of bacterial hosts as compared to a lower multiplicity of infection (MOI = 0.1).

In the case of the BRS-18 strain, a lower coefficient of specific growth rate was observed at MOI = 0.1 than MOI = 1.0. This may be due to the fact that the phage titer was slightly higher, resulting in a multiplicity of infection over 1.0. In this case, the lytic activity of the phage could be reduced because the highest efficiency is observed at a multiplicity of infection of 1.0 or slightly below (so on average, one bacterial cell accounts for one phage particle, and lysis can be more efficient). The above relates to the fact that bacteriophages in an environment with high density of

Table 2. Changes in the specific growth rate of the bacterial culture isolated from the broccoli sprouts after addition of the phage lysate, $n = 10$

No.	Bacterial strain code	Control sample $\mu \cdot h^{-1}$	MOI 1.0 $\mu \cdot h^{-1}$	MOI 0.1 $\mu \cdot h^{-1}$
1	BRS-02	0.027	0.018	0.020
2	BRS-03	0.025	0.011	0.013
3	BRS-06	0.036	0.018	0.019
4	BRS-10	0.041	0.016	0.019
5	BRS-14	0.036	0.029	0.033
6	BRS-18	0.058	0.031	0.026
7	BRS-19	0.038	0.010	0.012
8	BRS-24	0.029	0.012	0.023
9	BRS-25	0.042	0.018	0.024
10	BRS-28	0.029	0.024	0.024
11	BRS-38	0.048	0.017	0.024
12	BRS-39	0.057	0.017	0.027
13	BRS-45	0.045	0.017	0.023

Table 3. Changes in the coefficient of specific growth rate of bacterial cultures isolated from spinach leaves after addition of phage lysate, $n = 10$

No.	Bacterial strain code	Control sample $\mu \cdot h^{-1}$	MOI 1.0 $\mu \cdot h^{-1}$	MOI 0.1 $\mu \cdot h^{-1}$
1	SPL-07	0.040	0.021	0.033
2	SPL-08	0.064	0.027	0.051
3	SPL-20	0.080	0.029	0.036
4	SPL-23	0.065	0.037	0.047
5	SPL-30	0.062	0.019	0.027
6	SPL-33	0.052	0.015	0.015
7	SPL-36	0.054	0.028	0.031
8	SPL-37	0.067	0.046	0.056
9	SPL-40	0.055	0.042	0.052

Table 4. Changes in the coefficient of specific growth rate of bacterial cultures isolated from freshly squeezed carrot-celery juice after addition of phage lysate, $n = 10$

No.	Bacterial strain code	Control sample $\mu \cdot h^{-1}$	MOI 1.0 $\mu \cdot h^{-1}$	MOI 0.1 $\mu \cdot h^{-1}$
1	CCJ-04	0.022	0.016	0.021
2	CCJ-16	0.028	0.016	0.019
3	CCJ-21	0.030	0.019	0.025
4	CCJ-26	0.040	0.018	0.032
5	CCJ-27	0.022	0.011	0.018
6	CCJ-29	0.023	0.015	0.015
7	CCJ-35	0.017	0.009	0.011

bacterial population are more likely to infect bacterial cells, striving to dominate the environment. The use of multiplicity of infection 1.0 and 0.1 for BRS-28 strain did not show differences in the values of coefficients of specific growth rate. The BRS-19 strain culture, together with complementary phages of MOI equal to 1.0, was the most efficient in decreasing the coefficient of specific growth rate – almost four times more than the control sample.

The changes in the coefficient of the specific growth rate of the bacterial cultures isolated from spinach leaves (SPL) after adding a phage lysate are presented in Table 3. In each sample, a higher multiplicity of infection (1.0) inhibited the increase in the density of the culture more than in the MOI equal to 0.1. Nine competent phages were isolated from bacteria from spinach samples.

Table 4 shows changes in the coefficient of the specific growth rate of bacterial cultures isolated from freshly squeezed carrot-celery juice after the addition of phage lysate. Seven competent phages were isolated from the freshly squeezed carrot-celery juice (CCJ) samples.

