TESTING THE IN VITRO EFFECTIVENESS OF PHOSVITIN AND CARVACROL AGAINST FOUR PATHOGENS INVOLVED IN MAJOR FOODBORNE OUTBREAKS: SALMONELLA ENTERICA, LISTERIA MONOCYTOGENES, ESCHERICHIA COLI O157:H7, AND STAPHYLOCOCCUS AUREUS

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Abstract

Reformulation of food products with natural antimicrobials is a potential solution for several food-related problems: the growing consumers' request for natural products, the negative health impact due to the presence in foods of artificial antimicrobials, and the risk of emergence of new antimicrobial resistant pathogens. The assessment of minimum inhibitory concentration (MIC) has a significant impact on the choice of an antimicrobial strategy and represents the first step in selecting a new antimicrobial to be used in foods. Phosvitin or carvacrol were tested against Salmonella enterica, Listeria monocytogenes, Escherichia coli O157:H7, and Staphylococcus aureus. Brain heart infusion (BHI) broth with phosvitin (10, 20, 40, 60, 80 or 100 mg/ml) or carvacrol (0.09, 0.12, 0.14, 0.19, 0.38, or 0.75 mg/ml) was individually inoculated with selected pathogens (5.0 log10 CFU/ml per pathogen). Growth of each pathogen in BHI (35℃, 24 h) was monitored using the OD600 nm-values (using a Bioscreen C turbidometer) to determine the individual minimum inhibitory concentration for each selected antimicrobial. The MIC for tested antimicrobials and pathogens were: 80 mg/ml (phosvitin) for Salmonella enterica, Listeria monocytogenes, and Escherichia coli O157:H7, whereas the MIC of phosvitin for S. aureus is greater than 100 mg/ml. For Salmonella enterica and Listeria monocytogenes, the MIC of carvacrol was 0.14 mg/ml. The determined MIC of carvacrol on Escherichia coli O157:H7 and Staphylococcus aureus was 0.12 mg/ml.The use of phosvitin or carvacrol could be considered as natural alternatives to replace the chemical preservatives for the control and inactivation of pathogens in commercially produced foods.

Keywords: Minimum inhibitory concentration (MIC), *Salmonella enterica, Listeria monocytogenes, Escherichia coli* O157:H7, *Staphylococcus aureus*, phosvitin, carvacrol

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1. INTRODUCTION

There are valid expressed concerns among both governmental regulatory agencies and consumers regarding the worldwide high incidence of foodborne outbreaks. Among the pathogenic bacteria which are responsible for foodborne outbreaks that affect humans *Salmonella enterica, Listeria monocytogenes, Escherichia coli O157:H7, Staphylococcus aureus* were identified among the most prevalent causative agents of foodborne outbreaks. Recent studies performed by the United States Center of Diseases Control (CDC), European Food Safety Authority

(EFSA), and European Centre for Disease Prevention and Control (ECDC), the incidence of infections caused by *Listeria monocytogenes*, *Salmonella enterica* (i.e.: serovar *Enteridis*) and *Escherichia coli* O157:H7 remained almost unchanged or even increased despite the implemented food safety measures (EFSA and ECDC, 2021; Tack *et al.*, 2020; Taseen *et al.*, 2009). Also, worldwide, the staphylococcal food-borne disease still represents one of the most common diseases resulting from the contamination of food with *Staphylococcus aureus* and its toxin (Ercoli *et al.*, 2017; Kadariya *et al.*, 2014). According to the data published by EFSA and ECDC bacterial toxins were the second cause of foodborne outbreak in 2017, withan increasing trend in Italy (EFSA and ECDC, 2018).

The use of synthetic antimicrobialsas food safety interventions raises multiple health and environmental risks (European Food Safety Authority, 2021; World Health Organization, 2011; Food and Agriculture Organization, 2020). While most of the methods to control pathogens in foods still involve the use of synthetic antimicrobials and preservatives these chemicals are becoming less desirable to both consumers and food industry. In addition, as the food industry puts efforts into responding to consumers' and governmental regulatory bodies' pressure, researching for novel, natural antimicrobials is pertinent for several reasons, such as: i) curbing the rise of antimicrobial resistance of pathogens due to wide use of artificial and/or synthetic preservatives and antimicrobials, and ii) aligning their products with the "clean label" global trend which demands the removal of synthetic preservatives with naturally derived ones for promoting healthier foods (Nachay, 2017; Grant, 2017). Therefore, a decrease trend in the use of synthetic antimicrobials in foods has been noticed over the past years and a wide range of novel natural antimicrobials, either animal- or plant-origin are investigated for their possible effective utilization as antimicrobial agents or preservatives in foods (Gyawali, 2014; Mostafa, 2017; Muhammad, 2017; Fernández-López, 2005).

