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Original scientific paper

DECONTAMINATION OF *Salmonella enterica* spp. ON SHELL EGGS BY *Allium cepa* L. DRY SCALES EXTRACTS

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ABSTRACT. Salmonellae are a major global diarrheal diseases agent and are commonly found in a variety of foods. However, eggs appear to be one of the most important sources of infection ultimately leading to salmonellosis. Here we investigate a potential utilization of onion (*Allium cepa* L.) dry scales extracts and decoction for disinfection of eggshells contaminated with *Salmonella enterica* spp. Antimicrobial activity was screened by a microplate dilution method against 32 environmental and 1 reference *Salmonella* strains, at 10^6 and 10^3 CFU/ml starting cell concentration. Also, the efficacy of egg submersion treatment was tested. At high contamination level of 10^6 *Salmonella* cells per ml, extracts exhibited MIC and MBC values in the range of 0.08-2.50 mg/ml and 0.31-10.0 mg/ml, respectively. Ethanol extract had the most potent antibacterial activity followed by methanol, ethyl-acetate and acetone extracts. Pure decoction had MIC and MBC in the range of 250-500 mg/ml. When testing a lower level of contamination (10^3 CFU/ml) which is more similar to real life levels, MIC and MBCs were 8 to 14 times lower. Submersion of artificially contaminated eggs in ethanol extracts and decoctions resulted in the complete elimination of *Salmonella* after 8 minutes of exposure. Thus, a reduction of a minimum of 3.71 log units was achieved during the 8 minutes of treatment. Having in mind natural levels of *Salmonella* eggshell counts are far lower it can be implied that such treatment could be highly effective in practical application. Results show that tested onion dry scale decontamination solutions are an efficient, cost-effective and eco-friendly options for eggshell decontamination, and are especially promising for use in organic egg production.

Keywords: egg disinfection, *Salmonella*, salmonellosis, *Allium cepa*, antimicrobial activity, antibacterial activity.

INTRODUCTION

Salmonellae are a major global diarrheal diseases agents and are commonly found in a variety of foods. Genus *Salmonella* of the Enterobacteriaceae is currently taxonomically

divided into two species, namely *S. enterica* and *S. bongori* (LE MINOR and POPOFF, 1987; BRENNER *et al*., 2000). *S. enterica* further consists of *arizonae*, *diarizonae*, *enterica*, *houtenae*, *indica* and *salamae* subspecies (TINDALL *et al*., 2005). The three most frequently isolated serovars are Typhi, Typhimurium and Enteritidis. However, there are currently over 2500 recognized serovars (REEVES *et al*., 1989; BRENNER *et al*., 2000; GAST, 2007). Diarrheal diseases agents, in particular non-typhoidal *S. enterica* are the major contributor to the global and regional burden of food borne diseases (HAVELAAR *et al*., 2015). According to MAJOWICZ *et al*. (2010) estimation, 93.8 million cases of gastroenteritis and 155,000 deaths due to non-typhoidal *Salmonella* infections occur annually worldwide.

Consumption of eggs is considered one of the main risk factors leading to *Salmonella* Enteritidis infections (PATRICK *et al*., 2004; BRADEN, 2006; PERRY, 2010). The risk of salmonellosis is often increased by a range of inadequate handling procedures, such as storage at room temperature, a short cooking period, or close contact between eggs during the storage period leading to cross-contamination (PASSARO *et al*., 1996). Moreover, survival of *Salmonella* cells on eggshell depends on storage temperature and relative humidity and might be extended by the presence of moist organic content such as chicken droppings (GANTOIS ET AL., 2009). Therefore, in order to reduce the risk of eggs-related salmonellosis, washing eggs using antimicrobial agents became a standard procedure in the food processing industry.

