Food Research 6 (Suppl. 2) : 219 - 226 (2023)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Evaluation on the potential application of bacteriophages mixture to control the growth of *Escherichia coli* strains on selected vegetables

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Article history:
Received: 4 September 2021
Received in revised form: 29 October 2021
Accepted: 24 February 2022
Available Online: 8 March 2023

Keywords:
Bacteriophages,
Biological control,
Contamination,
Escherichia coli,
Foodborne

DOI:
https://doi.org/10.26656/fr.2017.6(S2).038

Abstract

Fresh vegetables are being identified as a source of foodborne outbreaks with increasing cases around the world. *Escherichia coli* has been used as one of the hygiene indicators to assess the hygiene status of a food product. Bacteriophages may offer a more natural means to eliminate or reduce microbial load to a safe level when compared to some commonly used conventional methods. This study evaluated the ability of bacteriophages mixture to reduce or eliminate mixed *E. coli* strains in selected vegetables. The bacteriophages mixture contained several types of bacteriophages isolates and was suspended in a phage buffer. An amount of 10 g of selected vegetables (cherry tomato, green mustard, bean sprout and lettuce respectively), was washed and subsequently added with high-density *E. coli* strains (10^8 CFU/mL) to create artificial contamination. Later, 100 µL of bacteriophages mixture (10^9 PFU/mL) was applied to each of the vegetables and allowed to dry for 15 min in a biosafety cabinet. Evaluation of the reduced number of viable *E. coli* cells (CFU/g) in each sample was done at 0 hr, 6 hrs, 24 hrs, 48 hrs and 72 hrs intervals, for two different storage conditions which were room temperature (30°C) and chilled temperature (4°C). The artificially contaminated sample not treated with bacteriophages mixture was used as a control. The overall results showed that the application of bacteriophage mixture was able to reduce viable *E. coli* cell numbers if compared with the control sample. Its application was found most efficient in reducing *E. coli* numbers in cherry tomatoes stored at room temperature if compared to other types of vegetables stored at either room or chilled temperature. It is safe to conclude that the efficiency of the bacteriophage mixture in controlling *E. coli* growth in the vegetables varied depending on the type of vegetables and also on the storage condition (chilled and room temperature). These findings will provide some preliminary data for the potential application of bacteriophage as a natural agent in controlling the growth of *E. coli* species in food, especially in minimally processed vegetables.

1. Introduction

Fresh vegetables are being identified as a source of foodborne outbreaks with increasing cases around the world (Siti Zaharah *et al*., 2020). The incidence of foodborne illness associated with the consumption of minimally processed ready-to-eat salad vegetables is also reported consistently increasing throughout the world (Romero *et al*., 2017). Fresh vegetables are vulnerable and easily contaminated by microorganisms at many points throughout the pre-harvest and post-harvest systems (Taban and Halkman, 2011). The contamination could happen via soil, water, animals, insects, equipment and human handling (Salinas *et al*., 2016). Some of the commonly found microorganisms contaminating the vegetables are *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Escherichia coli*, *Listeria* spp. and *Pseudomonas* spp. (De Silva, 2013). The increasing number of outbreaks due to the consumption of fresh vegetables and salad has indicated the importance of developing new antimicrobial strategies as an alternative to available methods to reduce microbial contamination (Arienzo *et al.,* 2020).