Phosvitin is a 35 kDa glycoprotein found exclusively in the granule fraction of chicken egg yolk, making up 7% of the yolk protein and 80% of the protein bound phosphorus (Samaraweera, 2011; Stadelman, 1977; Wallace, 1986). These 217-aminoacid peptides are composed of over 50% serine residues, of which 90% are phosphorylated (Byrne, 1984; Clark, 1985). Phosvitin contains between 3 to 6 iron atoms per molecule depending on the method of isolation from hens' eggs; however, it has a binding capacity of about 60 iron molecules (Albright, 1984; Taborsky, 1963; Webb, 1973). Phosvitin is a strong metal chelator and could be used as an antimicrobial agent (Samaraweera, 2011).

Carvacrol is an essential oil found in the leaves and flowering plant of both thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*) (Burt, 2004; Oussalah, 2007). The percent composition of carvacrol in oregano ranges from trace amounts to 82% and 2 to 45% in thyme (Arrebola, 1994; Burt, 2004; Lagouri, 1993).The hydrophobic phenolic compound in its structure is thought to be the main contributor toits antimicrobial properties (Veldhuizen, 2006).

Minimum inhibitory concentrations (MIC) are used often as a primary research tool to determine the *in vitro* activity of new antimicrobials, and data from such studies are essential for the potential subsequent applications for replacement of synthetic antimicrobials and for re-formulating foods with a cleaner label(Nachay, 2017; Grant, 2017; Cassiday, 2017).

The objective of our study was to evaluate the individual *in vitro* antimicrobial efficacy of phosvitin and carvacrol against four major foodborne illnesses causative agents, namely: *Salmonella enterica, Listeria monocytogenes, Escherichia coli* O157:H7, and *Staphylococcus aureus*.

2. MATERIALS AND METHODS

Bacterial cultures and culture conditions. *Listeria monocytogenes* (H7962-4b, H7762-4b, H7596-non4b, H7969-4b, and NADC-2045-4b),*Salmonella enterica* (Enteritidis-ATCC13076, Heidelberg, Typhimurium-ATCC 14802, Gaminara-8324, Oranienburg-9329), *Escherichia coli* O157:H7 (FRIK125, ATCC 35150, ATCC 43894, ATCC 43895, and 93-062), and *Staphylococcus aureus* (ATCC 6538, ATCC 25923, and BAA-44) were obtained from the culture collection of the Microbial Food Safety Laboratory, Iowa State University, Ames, IA. The stock cultures were kept frozen (-70ºC) in Brain Heart Infusion (BHI) broth (Difco, Becton, Dickinson, Sparks, MD) supplemented with 10% (vol/vol) glycerol. The individual stock cultures were activated in BHI broth (pH 7.2) and incubated at 35ºC. At least two consecutive 18 to 22-hour transfers of each stock culture were carried out before using the cells as inoculum for each experiment.

Preparation of individual inoculum. An equal volume of each individual working cultures of *L. monocytogenes*, *S. enterica*, *Escherichia coli* O157:H7 or *Staphylococcus aureus* were combined in a sterile centrifuge tube. The cells were harvested by centrifugation (10,000 x g; 10 min; 4°C) using a Sorvall Super T21 (American Laboratory Trading, Inc., East Lyme, CT) and washed once in 0.1% (wt/vol) peptone. The pelleted cells were suspended in fresh 0.1% (wt/vol) peptone to obtain a final viable cell concentration of approximately 10^9 CFU/ml. The viable counts of the washed cell suspensions were evaluated by surface plating

serially diluted (10-fold) samples on tryptic soy agar supplemented with 0.6% yeast extract. The individual cell suspensionswere used to inoculate the BHI broth samples for further testing.

Antimicrobials. Phosvitin was supplied by Dr. Dong Ahn from the Department of Animal Science at Iowa State University, Ames, IA. A commercial preparation of carvacrol was purchased fromSigma-Aldrich, USA (Aldrich W224502) and contained more than 98% of phenolic compounds according to the producer's declaration.

Preparation of treatment solutions for Bioscreen C Assay. BHI broth with added phosvitin (10, 20, 40, 60, 80, 100 mg/ml) or carvacrol (0.09, 0.12, 0.14, 0.19, 0.38, or 0.75 mg/ml) was filter sterilized using 0.22 µm pore size Millipore filters (Fisher Scientific, Pittsburgh, PA). Samples of the treatment solutions (2.5-ml) and control (BHI broth with no added antimicrobial) were each inoculated with 25 µl of diluted (1:100) *S. enterica, L. monocytogenes*, *E. coli* O157:H7 or *S. aureus* cell suspensions to obtain a final concentration of approximately 10^5 CFU/ml of sample.