A variety of methods/agents have been proposed so far to decontaminate eggshells, such as washing egg surfaces with cool water (JONES *et al*., 2005), treatment with chlorine (CAO *et al*., 2009; PARK *et al*., 2017), iodine-based compounds (KNAPE *et al*., 1999), hydrogen peroxide (PADRON, 1995), ozone (KOIDIS *et al*., 2020), ammonium compounds (WANG AND SLAVIK, 1998), electrolyzed water (RUSSELL, 2003; CAO *et al*., 2009), a combination of ozone and UV radiation (RODRIGUEZ-ROMO and YOUSEF, 2005) and pulsed UV light (KEKLIK *et al*., 2010). More recently, plant-derived antimicrobials, as well as hot water immersion, have also been proposed (UPADHYAYA *et al*., 2013, 2016; GEVEKE *et al*., 2016). Although promising, some of the methods mentioned above have not been successfully applied in industrial settings since they are prohibitively expensive. In addition, they appear to have limited antimicrobial activity, especially if organic matter is present, which is usually the case (MOATS, 1978; WANG and SLAVIK, 1998; AL-AJEELI *et al*., 2016). Moreover, the washing process without strictly controlled parameters such as water temperature, pH and disinfectant concentration might promote additional contamination by facilitating penetration of *Salmonella* cells into eggs and a loss of shell integrity (BARTLETT *et al*., 1993; HUTCHISON *et al*., 2003). Therefore, the application of an efficient disinfection agent/process is critically important for the prevention of salmonellosis (KUO *et al*., 1997; PARK *et al*., 2005). Ideally, an antimicrobial sanitizer should be able to efficiently reduce high counts of the targeted pathogen in a short period of time while maintaining its effectiveness even in the presence of organic compounds. Additionally, it should be safe for workers and the environment, as well as cost-efficient.

The antimicrobial efficacy of onion essential oil has previously been confirmed by many studies (ZOHRI *et al*., 1995; BENKEBLIA, 2004; DOBRE *et al*., 2011; BAG and CHATTOPADHYAY, 2015) as well as the effectiveness of extracts using different solvents obtained from fresh and dry bulbs (BAKHT *et al*., 2013, 2014). However, few studies investigated the antimicrobial potential of inedible onion dry scales. Škerget *et al*. (2009) reported that both acetone and ethanol extracts exhibited antibacterial and antifungal activity at 5.0, 10.0 and 20.0 mg/ml concentrations.

Here we propose the application of onion (*Allium cepa* L.) dry scales, traditionally used in south-eastern Serbia as a method for eggs sanitation. The objective was to investigate the anti-*Salmonella* activity of methanol, ethanol, ethyl acetate and acetone extracts, as well as water decoction obtained from onion dry scales. The minimal inhibitory (MIC) and bactericidal concentrations (MBC) against 33 strains of *Salmonella enterica* spp. have also been obtained. The ethanol extract and the water decoction were further investigated as the

most promising treatments. Since dry onion scales are a waste by-product in onion processing and are recyclable, the proposed method is both, cost-effective and environmentally friendly, and thus widely available to many egg manufacturers, especially in organic egg production where the use of some conventional chemical disinfectants is restricted. To the best of our knowledge, this is the first study on this subject.

MATERIALS AND METHODS

Preparation of onion (Allium cepa) dry scale extracts

Brown onions (*Allium cepa*) were supplied from the local market. Dry outer scales were separated from the bulbs. Scales were pulverized in a blender and measured into 60 g portions.

The extracts were prepared by pouring 300 ml of the chosen solvent – methanol, ethanol, acetone and ethyl acetate, over 60 g portions of the plant material. The bottles containing plant material and solvents were kept tightly sealed without agitation in dark, at room temperature, for seven days. After the extraction period, filtration was performed, and the solvents were removed by a rotary evaporator (IKA RV 10 digital, Germany). Obtained crude extracts were used to prepare the stock solutions of extracts (100 mg/ml) in sterile 10% dimethyl sulfoxide (DMSO) aqueous solution. Overview of tested solutions for each experimental procedure and yields of extracts are shown in Table 1.

Table 1. Overview of onion (*Allium cepa*) dry scale treatment solutions used in experiments.

	Analyses performed			
Treatment solution	**MIC/MBC[1]**	**MIC/MBC[2]**	**Egg submersion treatment**	**Extract yield (g per 100g of dry scales)**
Methanol extract	+	-	-	
Ethanol extract	+	+	+	3.36
Ethyl-acetate extract	+	-	-	1.52
Acetone extract	+	-	-	1.81
Pure decoction	+	+	+	n.a.*
25% decoction solution in 10% ethanol	-	+	-	n.a.
25% decoction solution in 20% ethanol	-	+	+	n.a.