Escherichia coli has been used as one of the hygiene indicators to assess the hygiene status of a food product. The presence of indicator bacteria in ready-to-eat food, although not inherently a hazard, however, can be indicative of poor practice during food processing and preparation. *E. coli* also has been known as one of the most common bacteria found in the intestinal tract of

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eISSN: 2550-2166
Published by Rynnye Lyan Resources

humans and warm-blooded animals. Their ability to survive outside the body for a longer period of time makes them an ideal indicator organism to test food and environmental samples for faecal contamination (Samuel *et al*., 2011). Even though *E. coli* species are generally known as harmless intestinal flora but some of them are pathogenic and have been identified as the serious causal agents of various illnesses. As an example, the strain *E. coli* O157:H7 has been identified since 1983 (O'Flynn *et al*., 2004) and since then has emerged as an important human pathogen (Wang *et al*., 2017). Pathogenic *E. coli* strains cause several types of human diarrhoea and their infection is a major cause of public health problems in developing countries (Yu and Tan, 2008). The health hazards associated with *E. coli* have become complicated by the fact that some of the causal agents have over the years, developed resistance against commonly used antibiotics (Campos *et al*., 2013; Arienzo *et al.,* 2020). Food safety is a public health priority worldwide as millions of people fall ill every year due to the consumption of unsafe food. In certain countries, rates of illnesses are increasing significantly every year. Most food-borne illness cases are resulting from the consumption of food, contaminated by pathogenic microorganisms including *E. coli* strains.

The process of decontaminating fruits and vegetables is considered a challenge. Some of the most common strategies used to control the growth of bacteria on fruits and vegetables are washing using water and also washing using solutions containing various antibacterial chemicals such as calcium hypochlorite and other chlorine-based chemicals. These approaches are generally effective and many researchers had written about the positive impact of synthetic chemical-based solutions in reducing microbial load. However, the extensive use of synthetic chemical sanitisers had led to the development of various bacterial resistance strains. These strains will become resistant to the application of common sanitisers which has resulted in a decline in the efficacy of the sanitiser (Sharif *et al*., 2017). The application of bacterial viruses or bacteriophages as an agent to prevent and treat infectious diseases of bacterial origin has a very long history of positive impact. However, their potential application as a biological agent in controlling pathogenic and indicator bacteria for improving the safety and hygiene of food only appears to gain interest and recognition quite recently. Bacteriophages or phages may offer natural means to eliminate or reduce microbial load to a safe level if compared to some common and conventional methods (Callaway *et al*., 2008). Bacteriophages can be isolated from various sources such as wastewater, sewage, human or animal faecal samples, polluted rivers, soil or some rotten foods (Cieplak *et al*., 2018).

Previous studies done by other researchers on the ability of bactericidal effects by bacteriophages to control several pathogenic pathogens have shown promising results. It is then suggested as an alternative way to commonly use synthetic chemical-based sanitisers and is believed as more natural, safer, non-toxic, and environmentally friendly. Bacteriophage is a bacterial virus that in its virulent state infects the bacterial cells, multiplies within them and eventually causes the cell to burst (lysis). Their activities cease once the bacterial cell is killed. Bacteriophages' activity is very specific and only applicable to their targeted bacterial species. They do not attack bacteria indiscriminately but instead, they usually attack a specific type and importantly, it does not infect human or animal cells (Stelios *et al*., 2011). This study was conducted to evaluate the ability of bacteriophages mixture to reduce or eliminate *E. coli* strains in selected vegetables. Commonly found vegetables in the market and regularly used by consumers were selected which were cherry tomato, green mustard, green bean sprout and lettuce.

2. Materials and methods

2.1 Escherichia coli strains preparation for challenge and simulation study

Three *E. coli* strains from the American Type Culture Collection (ATCC) namely ATCC®11303™, ATCC® 13706™ and ATCC® 43888™ were used in this study as representative of *E. coli* strains. The *E. coli* strains were initially stored in 20% (v/w) glycerol as glycerol stock at -20±2°C and were prepared for the study according to the method done by O'Flynn *et al*. (2004). They were revived prior to use by transferring 20 µL of the glycerol stock into 5 mL Tryptic soy broth (TSB, Oxoid, UK) and incubated at 37±2°C for 24 hrs. Later, 1 mL of these cultures was transferred into 10 mL TSB and again incubated at 37±2°C for 24 hrs. The bacterial suspension was then prepared to a turbidity of 0.5 McFarland standard (~1×10^8 colony-forming units per mL, CFU/mL) by adding the sterile distilled water into the suspension until the desired turbidity was achieved. The desired turbidity was determined by using a McFarland nephelometer (Becton Dickinson, USA). Each of the *E. coli* strains with the turbidity of 0.5 McFarland standard was then mixed with a 1:1:1 ratio prior to use.