Bioscreen C assay. Aliquots (200-µl) of inoculated samples were added in triplicate to the wells of microtiter plate for the Bioscreen C Turbidometer (Growth Curves, Piscataway, NJ), an automated microbial growth analyzer and incubator. Plates were incubated in the Bioscreen C at 35°C for 24 h and the instrument was programmed to record optical density (OD) at 600 nm every 30 min, with shaking of samples before each optical density reading. Individual BHI broth samples without phosvitin or carvacrol, inoculated with the individual test-pathogen, were used as controls. An initial reading of OD_{600} has been made for all samples (at t= 0 hours).

Significance of the results. Minimum inhibitory concentration (MIC) was the lowest treatment concentration that completely inhibited microbial growth for 24 h (OD_{600}<0.05-unit increment) for each antimicrobial and for each pathogen. The MIC was the lowest concentration of the tested antimicrobial agent giving complete inhibition of growth (an OD_{600} equal to the OD_{600} of initial cell suspension determined at time = 0 hours). The microplate assay was repeated three times for each antimicrobial-bacterium combination, and the MIC was reported as the median of three replications.

Results and Discussions

1.1. Determination of minimum inhibitory concentration (MIC) of phosvitin on Salmonella enterica, Listeria monocytogenes, Escherichia coli O157:H7 and Staphylococcus aureus.

The effect of various levels of phosvitin on the growth of *Salmonella enterica*, *Listeria monocytogenes*, *Escherichia coli O157:H7 and Staphylococcus aureus* in Brain Heart Infusion (BHI) broth at 35ºC was assessed by measuring

the OD_{600} values over 24 hours (Figures 1-4). Based on the 24-hour evolution of the OD_{600} values, the growth inhibition of selected pathogens was directly proportional with the increment of the antimicrobial concentrations. The initial OD_{600}values for all the cultures at time 0 were approximately 0.1 units.

The exposure of *S. enterica* to 0 mg/ml (control), 10 mg/ml, and 20 mg/ml of phosvitin in BHI broth led to a rapid increment of the OD_{600} values which within 18 hours reached between 1.2 and 1.3 units. A modest improved antimicrobial efficacy has been noted when phosvitin has been used in higher concentrations, namely 40 mg/ml and 60 mg/ml of phosvitin. While the addition of 40 mg/ml or 60 mg/mlphosvitin in BHI broth extended the pathogen's lag phase with 8 to 16 hours, the addition of 80 mg/ml and 100 mg/ml of phosvitin led to a complete inhibition of *S. enterica* growth over the 24 hours at 35℃ (Figure 1). At concentrations of 80 and 100 mg/ml phosvitin exerted a bacteriostatic effect on the pathogen and the determined minimum inhibitory concentration (MIC) of phosvitin for *S. enterica* was 80 mg/ml.

Similar growth and inhibition trends were noted for *L. monocytogenes* (Figure 2). Based on the readings of OD_{600} values, small inhibitory effect was noted for phosvitin concentrations of 10 mg/ml, 20 mg/ml, 40 mg/ml, and 60 mg/ml in BHI broth. For three phosvitin concentrations, namely for 10 mg/ml, 20 mg/ml, 40 mg/ml of phosvitin, modest differences in the prolongment of the pathogen's lag phase were noted when compared to control sample. For 60 mg/ml of phosvitin in BHI broth, a longer lag phase of 18 hours was reached. The phosvitin in concentrations of 80 and 100 mg/ml in BHI broth exerted a bacteriostatic effect on *L. monocytogenes* for 24 hours at 35℃ since no growth of bacteria was recorded. The determined MIC of phosvitin for *L. monocytogenes* was 80 mg/ml.

The effect of phosvitin on the growth of *Escherichia coli* O157:H7 and *Staphylococcus aureus* in Brain Heart Infusion (BHI) broth is shown in Figures 3 and 4.

