Animal Health Research Journal Vol. 7, No. 4, November 2019 pp. 842-851

ISSN: 2356-7767

# Biocontrol of *salmonella* Typhimurium in minced meat using its assigned Bacteriophage.

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Received in 12/09/2019
Accepted in 14/10/2019

**Abstract**

Biocontrol of food-borne pathogens in food is an issue that occupies a very important place because of their serious impact on the health of the consumer and the economy of the country. ***Salmonella*** is an important foodborne pathogen and a serious threat to human. Using bacteriophages in biocontrol of food is gaining increased acceptance as it considered as green and natural method. In this study, efficacy of a commercial preparation of ***Salmonella*** lytic bacteriophage (***PhageGuard S***) in reducing ***Salmonella*** populations in minced meat was evaluated. Minced meat was inoculated with ***Salmonella* Typhimurium** (~6 log CFU/g) and treated with bacteriophage ($10^9$ PFU/mL). Treated samples were stored aerobically at (4°c and 12°c). Treatment with phage significantly ($P < 0.05$) reduced ***Salmonella*** by 0.2, 0.9,1.3, 2, 2.5 and 2.9 log CFU/g after 1h, 1st ,2nd d, 3rd d, 4th d and 5th d of storage at 4ºC.The reduction increased at 12°c;0.3, 1.3 and 1.6 log CFU/g after 1h, 1st and 2nd days of storage. Sensory evaluation of minced meat before and after cooking revealed that bacteriophage didn't adversely affect meat characters. Bacteriological and Sensory evaluation indicate that, bacteriophage (***PhageGuard S***) provides effective biocontrol of ***S.*Typhimurium** under meat processing conditions.

***Keywords: Salmonella****, Bacteriophage, Minced meat*

## Introduction

Minced meat is popular form of beef, it is relatively cheap and quick-cooking and constitutes a major ingredient in many meat products such as sausages, patties, hamburgers, meatballs, and salami and in many Egyptian dishes and recipes such as pasta, Kofta, hawawshy. In Egypt we have no document about the percentage of minced meat consumed compared to beef sales but in the UK, minced beef accounts for around 38% of beef sales by volume and hence represents an important component of household cooking **(NBA, 2016)**. Mincing process disrupts the meat cellular structure, releasing tissue fluids and making minced meat a highly nutritious medium supporting bacterial growth. Also, grinding allows any bacteria present on the surface to be mixed throughout the meat **(USDA, 2012, Motjarem *et al.*, 2014)**.

Raw or undercooked food such as egg, milk, chicken, meat and meat products are important sources of foodborne salmonellosis **(Mead *et al.*, 2010)**. Salmonella infection (salmonellosis) is a common bacterial disease that affects the intestinal tract. Salmonella bacteria typically live in animal and human intestines and are shed through feces. Humans become infected most frequently through contaminated water or food.

***Salmonella*** is generally divided into two categories; typhoidal and Non-typhoidal. Typhoidal ***Salmonella***, which causes typhoid fever, is rare, and is caused by ***Salmonella* Typhi**, which is carried only by humans. Non-typhoidal ***Salmonella*** is the most common form, and is carried by both humans and animals and cause non-typhoidal Salmonellosis. Non-typhoidal ***Salmonella*** are the leading cause of bacterial foodborne illnesses causing

one million illnesses, 19,000 hospitalizations, and 380 deaths every year in the United States **(CDC, 2019).** It is responsible for approximately 30% of foodborne outbreaks in the United States and 23% in the European Union **(Gould *et al.,* 2013 and EFSA, ECDC, 2016)**.

The use of some strategies to minimize the microbial load of raw products as the use of antibiotics is restricted due to the negative impact on human health **(FAO, 2015)**. Other methods of preservation such as steam, dry heat and UV light (physical treatments) alter the organoleptic properties of meat. Also, the miss use of sanitizers can form resistant bacteria, rendering these procedures less effective. So, food researchers continuously investigate new strategies to avoid transmission of bacterial pathogens throughout the food chain, to fulfil consumer demands for minimally processed foods with fewer chemical preservatives.A promising field of application is the use of phages as natural antimicrobials in food to inhibit undesirable bacteria, which is likely to be acceptable to consumers **(Zachary *et al.,* 2018)**.