Changes in the onset of the logarithmic growth phase in phage-infected cultures were investigated by Zhao et al. (2017). They demonstrated that regardless of the multiplicity of infection, the bacterial cultures treated with phages began the log-phase significantly later than the control cultures.

In a study conducted by Mahmoud et al. (2018), the growth of *Salmonella* Kentucky infected with bacteriophages at MOI = 1.0 was delayed by all phages examined compared to the control cultures. After 24-h incubation, the phages completely inhibited the growth of the bacterial host strain. In turn, in an experiment carried out by Yu et al. (2016), the phage-infected cultures exhibited poorer growth compared to the control culture up to 24 h. In the subsequent 24 h, part of the culture showed a stronger growth than the control culture, which – according to the authors – was due to the fact that the bacteria became resistant to infection with the phages tested.

The results of the aforementioned experiments suggest that the preservation of lytic activity is strongly dependent on bacteriophage type. The analysis of the lysis rate of phage-infected bacterial cells in time enables preliminary estimation of the virulence of viruses.

The target stage of our research was the use of phage suspensions (which were a mixture of all 29 phage lysates) in the food products studied. It should be noted that the obtained phage cocktail contained viruses specific to various genera, species and strains of bacteria belonging to the Enterobacteriaceae family. The lack of phage isolated against the *Escherichia coli* SPL-27 strain (Table 1) proves the specificity of the phages (the phages were isolated against other *E. coli* strains). The application of phage cocktails to the products was carried out using three methods: by direct addition of the cocktail (in the case of freshly squeezed carrot-celery juice), by spraying or placing the product on an insert soaked with a mixture of phages (in the case of spinach leaves and broccoli sprouts).

Figure 1 presents how the time and the method of phage application influences the microbiological quality of broccoli sprouts. During a 48-hour storage period in the control sample, the initial log value from the TNB in 1 g was 8.81 and after completion, 9.16.

The use of spraying and an absorbent pad significantly reduced the growth of microorganisms in the product environment after 24 hours. After 48 h the log value from the number of CFU/g was respectively 8.47 and 8.45. During storage, the TNB in the control sample increased by less than half the log unit, while in the samples with phages it decreased by a similar value.

Figure 2 presents how the method of phage application influenced the microbiological quality of

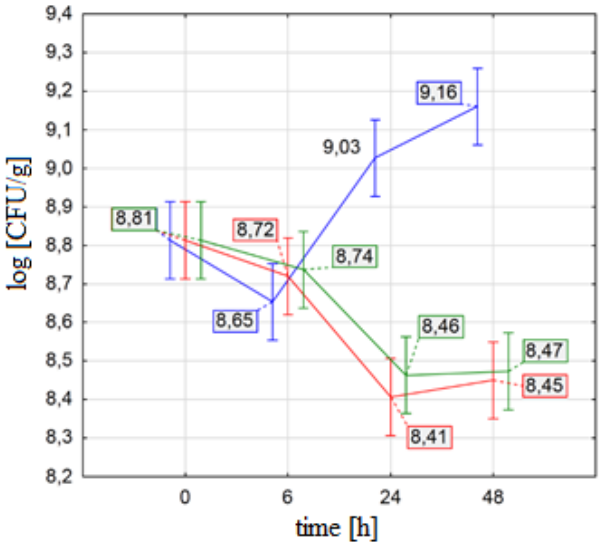

Fig. 1. Influence of the phage application method and storage time on the TNB of broccoli sprouts, n = 3: blue line – control sample, green line – spraying of phages, red line – absorbent pad soaked with phage mixture. Vertical bars in the charts represent 0.95 confidence intervals

spinach leaves in the course of the experiment. During a 48-hour storage period in the control sample, the log value from the TNB in 1 g increased from 7.17 to 8.83. Either spraying or using an absorbent pad significantly reduced the growth of microorganisms in the product environment after 6 hours (compared to the control sample). After 48 hours, the log value from the TNB in 1 g of the product was 8.09 for the absorbent pad and 8.33 for the spray. Compared with the control sample, the use of a phage cocktail on spinach leaves reduced the TNB by a half to almost one log unit depending on the method of application.