2.2 Bacteriophages mixture preparation for challenge and simulation study

Bacteriophages mixture was a solution containing several types of local bacteriophage isolates originating from raw meat with a density of approximately 10^9

https://doi.org/10.26656/fr.2017.6(S2).038

plaque-forming unit per mL (PFU/mL) as determined by phage assay technique in a polyethylene glycol and sodium chloride-based phage buffer. It was kept in a sterile bottle at a chilled temperature and was in a ready-to-use form and was previously prepared according to the method mentioned by Nur ilida *et al*. (2013).

2.3 Preparation of vegetables for simulation study

The vegetables (cherry tomato, green mustard, bean sprout and lettuce) used in this study were purchased from the local supermarket and transported back to the laboratory under chilled conditions. Each type of vegetable respectively was first washed with running tap water to remove soil and dirt. Each of them then was dipped into chlorinated water (20 ppm) and rinsed with distilled water before being left to dry in a biosafety cabinet to clean them from naturally occurring microflora prior to use.

2.4 Challenge study to evaluate the effectiveness of bacteriophages mixture to control the growth of Escherichia coli strains in laboratory broth

An amount of 100 mL of Luria Bertani (LB) broth in two different flasks was inoculated with an overnight culture of *E. coli* strains respectively and incubated in an incubator shaker at 100 rpm, 37±2°C for 1 hr. Then, a bacteriophage mixture was added to one of the flasks and the other flask that was not added with the bacteriophage mixture acted as a control. The incubation was continued inside the incubator shaker at 100 rpm, 37±2°C. The *E. coli* count was taken every hour for 24 hrs and was recorded as CFU/mL.

2.5 Simulation study to evaluate the effectiveness of bacteriophages mixture to reduce or eliminate the number of Escherichia coli strains on selected vegetables

An amount of 10 g of each type of vegetable that was previously prepared and left for air-dried respectively, were aseptically transferred into sterile polypropylene plastic container. Each type of vegetable was subsequently added with *E. coli* strains (10^8 CFU/mL) to create artificial contamination. Later, 100 µL of the bacteriophage mixture (10^9 PFU/mL) were applied to each of the vegetables and the containers were finally covered with their lid (treated samples). The samples were allowed to dry for 15 mins in a biosafety cabinet. The artificially contaminated samples not treated with bacteriophages mixture were used as a control. All samples were stored at room (30±2°C) and chilled (4±2° C) temperatures respectively.

The evaluation of the reduced number of viable *E. coli* cells in each treated and control sample was done at 0 hr, 6 hrs, 24 hrs, 48 hrs and 72 hrs intervals for each storage temperature (4±2°C and 30±2°C). The 10 g sample was homogenized with 90 mL Ringers solution (Oxoid) and a 10-fold serial dilution was performed for each sample. An amount of 1 mL homogenate was transferred onto 3M™ Petrifilm™ *E. coli* (3M, USA) and incubated at 37±2°C for 24 hrs. The cell count was recorded as CFU/g. The experiment was conducted in triplicate for each type of vegetable and all experimental results were expressed as mean values obtained from three replicates (n = 3)

2.6 Statistical analysis

All experimental results were expressed as mean values obtained from three replicates (n = 3) unless stated otherwise and the data were statistically analysed using SAS 9.3 statistical software. A one-way analysis of variance (ANOVA) was performed to evaluate significant differences between sample means followed by Duncan Multiple Range Test (DMRT). The level of significance was set at $P<0.05$.