For *E. coli* O157:H7, the OD_{600} value corresponding to the control sample increased from 0.1 (at 3h) to greater than 1.2 (at 17h) (Figure 3). The addition of phosvitin at a concentration of 10 mg/ml did not affect the growth of this pathogen and the DO_{600} values followed the same trend as in the control sample. All other tested concentrations, higher than 10 mg/ml of phosvitin, inhibited the growth of *E. coli* O157:H7.Phosvitin concentrations of 20 to 60 mg/ml only determined a prolongment of the lag phase and did not significantly affect the tested pathogen's ability to recover and grow; after 24 hours the DO_{600} reached values between 0.4 and 0.7 units. Phosvitin concentrations of 80 and 100 mg/ml were bacteriostatic for *E. coli*

O157:H7 and no changes of the DO_{600} values were noted between 0 and 24 hours at 35°C. Therefore, the determined MIC of phosvitin for *E. coli* O157:H7 was 80 mg/ml.

Growth inhibition and the extension of the lag phase of *S. aureus* varied direct proportionally with the phosvitin concentrations. However, *S. aureus* growth was not inhibited by any of the tested phosvitin concentrations (Figure 4). In this case, based on the evolution of the DO_{600} values over the 24 hours, phosvitin only affected the lag phase and slightly reduced the pathogen's ability to recover. The OD_{600} of control and BHI broth containing 10 and 20 mg/ml phosvitin samples increased rapidly and after 17 hours it exceeded an OD_{600} of 0.8 units. Phosvitin concentrations of 60, 80, and 100 mg/ml did not exert a cidal effect and the DO_{600} values for these samples, at 24 hours, varied between 0.2 and 0.6 units. For the highest phosvitin concentration, namely 100 mg/ml, at 24 hours, the DO_{600} value increased with 0.1 units which indicates that the MIC of phosvitin for *S. aureus* is greater than 100 mg/ml and phosvitin only sensitized this pathogen.

1.2. *Determination of minimum inhibitory concentration (MIC) of carvacrol on Salmonella enterica, Listeria monocytogenes, Escherichia coli O157:H7 and Staphylococcus aureus*

Inhibitory effect of carvacrol in BHI broth on the growth of *Salmonella enterica, Listeria monocytogenes, Escherichia coli O157:H7 and Staphylococcus aureus* in BHI supplemented with various concentrations of carvacrol is shown in Figures5-8.

For *S. enterica*, the OD_{600}value for control sample increased, within 16 h, from 0.1 unit to greater than 1.0 unit. Of all tested carvacrol concentrations, increment in OD_{600} values was observed only in the pathogen cultures with the lowest concentrations (0.09 and 0.12 mg/ml). At 24 hours the OD_{600} values of control, and BHI broth with 0.09 mg/ml and 0.12 mg/ml were 1.03, 0.68 and 0.39, respectively. All the other carvacrol concentrations (0.14 to 0.75 mg/ml) completely inhibited the growth of *S. enterica*. The determined MIC of carvacrol for *S. enterica* was 0.14 mg/ml (Figure 5).

For *L. monocytogenes*, except for 0.09 and 0.12 mg/ml of carvacrol in BHI broth, all other tested carvacrol concentrations completely prevented an increase in OD_{600} values in *L. monocytogenes* cultures (Figure 6). The BHI broth treated with 0.09 mg/ml of carvacrol had little to no effect on the growth of *L. monocytogenes* and the increases in OD_{600} values were very similar in trend with the OD_{600} values of the control sample. The MIC of carvacrol for *L. monocytogenes* was 0.14 mg/ml.