Figure 3 presents how the method of phage application influenced the microbiological quality of freshly squeezed carrot-celery juice in the course of the experiment. During a 48-hour storage period in the control sample, the TNB did not change significantly. Application of the phage suspension to the juice noticeably reduced the TNB after 6 hours. During storage, the TNB in the product infected with the phage mixture constantly decreased and after 48 hours its log value was 6.45. Probably the fluid environment of the

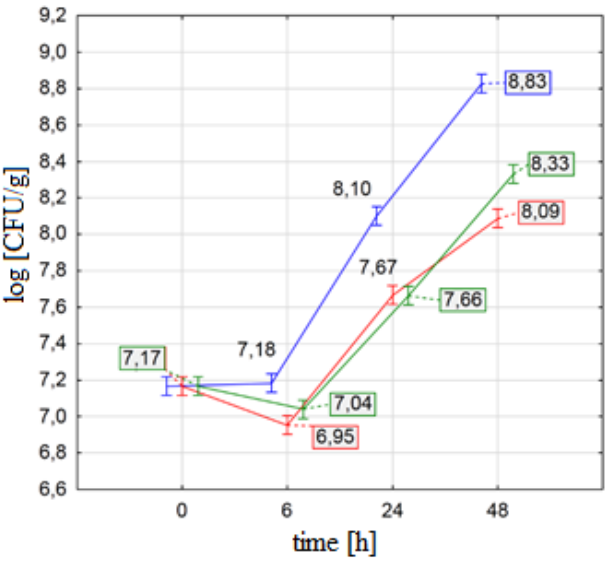

Fig. 2. Influence of the phage application method and storage time on the TNB of spinach leaves, n = 3: blue line – control sample, green line – spraying of phages, red line – absorbent pad soaked with phage mixture. Vertical bars in the charts represent 0.95 confidence intervals

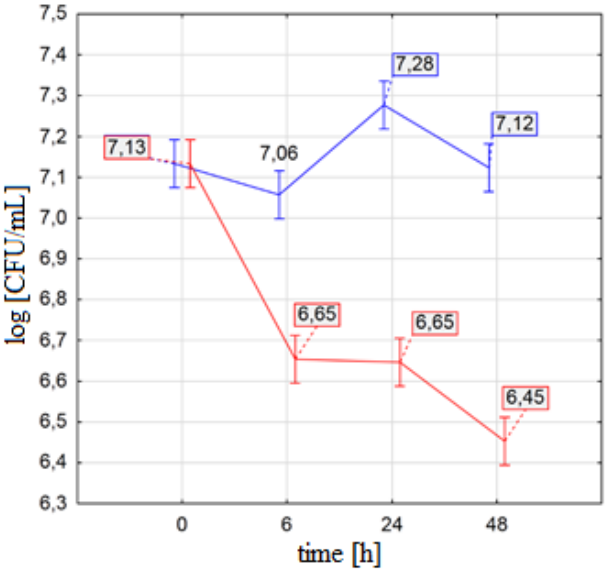

Fig. 3. Influence of the phage application method and storage time on the TNB of freshly squeezed carrot-celery juice, n = 3: blue line – control sample, red line – addition of phage cocktail. Vertical bars in the charts represent 0.95 confidence intervals

product, which is the juice (with high water activity, reaching almost 1.0), significantly facilitated both the spread of phages in the product and the effective infection of host bacterial cells.

It is worth noting that the phages included in the mix were isolated against bacterial hosts from other product batches than those for which they were ultimately used. Their effective action was therefore possible only if the product was contaminated with the same bacterial strains as in the production batches or the isolated phages displayed a wide spectrum of activity on different host species (or strains). Such a bacteriophage trait would be most desirable because the viral preparation would become more versatile.

We found only one study in which the effect of adding a bacteriophage suspension on the level of TNB was tested. In the experiment of Gientka et al. (2021), similarly to this article, various methods for applying phage suspensions were used. The spraying method was significantly more efficient on sprouts than a pad soaked with phage, and the maximum reduction effect after 48 h of incubation was 1.5 log CFU/g. Using pads soaked with phage lysate reduced the total number of bacteria to about 0.27–0.79 log CFU/g. In the study mentioned, unlike in our research, the absorbent pad did not show better distribution of phages during storage. Perhaps the structure of the insert itself made it difficult for the phages to be released into the product matrix.