3. Results and discussion

3.1 Challenge study

Through observation done during the challenged study, the result showed that LB broth containing bacteriophages mixture has a reduction in the *E. coli* cell count (CFU/mL) if compared to the control sample (not containing bacteriophages mixture). The laboratory broth used which was LB broth initially contained the *E. coli* cell count of approximately 10^8 CFU/mL was significantly reduced ($P<0.05$) up to five log (Log CFU/mL) after two hours of bacteriophages application at 37±2°C while the reduction to below detectable level was observed after 24 hrs of application (Figure 1). The result also showed that at chilled temperature (10°C), the bacteriophages mixture was also able to reduce the *E. coli* cell count in LB broth significantly ($P<0.05$) (Figure 2). This result suggested that the application of the bacteriophages mixture has affected the ability of *E. coli* cells to grow in the laboratory broth. This finding is in line with several other researchers including Coffey *et al*. (2011) and Mangieri *et al*. (2020). Coffey *et al*. (2011) observed that the initial *E. coli* cell concentrations used in their study (10^4 CFU/mL) were reduced almost immediately after exposure to the bacteriophage and they also suggested the potential to incorporate the bacteriophage into the food environment to control the growth of pathogenic bacteria. A similar finding was also reported by Mangieri *et al*. (2020) where an effective reduction of *E. coli* population in LB broth added with bacteriophage was observed during their challenge study.

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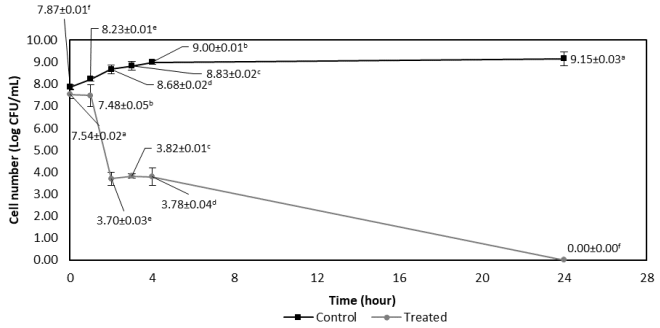

Figure 1. Effect of bacteriophage mixture on the growth of *E. coli* in LB broth at 37°C. Values with different superscripts indicate that the *E. coli* cell count reduction within the same treatment (control or treated at different time interval) are significantly different (P<0.05).

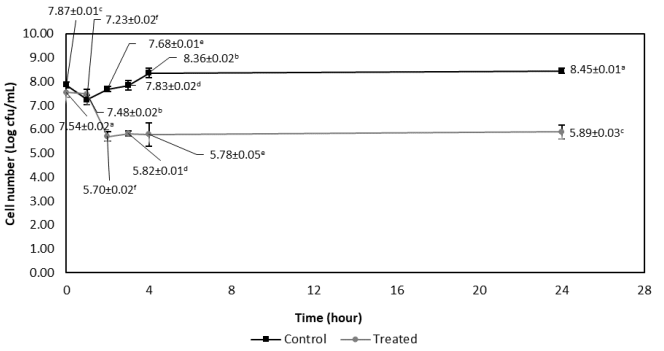

Figure 2. Effect of bacteriophage mixture on the growth of *E. coli* in LB broth at 10°C. Values with different superscripts indicate that the *E. coli* cell count reduction within the same treatment (control or treated at different time interval) are significantly different (P<0.05).

3.2 Simulation study

The overall result showed that the application of bacteriophages mixture was able to reduce viable *E. coli* cell count in artificially contaminated vegetables. The samples treated with bacteriophage mixture showed lower viable *E. coli* cell numbers if compared to the control sample not treated with the mixture. The reductions, however, were varied and very much dependent on the type of vegetables and also on their storage condition which were either chilled or room temperature. The effect of bacteriophage mixture on *E. coli* cell count on different types of vegetables after 72 hrs of storage at room and chilled temperature were summarised in Table 1.