Phage biocontrol is increasingly accepted as a natural and green technology, it considered effective as it specifically targeting bacterial pathogens in various foods in order to safeguard the food chain **(Sulakvelidze, 2013)**. Bacteriophage is a type of virus that infects bacteria. The word "bacteriophage" means "bacteria eater," because bacteriophages destroy their host cells. All bacteriophages are composed of a nucleic acid molecule that is surrounded by a protein structure **(Garcia *et al.*, 2008)**.

Bacteriophages provide an attractive alternative since phages are ubiquitous in different environments, unable to infect human cells and, consequently, they have great potential for use as biocontrol agents in foods **(Hudson *et al.,* 2005 and Billington *et al.*, 2005)**. The application of bacteriophages has already become an interesting tool to fight the emergence of antibiotic-resistant bacteria **(Kutter and Sulakvelidze, 2005)**. Phages are self-replicating and self-limiting and their replication occurs naturally as long as their host cells are present and they infect only their specific host. Their high specificity and lethal effect, and the relative ease of engineering their genomes and structures lend them to nanobiotechnological applications for food safety **(Billington *et al.*, 2014)**. Considerably, usage of phages as bio-preservative and therapy agents has been known to be safe and non-toxic. It considered having a great advantage over antibiotics since they target only the pathogens of interest **(Chanishvili *et al.*, 2001, Connerton, and Connerton, 2005 and Huff *et al.,* 2005).** The use of bacteriophage has many advantages; phages are natural control agents for bacteria and they do not affect the smell, taste, texture and color of foods, phages are host-specific and only affect the target bacterium. Bacteriophages are found everywhere in natural environmental conditions and are friendly to the environment. The total number of phages on Earth is estimated at $10^{30}$-$10^{32}$ and there are more than one hundred million types of bacteriophages. For this reason, phage therapy is much cheaper than developing new antimicrobials (**Kalkan *et al.,* 2011).** Finally, compared to other food safety interventions, the cost of applying bacteriophages is relatively low and is typically in the range of 1–4 cents per pound of food treated; whereas HPP treatment and irradiation typically cost 10–30 cents per pound **(Viator *et al.,* 2015)**. Bacteriophages have been used to control pathogenic bacteria in man and animals with varying degrees of success for over 80 years. They have also been a cornerstone of modern molecular biology and genetics **(Atterbury, 2009).**

Approval of ***Salmonella*** lytic bacteriophages for food processing by the **(FSIS USDA 2017)** has intensified the research on application of phages as antimicrobials during poultry and meat processing. The efficacy of different phage preparations to inactivate various foodborne pathogens including ***Salmonella*** has been studied by direct application in food (**Goode *et al.*, 2003; Whichard *et al.*, 2003; Fiorentin *et al.,* 2005; Higgins *et al.,* 2005; Hungaro *et al.*, 2013; Spricigo *et al.*, 2013; Zinno *et al.,* 2014; Sukumaran *et al.*, 2015 & 2016 and El-Shibiny *et al.,* 2017**). Bacteriophage was found to be effective agent in preserving food at both room temperature and chilling condition even at 1°C thus controlling

growth of pathogenic and spoilage bacteria on refrigerated foodswithout any adverse effect on the sensory quality **(Bigwood *et al.*, 2008; Li *et al.*, 2014 and Perera *et al.*, 2015 ).**

The aim of our study is to evaluate the effect of ***Salmonella*** phage as a biocontrol agent against ***Salmonella*** **Typhymurium** in minced meat under two different temperatures that used during meat production, and aerobic storage condition.

## Materials and Methods

### Sample Preparation

Two kilograms of fresh minced beef was obtained from a butcher shop and screened for ***Salmonella*** *spp*. to ensure that meat was not contaminated according to the method described by **ISO (2017)**. Then 600 grams of minced meat was aseptically divided into 4 portions each weighing about 150 grams and maintained at a temperature of 4± 1°C.