Other studies are mainly focused on the effective elimination of virulent bacterial strains which consists of the deliberate contamination of samples with a specific bacterial host strain to which specific phages are then added. In this way, the number of microorganisms can be reduced by at least two log units. In the experiments of Guenther et al. (2009), the effectiveness of selected phages specific to the specific strains of *Listeria monocytogenes* was examined. Samples were intentionally infected with bacteria and then with phages. In the case of cabbage samples (sliced fresh leaves), there was a reduction in the number of bacteria by 3–4 log units, and in the case of iceberg lettuce by 2 log units in comparison with the control samples (without phages). Leverentz et al. (2003) investigated the effect of lytic phages, specifically on *Listeria monocytogenes*, using two different ways of applying viruses: by spraying and by pipetting on intentionally contaminated fresh melon and apple fruit. The phage mixture reduced the *L. monocytogenes* population by 2.0 to 4.6 log units compared to the melon control sample and to below 0.4 log as compared to the apple control sample. In combination with nisin, a mixture of phages reduced the bacterial population by 5.7 and 2.3 log units, respectively, compared to the control samples. Both phage spray and phages with nisin reduced the number of bacteria to at least the same extent as the use of pipetting. The effectiveness of the phages depended on both the method of application and the initial concentration of *L. monocytogenes*. In other studies (Oliveira et al., 2014), the effect of Listex™ P100 on the inactivation of *Listeria monocytogenes* in fruit juices was investigated. It was shown that the use of phage preparation significantly reduced the bacterial population in pear and melon juices (about 1 and 3 log units, respectively, compared to the control samples, without phages). When Listex™ P100 was applied to apple juice, no statistically significant reduction in bacteria was observed. This may be related to the pH of the product environment (pH 3.7), which both limited the growth of bacteria during storage and reduced the lytic activity of bacteriophages.

Commercial bacteriophage preparations targeted at the main food pathogens are already available for sale, and their effectiveness has been repeatedly confirmed in scientific studies. The high quality of minimally processed food can be assured not only by the elimination of pathogenic bacteria, but by generally limiting the number of microorganisms, including saprophytic ones. Therefore, successive research should focus on developing such a biological agent that will also have an effect on saprophytic microorganisms.

CONCLUSION

Due to the increasingly common problem of acquiring bacterial resistance to applied methods of food preservation, research centers around the world are trying to biologically control food and the surfaces of direct contact with food products using phage mixtures.

In the technology of preservation of minimally processed food, the producers neither intentionally contaminate food nor examine what bacterial microflora dominates in a given production batch. Therefore, the phage cocktails used must be "universal", i.e., contain a wide range of phages selected with the

aim of affecting many bacteria strains, species and types, both pathogenic and saprophytic. Such a mixture should both guarantee the safety of food and extend the shelf life of minimally processed products.

The obtained results indicate that using a phage cocktail to preserve minimally processed products can bring the expected reduction in the contamination with accompanying microflora, regardless of the application method used. In the case of broccoli sprouts, the application methods applied decreased the TNB to a similar level (about 0.7 log units), while in the case of spinach leaves, the use of an absorbent pad (reduction by about 0.8 log units) proved to be more beneficial. The application of a phage suspension to freshly squeezed carrot-celery juice reduced the degree of contamination of the product by about 0.7 log units compared to the control sample. The developed phage mixture provides the possibility of extending the shelf life of minimally processed food products.

In further studies, attention should be paid to recognizing phages with the broadest possible spectrum of activity. Phages in lysogenic development are more prone to host resistance thus searching for phages capable only of the lytic cycle is desirable.