[1] determination at 10^6 CFU/ml starting cell count;
[2] determination at 10^3 CFU/ml starting cell count;
*n.a. – not applicable.

A pure decoction was freshly prepared before experiments by pouring 1500 ml of cold water over 100 g of pulverized dry onion scales. The mixture was heated to a boiling point for 30 minutes followed by filtration through sterile gauze. The final solution was adjusted to a volume of 1500 ml using cold sterile water.

Salmonella isolates

We tested the antimicrobial activity of the onion scale extracts and decoction against environmental and reference *Salmonella* strains. Environmental isolates (a total of 32 strains)

were isolated from soil and sediment samples in the Republic of Serbia (ČUČAK *et al*., 2018). Detailed characteristics of environmental isolates are given in Table 2.

Table 2. Environmental *Salmonella enterica* strains on which antimicrobial activity of *Allium cepa* dry scale decoction and extracts was tested. Strains were isolated and characterized in detail in the previous study (ČUČAK *et al*., 2018).

Strain number	Strain mark in Čučak *et al.* (2018)*	*Salmonella enterica* subspecies	Serovar	Origin
1	1	diarizonae	-	sediment
2	10	enterica	Enteritidis	soil
3	20	salamae	-	sediment
4	21	enterica	Enteritidis	sediment
5	64	salamae	-	soil
6	97	enterica	Isaszeg	soil
7	134	enterica	Brandenburg	soil
8	137	enterica	Brandenburg	soil
9	150	enterica	Wien	soil
10	180	salamae	-	soil
11	189	salamae	-	soil
12	224	salamae	-	soil
13	233	salamae	-	soil
14	255	salamae	-	soil
15	451	enterica	Brandenburg	soil
16	530	enterica	Bispebjerg or Tinda	soil
17	536	enterica	Sunnycove	soil
18	542	enterica	Wien	soil
19	566	enterica	Athinai	soil
20	613	salamae	-	soil
21	674	enterica	Azteca	soil
22	722	enterica	Brandenburg	soil
23	754	salamae	-	soil
24	773	enterica	Athinai, Bonariensis or Aba	soil
25	811	diarizonae	-	soil
26	860	enterica	Molade	soil
27	869	enterica	Wien	sediment
28	882	enterica	Enteritidis	soil
29	884	enterica	Enteritidis	soil
30	903	enterica	Szentes	soil
31	907	enterica	Enteritidis	sediment
32	943	enterica	Athinai, Bonariensis or Aba	soil

*****Čučak, D., Babić, O., Tamaš, I., Simeunović, J., Karaman, M., Kovač, D., Novaković, M., Markov, S., Knežević, P., Stojanov, I., Obradović, V., Radnović, D. (2018): Prevalence, Antibiotic Resistance and Diversity of *Salmonella* Isolates from Soils and Sediments in Serbia. *Int. J. Environ. Res*. **12**, 829–841. https://doi.org/10.1007/s41742-018-0138-3.

In addition, we tested a reference strain *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076.

These environmental strains of *Salmonella* were chosen for the analysis as opposed to e.g. clinical human isolates, as there is a higher possibility such isolates could be encountered as contamination of eggs in organic chicken farms and free-range chicken farms, which would be prime users of our type of natural egg disinfection solutions.

Determination of MIC and MBC of onion scale extracts and decoction by a microplate dilution method