### Preparation of *Salmonella* Inoculum

***Salmonella*** **Typhimurium** (ATCC 14028), was obtained from Food Hygiene Department, Animal Health Research Institute, Egypt. The strain was recovered by thawing freeze-dried pellets for approximately 2 min in water bath at 37°C and subsequently transferring the entire content of the vial to a sterile test tube containing 5 mL of tryptic soy broth (TSB), which were incubated overnight at 37°C. Recovered culture was streaked on xylose lysine deoxycholate (XLD) agar plates. Plates were incubated aerobically at 37°C to ensure that strains were live and viable. Culture ($10^9$ CFU/mL) was prepared in sterile 10 mL tryptic soy broth by adding a single colony into the tube and incubating overnight at 37°C. The cultures were pelleted by centrifugation at 3,300 × g for 10 min. The supernatant was discarded and the pellets were suspended in fresh 10 mL sterile 0.1% peptone water and the concentrate was estimated with a spectrophotometer. The desired concentration was prepared by serially diluting (10 fold) in sterile 0.1% peptone water **(Higgins *et al.* 2005).**

### 3-Lytic Phage Preparation

***Salmonella*** lytic bacteriophage preparation (phage guard S) approved by the Food and Drug Administration and U.S. Department of Agriculture's Food Safety and Inspection Service as GRAS GRN. 000468 (Generally recognized as safe) food processing aid against Salmonella was obtained from Micreos Food Safety (Wageningen, The Netherlands)**.** Phage S stock concentration in saline was approximately $10^{11}$ PFU=mL by plaque formation assay. Phage S stock solution was serially diluted in physiological saline for preparing the $10^9$P-FU=mL concentration to simulate industry practices.

### Experimental design:

### 4-1-*Salmonella* Typhimurium inoculation

***Salmonella*** inoculum was prepared as mentioned above. 1.5 mL of the desired inoculums concentration ($10^8$cfu/ml) was uniformly pipetted on each portion of minced meat (150 grams) and mixed well in a sterile bag in order to achieve ~6 log CFU/g. After inoculation, the samples were left in a biosafety cabinet for 15 minutes at room temperature to allow the surface bacterial attachment.

### 4-2-Antibacterial effect of Salmonella Phage at chilling storage (4°C)

One portion of inoculated minced meat was then mixed well with 3 mL of bacteriophage solution ($10^9$ PFU/mL) by stomaching in stomacher and subdivided into 6 portions each weighing 25 grams in sterile bags. The second portion (Control group) was mixed with 3 ml distilled saline instead of bacteriophage and subdivided into 6 portions each weighing 25 grams in sterile bags. All the samples were stored aerobically at chilling temperature (4±1° C) and microbiological analysis was carried out after one hour, one, two, three, four and five days of storage.

### 4-3-Antibacterial effect of Salmonella Phage at (12°C)

The same procedure as (4-2) were applied on the other 2 minced meat portions then each portion was subdivided into 3portions each weighing 50 grams in sterile bags and stored aerobically at temperature (12±1°C) and microbiological analysis was carried out after one hour, one and two days of storage.

**4-4-Enumeration of *S.* Typhimurium from minced meat:** as recommended by **Sukumaran *et al.*, (2016).**
25 grams of each sample was stomached with 225 mL of sterile 0.1% peptone water in a stomacher. To avoid plating the bacteriophage, 10 mL samples from the homogenate were centrifuged at 10,000 × g for 5 min and supernatant containing phages was discarded and pellets were re-suspended in 10 mL of sterile 0.1% PW **(Soni *et al.*, 2010)**. For each sample 250 µl homogenate was plated on to 4 XLD agar plates and incubated at 37°C for 24 h. Salmonella counts were converted to log CFU/g.

**5- Sensory evaluation of minced meat**
**5-1-Before cooking**
The sensory panelconsisted of twelve expert assessors **(ISO 8586-1:1993).** On the evaluation day, Two kilograms of fresh minced meat were divided into 2 portions, 1st (treated) portion was mixed with 2% Salmonella phage (Phage guard S) $10^9$ PFU/g and the 2nd portion was (control) mixed with 2% distilled water and submitted to the assessors. The analysis was performed under daylight at ambient temperature to evaluate color, odor and overall - acceptability using 5 point scale for grading the quality of samples 1.very poor; 2.poor; 3.common; 4.good and 5.very good **(Szczesniak, 1987)**.

**5-2- After cooking**
Fresh minced meat (control and treated) were mixed with (salt 1.5% and white paper 0.2%). Each meat portion was shaped using a commercial burger maker into disc pieces of 50 g and diameter of 9 cm and a thickness of 1 cm to obtain burger. The burgers were cooked for 20 min at 160°C in an electric oven until the internal temperature attains 80°C as measured at the center using a digital probe thermometer. At intervals of 10 min, the burgers were turned upside down to ensure uniformity of cooking. The burgers were assessed for a number of sensory characteristics by twelve expert assessors as described in **(America Meat Science Association, 2015)** to evaluate color, flavour, odor and overall- acceptability. using 5 point scale for grading the quality of samples 1.very poor; 2.poor; 3.common; 4.good and 5.very good **(Szczesniak, 1987)**.