REFERENCES

Alves, D., Cerqueira, M. A., Pastrana, L. M., Sillankorva, S. (2019). Entrapment of a phage cocktail and cinnamaldehyde on sodium alginate emulsion-based films to fight food contamination by *Escherichia coli* and *Salmonella* Enteritidis. Food Res. Int., 108791. https://doi.org/10.1016/j.foodres.2019.108791

Arthur, T. M., Kalchayanand, N., Agga, G. E. (2016). Evaluation of bacteriophage application to cattle in lairage at beef processing plants to reduce *Escherichia coli* O157:H7 prevalence on hides and carcasses. Foodborne Pathog. Dis., 14(1), 17–22. https://doi.org/10.1089/fpd.2016.2189

Aymerich, T., Picouet, P. A., Monfort, J. M. (2008). Decontamination technologies for meat products. Meat Sci., 78(1–2), 114–129. https://doi.org/10.1016/j.meatsci.2007.07.007

Baenas, N., Gómez-Jodar, I., Moreno, D. A., García-Viguera, C., Periago, P. M. (2017). Broccoli and radish sprouts are safe and rich in bioactive phytochemicals. Postharv. Biol. Technol., 127, 60–67. http://dx.doi.org/10.1016/j.postharvbio.2017.01.010

Berthold-Pluta, A., Garbowska, M., Stefańska, I., Pluta, A. (2017). Microbiological quality of selected ready-to-eat leaf vegetables, sprouts and non-pasteurized fresh fruit-vegetable juices including the presence of *Cronobacter* spp. Food Microbiol., 65, 221–230. https://doi.org/10.1016/j.fm.2017.03.005

EFSA (2016). Evaluation of the safety and efficacy of Listex™ P100 for reduction of pathogens on different ready-to-eat (RTE) food products. EFSA Panel on Biological Hazards. EFSA J., 14(8), 1–94. https://doi.org/10.2903/j.efsa.2016.4565

Garriga, M., Grèbol, N., Aymerich, M. T., Monfort, J. M., Hugas, M. (2004). Microbial inactivation after high-pressure processing at 600 MPa in commercial meat products over its shelf life. Innov. Food Sci. Emerg. Technol., 5(4), 451–457. https://doi.org/10.1016/j.ifset.2004.07.001

Gientka, I., Wójcicki, M., Żuwalski, A. W., Błażejak, S. (2021). Use of phage cocktail for improving the overall microbiological quality of sprouts – two methods of application. Appl. Microbiol., 1(2), 289–303. https://doi.org/10.3390/applmicrobiol1020021

Guenther, S., Huwyler, D., Richard, S., Loessner, M. (2009). Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. Appl. Environ. Microbiol., 75(1), 93–100. https://doi.org/10.1128/AEM.01711-08

Iacumin, L., Comi, G. (2019). Microbial quality of raw and ready-to-eat mung bean sprouts produced in Italy. Food Microbiol., 82, 371–377. https://doi.org/10.1016/j.fm.2019.03.014

Islam, M. S., Zhou, Y., Liang, L., Nime, I., Yan, T., Willias, S. P., ..., Li, J. (2020). Application of a broad range lytic phage LPST94 for biological control of *Salmonella* in foods. Microorganisms, 8(2), 247. https://doi.org/10.3390/microorganisms8020247

Kahn, L. H., Bergeron, G., Bourassa, M. W., De Vegt, B., Gill, J., Gomes, F., ..., Topp, E. (2019). From farm management to bacteriophage therapy: strategies to reduce antibiotic use in animal agriculture. Ann. NY Acad. Sci., 1441, 1 [special issue: Antimicrobial resistance from food animal production], 31–39. https://doi.org/10.1111/nyas.14034

Leverentz, B., Conway, W. S., Camp, M. J., Janisiewicz, W. J., Abuladze, T., Yang, M., ..., Sulakvelidze, A. (2003). Biocontrol of *Listeria monocytogenes* on freshcut produce by treatment with lytic bacteriophages and a bacteriocin. Appl. Environ. Microb., 69(8), 4519–4526. https://doi.org/10.1128/AEM.69.8.4519-4526.2003

Lewis, R., Bolocan, A. S., Draper, L. A., Ross, R. P., Hill, C. (2019). The effect of a commercially available bacteriophage and bacteriocin on *Listeria monocytogenes* in Coleslaw. Viruses, 11(11), 977. https://doi.org/10.3390/v11110977