Antibacterial activity of the obtained extracts and decoction was evaluated against 33 environmental and reference *Salmonella* strains using a modified CLSI microdilution assay (CLINICAL AND LABORATORY STANDARDS INSTITUTE, 2009). The inocula of the bacterial strains were prepared from overnight cultures. Suspensions were made in sterile saline solution (0.9% NaCl) followed by adjusting optical density to 0.5 McFarland, corresponding to 10^8 CFU/ml. Stock solutions of the extracts (100 mg/ml) were prepared in a sterile 10% dimethyl sulfoxide (DMSO) aqueous solution. Serial doubling dilutions of the stock extracts (0.002–10.00 mg/ml) and the decoction (0.10-500 µl/ml) in 96 well microtiter plates containing inoculated Mueller-Hinton broth were prepared. The final volume per well was 100 µl with a cell concentration of 10^6 CFU/ml. The plates were incubated for 24 h at 37°C. All experiments were performed in triplicates. The experiment included two controls: growth control using the same medium supplemented with 10% aqueous DMSO inoculated with strains, and negative control in which strains were not inoculated and sterility of used solutions were tested. The bacterial growth was detected by adding 20 µl of 0.5% triphenyltetrazolium chloride (TTC) aqueous solution into each well. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of the extract inhibiting visible growth detected by the presence of a red colored pellet in the bottom of the wells after TTC addition. To determine the minimal bactericidal concentration (MBC), before the addition of TTC, broth was taken from each well without visible growth and inoculated onto Mueller Hinton Agar (MHA) for 24 h at 37ºC to detect the persistence of viable cells. MBC was defined as the lowest concentration of treatment solution that killed 99.9% of bacterial cells.

In addition, the antimicrobial activity of selected treatment solutions (ethanol extract, pure decoction, 25% decoction solution in 10% ethanol, 25% decoction solution in 20% ethanol) was evaluated against a lower starting cell concentration of 10^3 CFU/ml of reference *Salmonella* strain ATCC 13076, which is closer to counts recorded in real conditions of intact, naturally infected eggs (1-100 cells per egg) (DUGUID and NORTH, 1991).

Submersion treatment for decontamination of Salmonella on eggshell surfaces

Eggs were purchased at the local market. Using sterile swabs, samples were taken from eggshells and inoculated onto selective XLD agar to determine if the eggs were naturally contaminated by *Salmonella*. Eggs were surface sterilized by wiping with ethanol (75%) and a subsequent 30 minutes exposure to UV light. Overnight cultures of *S.* Enteritidis (ATCC 13076) on MHA were used for preparing 0.5 McFarland suspensions (Grant Bio DEN-1 densitometer, corresponding to ~ 10^8 CFU/ml, followed by dilution in sterile saline solution to the actual turbidity corresponding to 10^3 CFU/ml. Sterile eggs were then soaked in 35 ml of the prepared bacterial suspension for 30 minutes to enable the attachment of bacterial cells to shell surfaces. After inoculation, eggs were dried on a sterile filter paper and transferred into 35 ml of the pure onion dry scales decoction, a 25% ethanol decoction solution, as well as the ethanol extract solution. The eggs were treated for 2, 4, 6, 8 and 10 minutes. After completion of the treatment, eggs were transferred into 35 ml of a sterile saline solution where they were kept for 10 minutes with mild shaking. From each suspension 100 µl aliquots were taken and

plated on XLD agar plates to determine the count of surviving *Salmonella* cells that have detached from the egg surface. The plates were incubated for 18 h at 37°C and subjected to CFU counting. Also, swabs of eggshell surface were taken and plated on the XLD agar to determine the count of viable *Salmonella* cells still attached to the egg surface.

All tests were performed in triplicates. The experimental design included two controls: sterility control and negative control (eggs inoculated with *Salmonella*, but treated with 20% ethanol instead of treatment solutions).

Potential for multiple use of treatment solutions

To investigate whether the extract and decoction solutions could be reused in multiple treatments, right after the treatments 100 µl of all tested solutions were spread plated on non-selective MHA in triplicate to determine bacterial counts. All colonies formed on MHA were then transferred to XLD agar to determine *Salmonella* presence. The remaining decoction and decoction dissolved in ethanol (10% and 20%) were kept in sterile Erlenmeyer flasks (in quintuplicate) at room temperature for two months. After that period, fungal contamination was detected by visual inspection.

RESULTS

Minimal inhibitory and bactericidal concentration (MIC and MBC) of onion scale extracts and decoction against Salmonella strains

Antimicrobial activity of *Allium cepa* extracts and decoction against 32 environmental and 1 reference *Salmonella* strain was screened by a microplate dilution method.