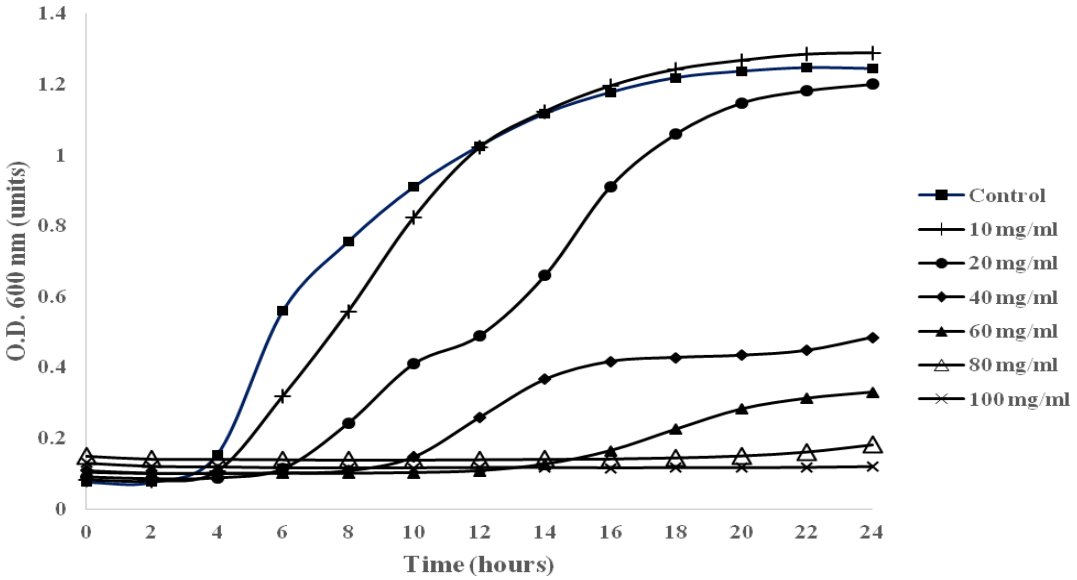

Figure 1. Growth of *Salmonella enterica* in BHI broth (35^{0}C) supplemented with various concentrations of phosvitin.

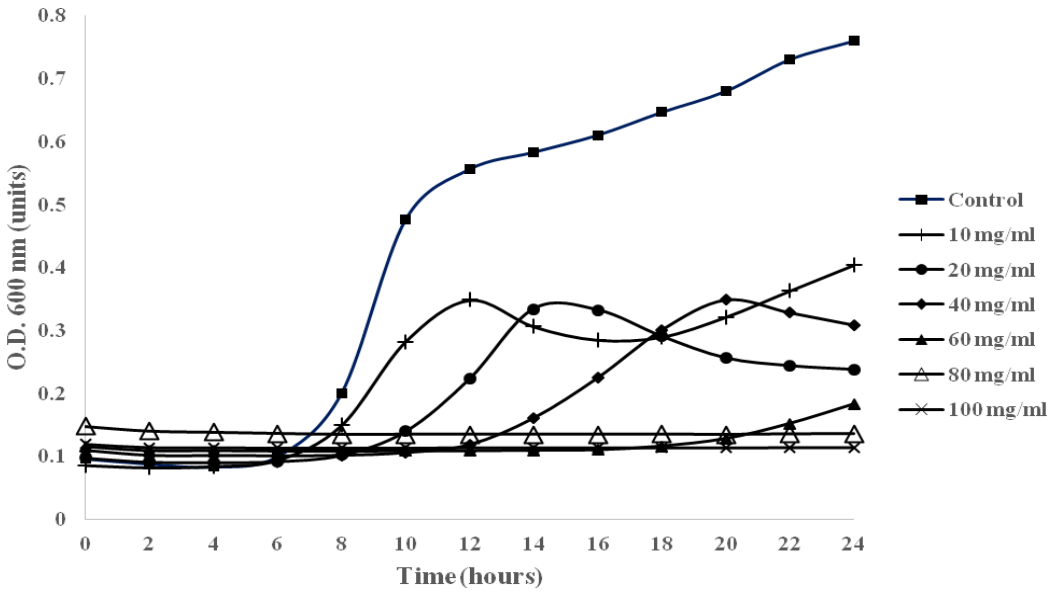

Figure 2. Growth of *Listeria monocytogenes* in BHI broth (35^{0}C) supplemented with various concentrations of phosvitin.