The application of bacteriophages mixture on cherry tomato showed the highest reduction number compared to other types of vegetables. At room temperature, the bacteriophages mixture was able to reduce the *E. coli* cell count after 72 hrs of storage (Figure 3) while at chilled temperature, the *E. coli* cell count was reduced from the initial cell count of approximately Log 7.40±0.02 CFU/g to Log 1.80±0.03 CFU/g significantly (P<0.05) (Figure 4). Observation at room temperature also showed that the control sample (without bacteriophages mixture) with the initial *E. coli* cell count of Log 7.40±0.01 CFU/g was increased to Log 7.75±0.03 CFU/g after 72 hrs. However, at chilled temperature, the control sample showed that the initial count was increased from Log 7.40±0.02 CFU/g to Log 7.70±0.06 CFU/g. Through this observation, it can be concluded that the reduction number was better at room temperature if compared to chilled temperature. O'Flynn *et al*. (2004) have also conducted a similar simulation test and evidenced that the reduction in *E. coli* cell number was also better at higher temperatures (37°C and 30°C respectively) if compared to the study done at 4°C. They concluded that it happens due to the inability of *E. coli* cells to grow well at low temperatures, thus lysis activity by the bacteriophages also slows down. Bacteriophage activity was dependent on their targeted bacteria and if the targeted bacteria is not growing well, the lysis activity will be affected too (Mangieri *et al*., 2020).

In the green mustard sample, the application of the bacteriophages mixture was able to reduce the *E. coli* cell count from the initial count of Log 8.08±0.02 CFU/g to Log 3.30±0.05 CFU/g significantly (P<0.05) after 72 hrs of storage at room temperature (Figure 5). On the other hand, the reduction was also observed from Log 8.08±0.03 CFU/g to Log 6.52±0.03 CFU/g significantly

Table 1. Effect of bacteriophage mixture on *E. coli* cell count on different types of vegetables after 72 hrs of storage at room and chilled temperature.

Type of vegetables	Storage temperature	Initial *Escherichia coli* cell count (CFU/g) at 0 hr	*Escherichia coli* cell count (CFU/g) after 72 hrs of storage
Cherry tomato	Room	7.40±0.01	$0.00±0.00^{h}$
Cherry tomato	Chilled	7.40±0.02	$1.80±0.03^{g}$
Green mustard	Room	8.08±0.02	$3.30±0.05^{f}$
Green mustard	Chilled	8.08±0.03	$6.52±0.03^{b}$
Bean sprout	Room	8.40±0.03	$7.30±0.05^{a}$
Bean sprout	Chilled	8.40±0.03	$5.70±0.06^{d}$
Lettuce	Room	8.08±0.01	$6.30±0.03^{c}$
Lettuce	Chilled	8.08±0.02	$5.28±0.06^{e}$

Values are presented as mean±SD of triplicates. Values with different superscripts indicate that the *E. coli* cell count is significantly different (P<0.05).

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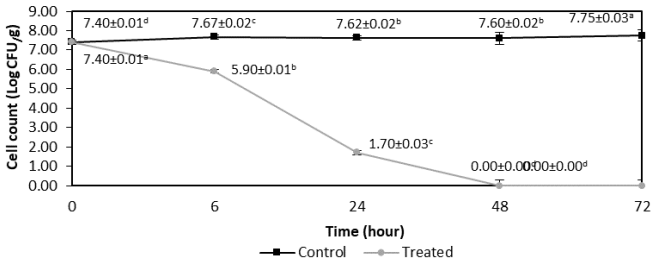

Figure 3. Effect of bacteriophage mixture on *E. coli* cell count in cherry tomato at room temperature. Values are presented as mean±SD of triplicates. Values with different superscripts indicate that the *E. coli* cell count reduction within the same treatment (control or treated at different time interval) are significantly different (P<0.05).