Our results indicate that the extracts exhibited an inhibitory effect against the tested *Salmonella* strains when applied in concentrations from 0.08-2.50 mg/ml, while bactericidal concentrations were achieved in the range from 0.31 – >10.0 mg/ml (Table 3). Ethanol extract had the most potent antibacterial activity followed by methanol, ethyl-acetate and acetone extracts. Specifically, ethanol extract MICs ranged from 0.08 to 1.25 mg/ml and MBCs from 0.31 to >10 mg/ml, while MIC range of methanol extract was from 0.16 to 2.5 mg/ml and MBC range was 0.31 to 10 mg/ml. Moreover, the majority of tested strains were more susceptible to the ethanol extract achieving both inhibitory and bactericidal effects at lower concentrations. In 10-20% of the tested strains, there was no bactericidal activity even at the highest tested concentration of ethanol, ethyl-acetate and acetone extract tested. The extracts had inhibitory activity against reference strain *S.* Enteritidis ATCC 13076 when applied in concentrations from 0.63 to 1.25 mg/ml, whereas the bactericidal effect was achieved when applying a 5.00 to 10 mg/ml solution. MICs and MBCs of the extracts did not differ notably between the ATCC strain and the environmental isolates. The sensitivity of environmental strains to different extracts was mostly well correlated. Strains no. 18, 25 and 29 were the most susceptible. Strain's serovar was not correlated to its susceptibility. The antimicrobial activity of the decoction was relatively uniform for all the strains, having both inhibitory and bactericidal effects at concentrations from 250 to 500 µl/ml.

Based on the highest obtained activity, non-toxicity of the solvent, as well as commercial potential, ethanol extract and the decoction were chosen for further analysis using the reference *S. enterica* ssp. *enterica* serovar Enteritidis ATCC 13076. In further analysis, the strain was inoculated at a lower starting cell concentration of 10^3 CFU/ml, which more closely resembles counts in naturally contaminated eggshells. Selected treatment solutions included: ethanol extract, pure decoction, 25% decoction solution in 10% ethanol and 25% decoction solution in 20% ethanol. Dilutions of decoction in ethanol were tested because the pure

decoction can change the shell color to dark brown, which could affect its further practical application.

Table 3. Antimicrobial activity of *Allium cepa* dry scales extracts and decoction against *Salmonella* at 10^6 CFU/ml starting cell count (microdilution assay).

***Salmonella* strain No.**	**MIC/MBC* (mg/ml for extracts and µL/ml for decoction)**				
	Methanol extract	**Ethanol extract**	**Ethyl-acetate extract**	**Acetone extract**	**Pure decoction**
1	2.50/10.00	1.25/10.00	0.63/>10.00	0.63/>10.00	250.0/250.0
2	1.25/10.00	0.63/>10.00	0.63/5.00	0.63/10.00	250.0/250.0
3	0.63/10.00	0.31/10.00	0.31/10.00	0.63/5.00	500.0/500.0
4	1.25/10.00	0.63/2.50	1.25/5.00	0.63/10.00	500.0/500.0
5	1.25/5.00	0.31/5.00	0.63/5.00	1.25/10.00	500.0/500.0
6	1.25/5.00	0.31/5.00	0.31/5.00	0.16/2.50	500.0/500.0
7	0.63/10.00	0.63/5.00	1.25/5.00	0.63/5.00	500.0/500.0
8	2.50/10.00	0.63/10.00	1.25/10.00	1.25/10.00	500.0/500.0
9	0.63/10.00	0.31/10.00	0.31/10.00	0.63/10.00	250.0/250.0
10	2.50/10.00	1.25/>10.00	2.50/5.00	2.50/>10.00	500.0/500.0
11	1.25/10.00	1.25/10.00	2.50/5.00	1.25/5.00	500.0/500.0
12	1.25/10.00	0.31/10.00	0.63/5.00	0.63/10.00	500.0/500.0
13	1.25/10.00	0.63/10.00	1.25/>10.00	0.63/10.00	500.0/500.0
14	0.63/10.00	0.63/10.00	0.63/>10.00	0.63/10.00	500.0/500.0
15	1.25/10.00	1.25/10.00	1.25/10.00	0.63/>10.00	500.0/500.0
16	0.31/5.00	0.63/>10.00	0.63/10.00	0.63/5.00	500.0/500.0
17	0.63/10.00	1.25/10.00	0.63/5.00	0.63/>10.00	500.0/500.0
18	0.31/0.31	0.08/0.31	0.16/0.63	0.16/0.63	250.0/250.0
19	0.63/10.00	0.63/>10.00	0.63/5.00	0.63/5.00	500.0/500.0
20	1.25/10.00	0.31/5.00	0.63/10.00	0.63/>10.00	500.0/500.0
21	2.50/10.00	1.25/10.00	1.25/10.00	2.50/10.00	500.0/500.0
22	2.50/10.00	0.63/10.00	1.25/5.00	0.63/>10.00	500.0/500.0
23	2.50/10.00	0.31/0.63	0.63/0.63	0.63/0.63	500.0/500.0
24	2.50/10.00	0.63/5.00	1.25/10.00	1.25/10.00	500.0/500.0
25	0.16/2.50	0.16/2.50	0.16/5.00	0.16/5.00	500.0/500.0
26	2.50/10.00	0.63/10.00	1.25/10.00	1.25/10.00	500.0/500.0
27	1.25/10.00	1.25/10.00	2.50/5.00	1.25/5.00	500.0/500.0
28	1.25/10.00	0.63/10.00	1.25/10.00	0.63/10.00	500.0/500.0
29	0.31/1.25	0.31/0.63	0.31/0.63	0.31/0.63	500.0/500.0
30	2.50/10.00	0.63/10.00	1.25/10.00	1.25/10.00	500.0/500.0
31	1.25/5.00	0.63/5.00	1.25/5.00	0.63/2.50	500.0/500.0
32	0.63/10.00	1.25/>10.00	1.25/>10.00	1.25/>10.00	500.0/500.0
ATCC 13076	0.63/10.00	0.63/5.00	0.63/5.00	1.25/5.00	250.0/250.0
MIC range	**0.16-2.5**	**0.08-1.25**	**0.16-2.5**	**0.16-2.5**	**250-500**
MIC median	**1.25**	**0.63**	**0.63**	**0.63**	**500**
MBC range	**0.31-10**	**0.08->10**	**0.63->10**	**0.63->10**	**250-500**