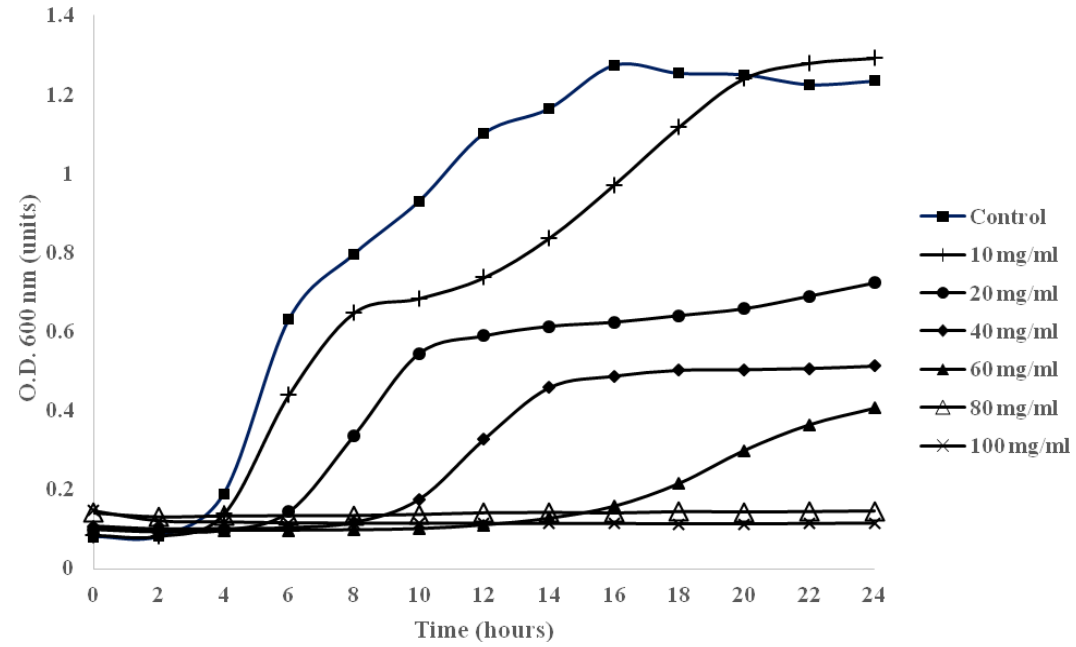

Figure 3. Growth of *Escherichia coli* O157:H7 in BHI broth (35^{0}C) supplemented with various concentrations of phosvitin.

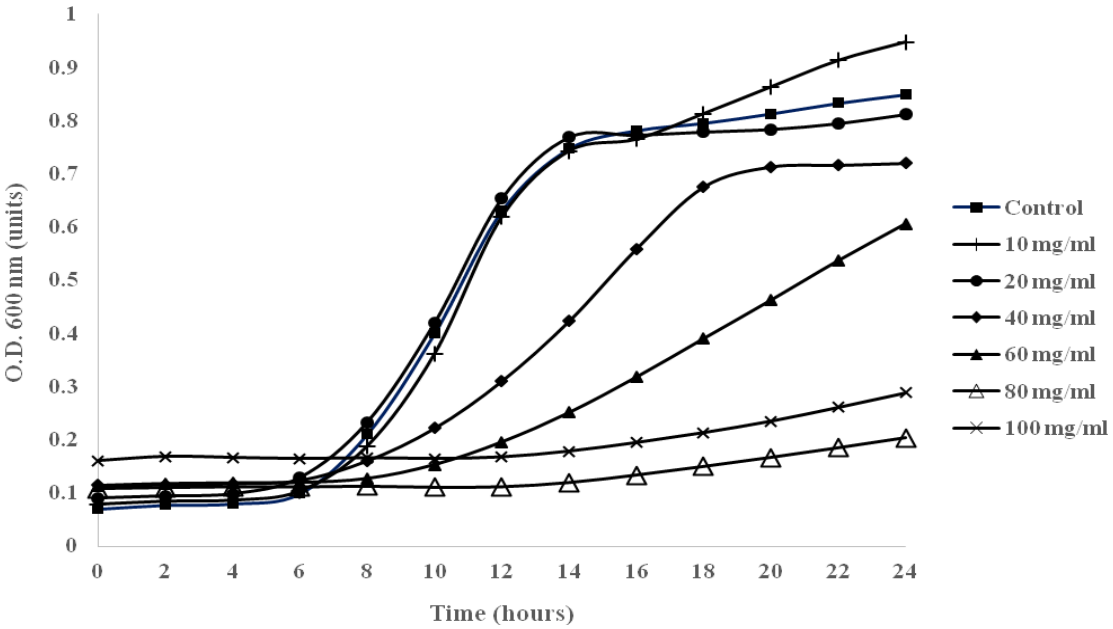

Figure 4. Growth of *Staphylococcus aureus* in BHI broth (35°C) supplemented with various concentrations of phosvitin.

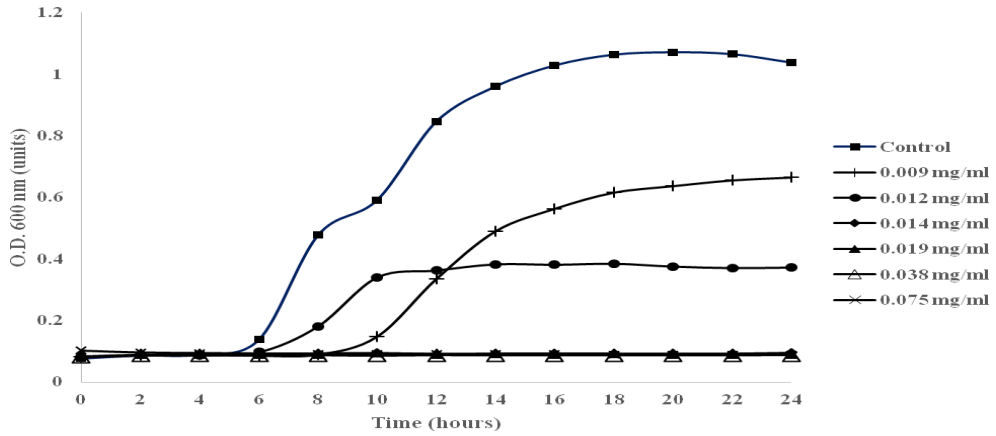

Figure 5. Growth of *Salmonella enterica* in BHI broth (35^{0}C) supplemented with various concentrations of carvacrol.