**Statistical Analysis**
The experiments were replicated 3 times. The results were expressed as mean values and standard errors. The data were statistically analyzed by ANOVA. Statistical significance was acceptedat a level of $P<0.05$ ***(*SAS Institute, 1988*).***

## Results

**Table (1).** Effect of phage in reducing the count of *S.* Typhimurium in treated minced meat.

| Sample type | | 1h | 1st d | 2nd d | 3rd d | 4th d | 5th d |
|---|---|---|---|---|---|---|---|
| 4ºc | Control | $6.2\pm0.05^{Aa}$ | $6.3\pm0.03^{Aa}$ | $6.4\pm0.08^{Ab}$ | $6.6\pm0.30^{Ac}$ | $6.5\pm0.07^{Ac}$ | $6.4\pm0.15^{Ab}$ |
| | Treated | $6\pm0.07^{Aa}$ | $5.4\pm0.25^{Bb}$ | $5.1\pm0.10^{Bc}$ | $4.6\pm0.05^{Bd}$ | $4\pm0.28^{Be}$ | $3.5\pm0.08^{Bf}$ |
| 12ºc | control | $6.3\pm0.51^{Ca}$ | $6.5\pm0.06^{Cb}$ | $6.6\pm1.05^{Cc}$ | Not applied | Not applied | Not applied |
| | Treated | $6\pm0.23^{Da}$ | $5.2\pm0.41^{Eb}$ | $5\pm1.01^{Fc}$ | Not applied | Not applied | Not applied |

Mean ± standard error. Statistical analysis was applied using two-way ANOVA. Upper case alphabet was used as indicator to comparing control and treated groups. Lower case alphabet was used as indicator to comparing different storage time inside the same control and treated groups. Mean values with different litter within the same raw and column are significantly difference ($P<0.05$).

**Table (2).** Effect of storage temperature and Phage treatment on reduction of *Salmonella* Typhymurium count.

| Temperature | | 1 h | 1st d | 2nd d | 3rd d | 4th d | 5th d |
|---|---|---|---|---|---|---|---|
| 4ºc | Reductioncount | 0.2 | 0.9 | 1.3 | 2 | 2.5 | 2.9 |
| | Reduction % | 3.2 | 14.3 | 20.3 | 30.3 | 38.5 | 45 |
| 12ºc | Reduction count | 0.3 | 1.3 | 1.6 | ____ | ____ | ____ |
| | Reduction % | 4.7 | 20 | 24.2 | ____ | ____ | ____ |

Reduction count = Mean of control – Mean of treated sample.
Reduction % = (Mean of control – Mean of treated sample) × 100.
Mean of control