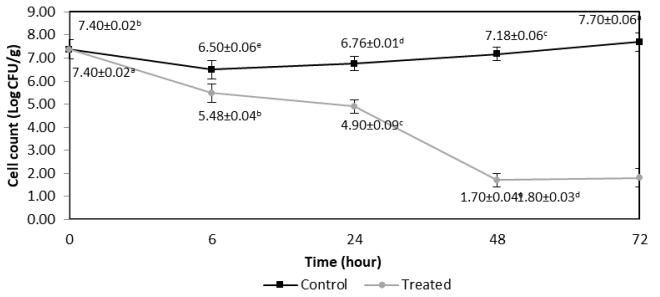

Figure 4. Effect of bacteriophage mixture on *E. coli* cell count in cherry tomato at chilled temperature. Values are presented as mean±SD of triplicates. Values with different superscripts indicate that the *E. coli* cell count reduction within the same treatment (control or treated at different time interval) are significantly different (P<0.05).

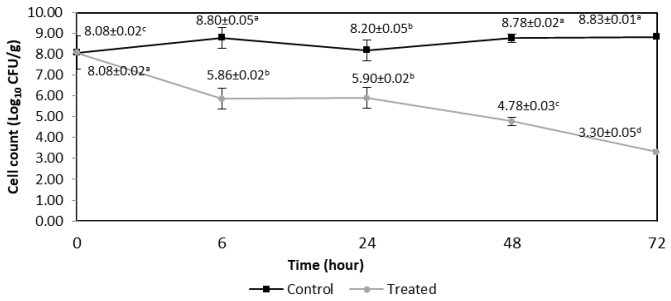

Figure 5. Effect of bacteriophage mixture on *E. coli* cell count in green mustard (*sawi*) at room temperature. Values are presented as mean±SD of triplicates. Values with different superscripts indicate that the *E. coli* cell count reduction within the same treatment (control or treated at different time interval) are significantly different (P<0.05).

(P<0.05) at chilled temperature (Figure 6). The observation pattern in green mustard is similar to the application of bacteriophages mixture on cherry tomato where reduction of *E. coli* cell number was better at room temperature if compare to chilled temperature.

Another pattern of *E. coli* growth reduction was observed in bean sprouts. The initial *E. coli* cell count was at Log 8.40±0.03 CFU/g and was reduced significantly (P<0.05) after 6 hrs of exposure to the bacteriophage mixture at room temperature (Figure 7). However, after 24 hrs of storage, the cell count was increased to Log 6.58±0.04 CFU/g and later remain high until 72 hrs of storage where the cell number increased significantly (P<0.05) to Log 7.30±0.05 CFU/g. Meanwhile, the control sample not treated by bacteriophages mixture contained Log 8.82±0.02 CFU/g after 72 hrs of storage at room temperature. The observation was done at chilled temperature however showed the reduction of *E. coli* by bacteriophages mixture was better as after 72 hrs, the bacterial count was reduced significantly (P<0.05) to Log 5.70±0.06 CFU/g, while the control sample was not treated with bacteriophages mixture, contained Log 7.75±0.07 CFU/g (Figure 8) of *E. coli* cell. This pattern might be happening due to the nature of green bean sprouts which usually have a shorter shelf life at room temperature if compare to chilled temperature. At room temperature, the growth of other bacterial species including spoilage microorganisms has rotten the bean sprout structure and thus might have hindered the ability of bacteriophages to get in contact with the *E. coli* cells.