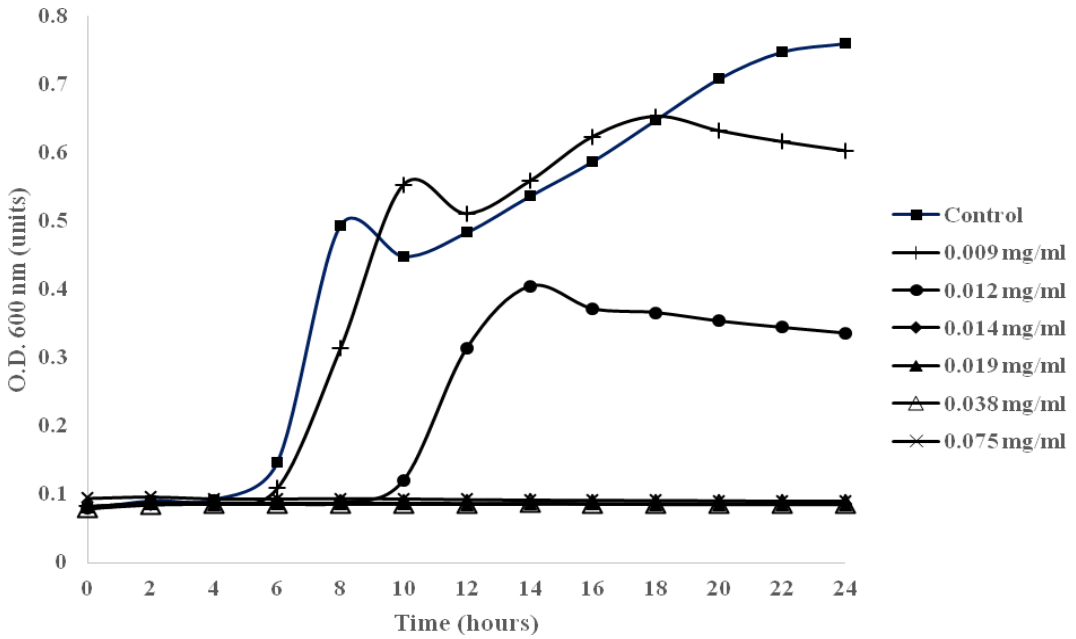

Figure 6. Growth of *Listeria monocytogenes* in BHI broth (35^{0}C) supplemented with various concentrations of carvacrol.

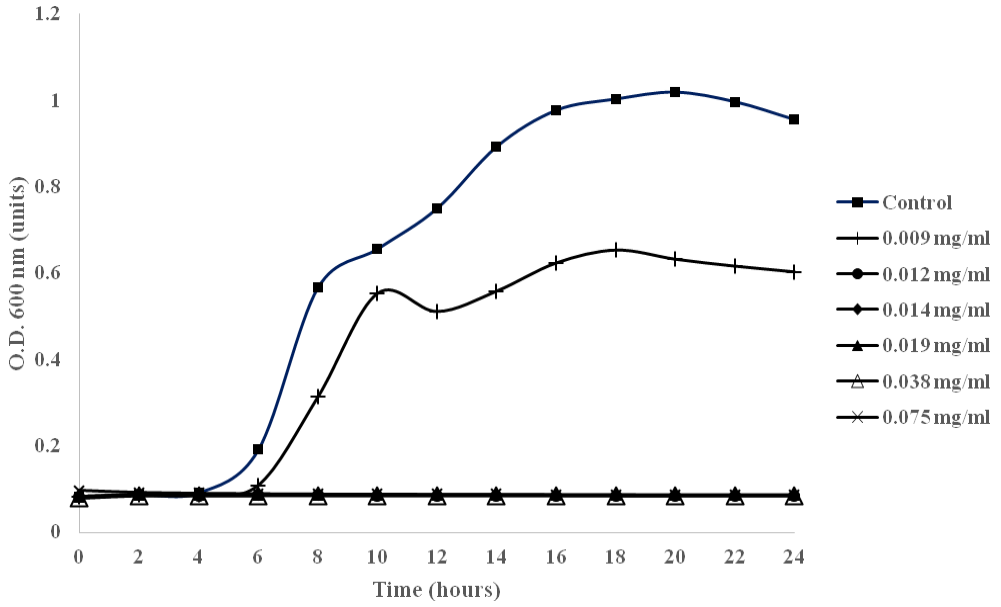

Figure 7. Growth of *Escherichia coli* O157:H7 in BHI broth (35^{0}C) supplemented with various concentrations of carvacrol.