Lopez-Galvez, G., Saltveit, M., Cantwell, M. (1996). The visual quality of minimally processed lettuces stored in air or controlled atmosphere with emphasis on romaine and iceberg types. Postharv. Biol. Tech., 8(3), 179–190. https://doi.org/10.1016/0925-5214(95)00002-X

Mahmoud, M., Askora, A., Barakat, A. B., Rabie, O. E.-F., Hassan, S. E. (2018). Isolation and characterization of polyvalent bacteriophages infecting multi drug resistant *Salmonella* serovars isolated from broilers in Egypt. Int. J. Food Microbiol., 266, 8–13. https://doi.org/10.1016/j.ijfoodmicro.2017.11.009

Mirzaei, M. K., Nilsson, A. S. (2015). Isolation of phages for phage therapy: A comparison of spot tests and efficiency of plating analyses for determination of host range and efficacy. PLoS ONE, 10(5), e0127606. https://doi.org/10.1371/journal.pone.0118557

Moye, Z. D., Woolston, J., Sulakvelidze, A. (2018). Bacteriophage application for food production and processing. Viruses, 10(4), 205. https://doi.org/10.3390/v10040205

Ohshima, T., Tamura, T., Sato, M. (2007). Influence of pulsed electric field on various enzyme activities. J. Electrostat., 65(3), 156–161. https://doi.org/10.1016/j.elstat.2006.07.005

Oladunjoye, A. O., Oyewole, S. A., Singh, S., Ijabadeniyi, O. A. (2017). Prediction of *Listeria monocytogenes* ATCC 7644 growth on fresh-cut produce treated with bacteriophage and sucrose monolaurate by using artificial neural network. Food Sci. Technol., 76, 9–17. https://doi.org/10.1016/j.lwt.2016.10.042

Oliveira, M., Viñas, I., Colàs, P., Anguera, M., Usall, J., Abadias, M. (2014). Effectiveness of a bacteriophage in reducing *Listeria monocytogenes* on fresh-cut fruits and fruit juices. Food Microbiol., 38, 137–142. https://doi.org/10.1016/j.fm.2013.08.018

Ragaert, P., Devlieghere, F., Debevere, J. (2007). Role of microbiological and physiological spoilage mechanisms during storage of minimally processed vegetables. Postharves Biol. Tec., 44(3), 185–19. https://doi.org/10.1016/j.postharvbio.2007.01.001

Svircev, A., Roach, D., Castle, A. (2018). Framing the future with bacteriophages in agriculture. Viruses, 10(5), 218. https://doi.org/10.3390/v10050218

Tirpanalan, O., Zunaboovic, M., Doming, K. J., Kneifel, W. (2011). Mini review: antimicrobial strategies in the production of fresh-cut lettuce products. Science against microbial pathogens: communicating research and technological advances, 1, 176–188.

Wójcicki, M., Błażejak, S., Gientka, I., Brzezicka, K. (2019). The concept of using bacteriophages to improve the microbiological quality of minimally-processed foods. Acta Sci. Pol. Technol. Aliment., 18(4), 373–383. http://dx.doi.org/10.17306/J.AFS.2019.0695

Yu, J.-G., Lim, J. A., Song, Y.-R., Heu, S., Kim, G. H., Koh, Y. J., Oh, C.-S. (2016). Isolation and characterization of bacteriophages against *Pseudomonas syringae* pv. *actinidiae* causing bacterial canker disease in kiwifriut. J. Microbiol. Biotechnol., 26(2), 385–393. https://doi.org/10.4014/jmb.1509.09012

Zhao, J., He, L., Pan, L., Liu, Y., Yao, H., Bao, G. (2017). Effect of a lytic bacteriophage on rabbits experimentally infected with pathogenic *Escherichia coli*. World Rabbit Sci., 25, 273–279. https://doi.org/10.4995/wrs.2017.6395

Zhao, W., Yang, R., Xiao, H., Zhang, W., Tang, Y., Chen, T. (2010). Inactivation of polyphenoloxidase of pear by pulsed electric fields. Int. J. Food Eng., 6(3), 1–15. https://doi.org/10.2202/1556-3758.1853