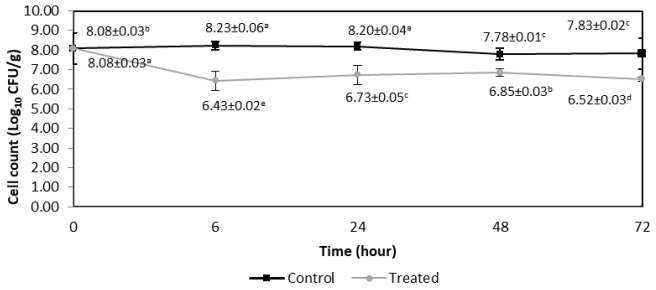

Figure 6. Effect of bacteriophage mixture on *E. coli* cell count in green mustard (*sawi*) at chilled temperature. Values are presented as mean±SD of triplicates. Values with different superscripts indicate that the *E. coli* cell count reduction within the same treatment (control or treated at different time interval) are significantly different (P<0.05).

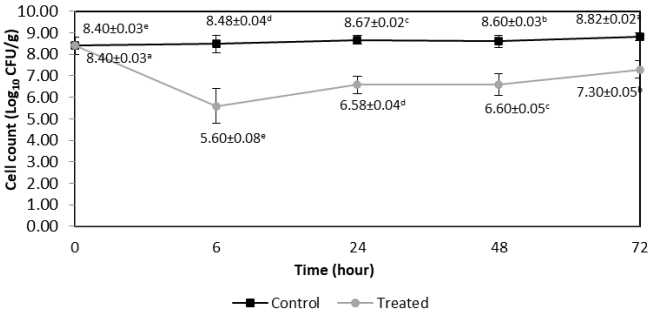

Figure 7. Effect of bacteriophage mixture on *E. coli* cell count in bean sprout at room temperature. Values are presented as mean±SD of triplicates. Values with different superscripts indicate that the *E. coli* cell count reduction within the same treatment (control or treated at different time interval) are significantly different (P<0.05).

As for the observation done in lettuce, the result showed that after 72 hrs of storage at room temperature, the *E. coli* cell count was reduced significantly (P<0.05) from Log 8.08±0.01 CFU/g to Log 6.30±0.03 CFU/g

https://doi.org/10.26656/fr.2017.6(S2).038

(Figure 9) in the sample treated with bacteriophages mixture and at chilled temperature, the amount was reduced significantly (P<0.05) from Log 8.08±0.01 CFU/g to Log 5.28±0.06 CFU/g (Figure 10). At the same time, after 72 hrs of storage at room temperature, the *E. coli* count in the control sample for lettuce (lettuce not treated with bacteriophages mixture) was at Log 8.83±0.02 CFU/g while at chilled temperature the count was at Log 8.20±0.03 CFU/g. This result showed that the bacteriophages mixture was able to reduce *E. coli* count in lettuce better at a chilled temperature whereas the same pattern has also been observed in bean sprouts. The room storage temperature was detrimental to lettuce as well as the bean sprout where the growth of other microorganisms especially the spoilage group was rapid and their growth might have interrupted the ability of bacteriophages mixture to perform on *E. coli* in lettuce at room temperature environment. A similar observation was found by Sharma *et al*. (2009) on the bacteriophage mixture being more effective at a lower temperature (4° C) than at a higher temperature (20°C) inactivating the *E. coli* populations on cantaloupes. In their discussion, they suggested that the bacteriophages focus on attacking *E. coli* strains better at lower temperatures due to the lack of growth of other bacterial populations at lower temperatures on the cantaloupes. Sharma *et al*. (2009) also concluded that unexamined factors have contributed to the better efficacy of the bacteriophage mixture in cantaloupes at 4°C temperature if compared with 20°C. The unexamined factor mentioned by them was including superior survival of bacteriophages at chilled temperatures while *E. coli* cells have a slower growth rate at that condition, thus more bacteriophages were available to infect the *E. coli.* Other researchers also have reported on the ability of bacteriophages to inactivate or reduce the targeted bacterial count in many types of vegetables. These included Ye *et al*. (2010) who reported on the ability of bacteriophages to inactivate *Salmonella* growth on bean sprouts and Abuladze *et al*. (2008) on spinach and broccoli. They have also concluded that the efficacy of bacteriophages activity varied depending on several factors including the type of vegetables involved, storage temperature and also the density of the bacterial cell and bacteriophage within the vegetables.