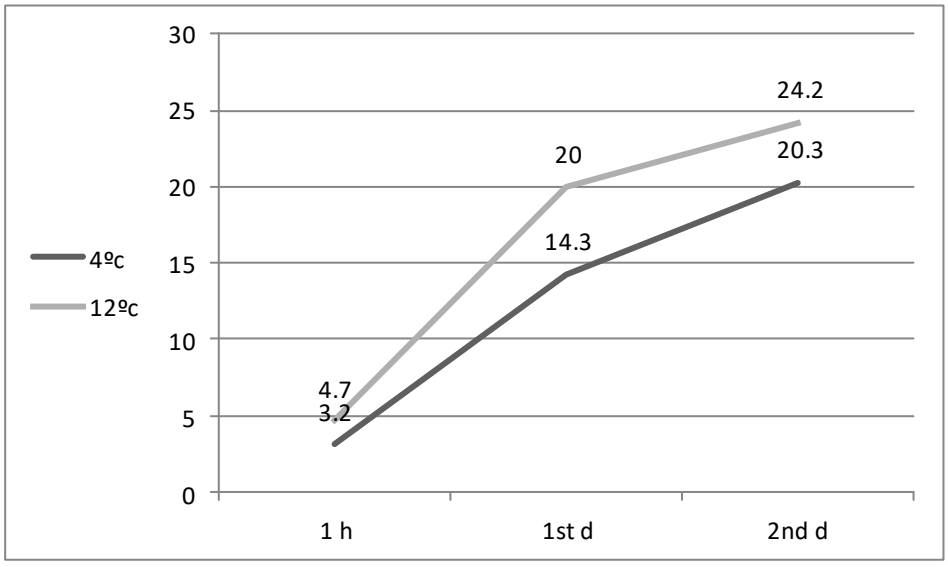


**Figure (1).** Reduction % of Salmonella populations at 4ºc and 12ºc

**Table (3).** Sensory evaluation of minced meat before cooking.

| Sample | Colour | Odour | Overall-acceptability |
|---|---|---|---|
| **Control** | 3.1±0.49[a] | 3.8±0.68[a] | 3.9±1.0[a] |
| **Treated** | 3.2±0.7[a] | 3.9±0.5[a] | 3.6±0.7[b] |

Mean values with different litter within the same column are significantly difference (*P<0.05*).

**Table (4)** Sensory evaluation of minced meat after cooking.

| Sample | Colour | Odour | Flavour | Overall-acceptability |
|---|---|---|---|---|
| **Control** | 4.09±0.27[a] | 3.18±0.36[a] | 4.4±0.42[a] | 4.5±1.0[a] |
| **Treated** | 4.18±0.5[a] | 3.27±0.15[a] | 4.6±1.0[a] | 4.4±0.7[a] |

Mean values with different litter within the same column are significantly difference (*P<0.05*).

## Discussion

In recent years, it has become widely recognized that, bacteriophages have several potential applications in the food industry. They have been proposed as alternatives to antibiotics in animal health, as bio-preservatives in food and as tools for detecting pathogenic bacteria throughout the food chain.

## Bacteriological evaluation

The experiment was carried out at two incubation temperatures, that is, 4°C and 12ºC to simulate the condition used during meat processing and storage. Bacteriophage treatment was significantly ($P < 0.05$) effective in reducing the populations of ***Salmonella*** by 0.2, 0.9, 1.3, 2, 2.5 and 2.9 log CFU/g after 1h, 1st, 2nd d, 3rd d, 4th d and 5th d of storage at 4ºC, respectively**.** Count of ***Salmonella*** in treated samples was decreased by more than one log on 3rd d. of storage at chilling condition, and the reduction increased with the prolonged storage time, reaching to 2.9 log reductions on the 5th day of storage. At 12ºC Salmonella population were decreased significantly by 0.3, 1.3 and 1.6 log CFU/g after 1h, 1st and 2nd days of storage **(Table 1).** From the data obtained in **(Table 2 and Figure 2)** the reduction % in Salmonella populations at the two studied temperatures (4ºC and 12ºC) were; (3.2 and 4.7), (14.3 and 20%) and (20.3 and 24.2%) respectively Maximum reduction (45%) was obtained at the 5th day of storage at 4ºC **(Table 2)** which suggests the influence of storage time and temperature on the antibacterial activity of bacteriophage similar findings were obtained by **Sukumaran *et al.*, (2016)** who stated that reduction of Salmonella populations was increased from 0.5 to 1.3 log CFU/g up on 7 days of storage period.In a study of The ability of host specific bacteriophages FSP-1 and FSP -3 to lyse Salmonella in artificially contaminated chicken meat, Salmonella viable counts were lowered by log (2.4 and 2.1), (3.9 and 3.4) and (1.9 and 2.3) CFU/ml on day 3 of storage at 4°C, 28°C and 37°C respectively**,** they noted the maximum reduction at room temperature, 92% **Augustine and Bhat, (2014). Bigwood *et al.*, (2008)** obtained 2 log (10) $cm^2$ at 5ºC and >5.9 log (10) $cm^2$ at 24ºC of *Salmonella* Typhimurium reduction in meat compared to phage-free controls using the Salmonella phage under optimal conditions (high host cell density and MOI). Similarly workers from Korea demonstrated the ability of phage wksl3 on Salmonella strains (log10 3.25 CFU cm2 of skin) inoculated on chicken skin ata of 8°C to completely eliminate the host cells over the 1-week test period **Kang *et al.*, (2013). Zinno *et al.*, (2014)** reported up to2- 3 log cycle reductions of ***Salmonella*** loads in liquid-eggs, chicken breast and ground chicken after 48 h at 4ºC. Approximately 1 log cfu/g reduction in ***Salmonella*** population was achieved in four meat matrixes (beef, pork, chicken, and turkey) when applying bacteriophages on trim and thighs prior to grinding by **Yeh *et al.*, (2017) and Yeh *et al.*, (2018).** In another