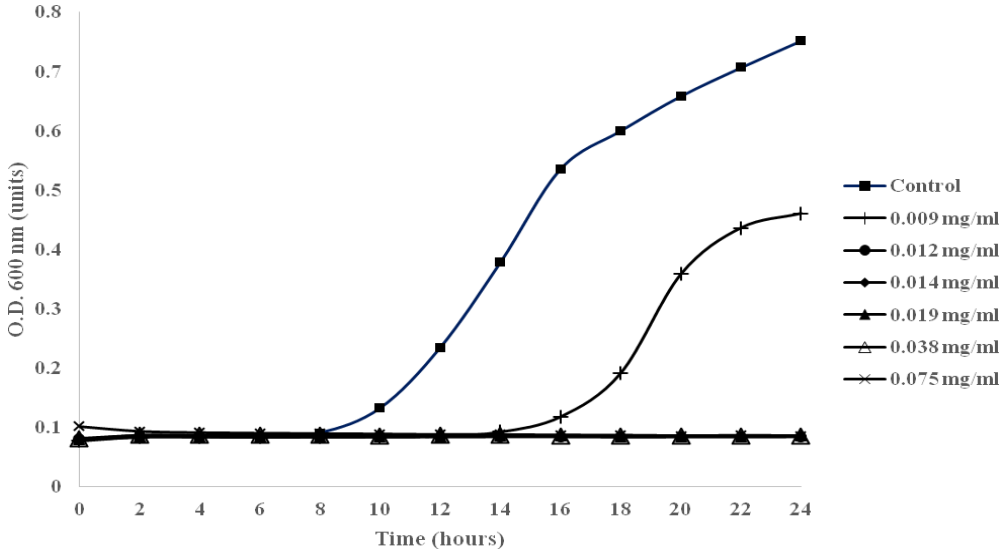

Figure 8. Growth of *Staphylococcus aureus* in BHI broth (35^{0}C) supplemented with various concentrations of carvacrol.

Growth of *S. aureus* and *E. coli* O157:H7 in BHI broth supplemented with various concentrations of carvacrol is shown in Figures 7 and 8. The OD_{600} values of control increased from 0.1 initial value to a greater value, respectively greater than 0.6 units within 18 hours.

For *E. coli* O157:H7, BHI broth with 0.09 mg/ml carvacrol slowed the growth of this pathogen when compared to the control. At 18 hours the OD_{600} of control and carvacrol (0.09 mg/ml) was ~ 1.0 and 0.65, respectively. However, except for 0.09 mg/ml carvacrol, all other tested higher carvacrol concentrations completely inhibited the growth of *E. coli* O157:H7 and the determined MIC of carvacrol for *E. coli* O157:H7 was 0.12 mg/ml (Figure 7).

When *S. aureus* was exposed to carvacrol, increment in OD_{600} value occurred only in BHI broth treated with 0.09 mg/ml carvacrol (Figure 8). After 24 hours, the control sample and the broth sample containing 0.09 mg/ml carvacrol and *S. aureus* reached greater OD_{600} values of 0.7 and 0.45, respectively. All other concentrations of carvacrol (0.12 - 0.75 mg/ml) completely inhibited the growth of*S. aureus*. The MIC of carvacrol for *S. aureus* was 0.12 mg/ml.

4. CONCLUSION

Food industry is under great pressure both from the consumers and from the governmental regulators to replace the use of synthetic antimicrobials with natural antimicrobial agents for reducing the incidence of foodborne outbreaks due to the presence of pathogenic bacteria. Additionally, determining the inhibitory effect of a particular antimicrobial, or of a combination of antimicrobials, using solely the *in vivo* traditional methods is time- and resource-consuming. Our selected animal- and plant-derived antimicrobial agents, namely phosvitin and carvacrol, respectively, proved to be effective under *in vitro* conditions against *Salmonella enterica, Listeria monocytogenes, Escherichia coli* O157:H7, -or *Staphylococcus aureus*. The MIC values for phosvitin and carvacrol, as determined in our study, could provide a valuable reference point for further predicting the antimicrobial efficacy of those two natural antimicrobials when used, alone or even in combinations, in different food systems.

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