4. Conclusion

The overall result showed that the application of bacteriophages mixture was able to significantly reduce viable *E. coli* cell numbers that has been artificially contaminated on selected vegetables (cherry tomato, green mustard, bean sprout and lettuce) and stored at both rooms and chilled temperature storage respectively if compared to control sample not treated with bacteriophage. The application of bacteriophages mixture was evaluated as most efficient in reducing *E. coli* count in cherry tomatoes stored at room temperature while the application was less effective in bean sprouts stored at room temperature as the log reduction number of the *E. coli* count was only able to be reduced for approximately one log.

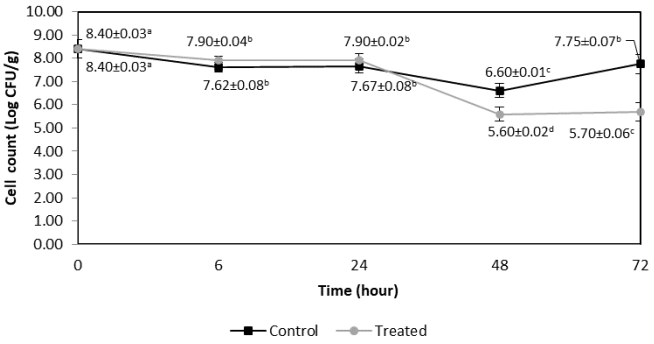

Figure 8. Effect of bacteriophage mixture on *E. coli* cell count in bean sprout at chilled temperature. Values are presented as mean±SD of triplicates. Values with different superscripts indicate that the *E. coli* cell count reduction within the same treatment (control or treated at different time interval) are significantly different (P<0.05).

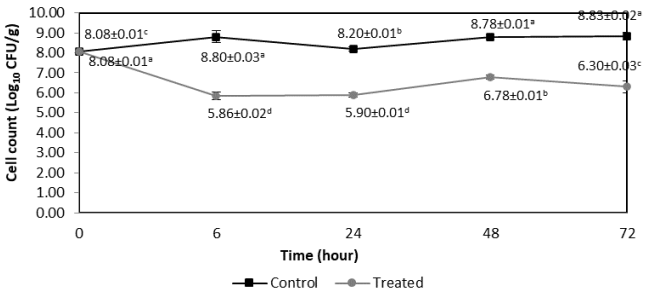

Figure 9. Effect of bacteriophage mixture on *E. coli* cell count in lettuce at room temperature. Values are presented as mean±SD of triplicates. Values with different superscripts indicate that the *E. coli* cell count reduction within the same treatment (control or treated at different time interval) are significantly different (P<0.05).

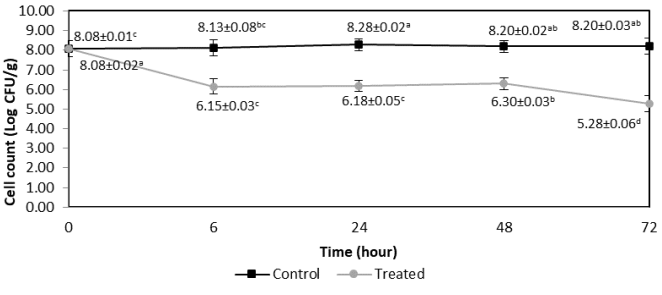

Figure 10. Effect of bacteriophage mixture on *E. coli* cell count in lettuce at chilled temperature. Values are presented as mean±SD of triplicates. Values with different superscripts indicate that the *E. coli* cell count reduction within the same treatment (control or treated at different time interval) are significantly different (P<0.05).

https://doi.org/10.26656/fr.2017.6(S2).038

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The team would like to acknowledge the Ministry of Science, Technology and Innovation (MOSTI) for funding the project through the ScienceFund Grant (05-03-08-SF1122).

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