study, ***Salmonella*** phage Felix-O1 was tested in biocontrol experiments for ***S.*Typhimurium** on chicken frankfurters contaminated with 300 C.F.U. and reductions of 2.1 log units were achieved **(Whichard *et al.*, 2003). Hungaro *et al.*, (2013)** also reported a one log CFU/cm$^2$ reduction of ***S.*Enteritidis** in experimentally contaminated chicken skin with $10^5$ CFU/cm$^2$ and treated by immersion in$10^9$ PFU/mL bacteriophage suspension cocktail for 30 min. the data suggest that bacteriophages can be employed as an alternative agent to reduce ***S. enteritidis*** contamination of poultry carcasses in industrial conditions. **El-Shibiny *et al.*, (2017)** obtained 2 $\log_{10}$ reduction in number of ***S.* enterica** ATCC 25566 when phage ZCSE1 applied to chicken skin and the surface of eggs, and to undetectable levels 1 day after treatment. .In a study conducted by **(Spricigo *et al.*, 2013)** significant decreases in the concentration of ***S.* Typhimurium and *S.* Enteritidis** in chicken breasts dipped for 5 min in a solution containing the bacteriophage cocktail and then refrigerated at 4°C for 7 days (2.2 and 0.9 log10 cfu/g, respectively; $p \leq 0.0001$) on the other hand they observed higher reduction (>4 and 2 log/cm$^2$) for ***S.* Typhimurium and *S.* Enteritidis**, respectively; $p \leq 0.005$) in pig skin sprayed with the bacteriophage cocktail and then incubated at 33°C for 6h which could suggest the influence of storage temperature on the lytic activity of bacteriophages. Bacteriophage applications were found efficient in decreasing several ***Salmonella*** strains in poultry carcasses and parts **(Fiorentin *et al.* 2005; Higgins *et al.*, 2005; Bielke *et al.*, 2007; Sharma *et al.*, 2015 and Sukumaran *et al.*, 2015). Bigot *et al.*, (2011)** obtained 2.5 $\log_{10}$ CFU /cm reduction in Listeria concentration following addition of specific phages at $5.2 \times 10^7$ PFU/ ml on the surface of vacuum-packed ready-to-eat chicken breast roll. **(Spricigo *et al.*, 2013 and Hooton *et al.*, 2011).** This work was carried out with meats and so most of the meat trials were carried out at chill temperatures due to the nature of the products.

**Sensory evaluation**

Sensory evaluation of minced meat before cooking (Colour, Odour and Overall-acceptability) and after cooking (Colour, Odour, Flavour and Overall-acceptability) revealed that there is no significant differences between control and phage treated samples **(Table 3& Table 4). Greer, (2005)** and **Kalkan *et al.*, (2011)** reported that Phages are natural control agents for bacteria and they do not affect the smell, taste, texture and color of foods.Similar findings were obtained by **Li *et al.*, (2013)** concluded that the bacteriophage Spp001 offered effective biocontrol of *S.* putrefaciens under chilled conditions, retaining the quality fish fillets, and thus could be a potential candidate for use in chilled fish fillet biopreservation. Also **Perera *et al.*, (2015)** Found that the organoleptic quality of salmon fillets was not affected by application of ListShield™, as no differences in the color, taste, or appearance were detectable.

**In conclusion**, the obtained results showed the potential effectiveness of Salmonella phage (Phage guard S) used in our study as a bio control agent of ***S.*Typhimurium**. Phage guard S was able to reduce ***Salmonella*** populations in minced meat during storage period by; 0.2, 0.9, 1.3, 2, 2.5 and 2.9 log CFU/g after 1h, 1$^{st}$, 2$^{nd}$ d, 3$^{rd}$ d, 4$^{th}$ d and 5$^{th}$ d of at 4ºC and at 12ºC the reduction in ***Salmonella*** populations were; 0.3, 1.3 and 1.6 log CFU/g after 1h, 1$^{st}$ and 2$^{nd}$ days of storage. The findings showed that salmonella phage was active in reducing *S.* Typhimurium population in conditions used in food processing industry. Sensory characters of minced meat before and after cooking didn't affected by phage treatment. The results suggest that the studied salmonella phage preparation may be applied to reduce the numbers of ***Salmonella*** Typhimuriumin meatindustry without affecting meat quality**.** Further studies are recommended for studying the biocontrol of other food poisoning bacteria by bacteriophages.

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