*****MIC – minimal inhibitory concentration, MBC - minimal bactericidal concentration, control with 10% aqueous DMSO did not have inhibitory nor bactericidal effect on *Salmonella*.

As expected, when treatments were applied to a lower-level *Salmonella* contamination, all tested solutions achieved both inhibitory and bactericidal effects at much lower concentrations (Table 4). The ethanol extract exhibited almost nine times higher inhibitory potential (MIC/MBC=0.071/4.56 mg/ml). The pure decoction showed inhibition at a concentration of 17.82 µl/ml (1.78%), which is 14 times lower compared to the MIC obtained for starting cell density of 10^6 CFU/ml. Both 25% decoction solutions in ethanol (10% and 20%) were inhibitory at 31.25 µl/ml (3.12%). No inhibition of cell growth was observed in control wells containing the solvent (10% and 20% ethanol) only.

Table 4. Antimicrobial activity of *Allium cepa* dry scale extracts and decoction against the reference *Salmonella* Enteritidis ATCC 13076 strain at 10^3 CFU/ml starting cell count (microdilution assay).

Strain	MIC/MBC* (mg/ml for extract and µL/ml for decocts)				Control
	Ethanol extract	Pure decoction	25% decoction solution in 10% ethanol	25% decoction solution in 20% ethanol	Ethanol (20%)
ATCC 13076	0.071/4.56	17.82/100.00	31.25/250.00	31.25/250.00	No inhibitory nor bactericidal effect

*****MIC – minimal inhibitory concentration, MBC – minimal bactericidal concentration.

Submersion treatment for decontamination of Salmonella on eggshell surfaces

Based on the results of the microdilution assay and practical applicability issues, ethanol extract, pure decoction and 25 % decoction in 20% ethanol were selected for egg submersion treatment. Submersion of artificially contaminated eggs in ethanol extracts and decoctions was highly effective in decontaminating *Salmonella* and was dependent on the period of exposure (Table 5).

Table 5. Effect of submersion in different *Allium cepa* treatment solutions on *Salmonella* cell count on eggshells.

Time (min)	Treatment solution							
	Ethanol extract		Pure decoction		25% decoction solution in 20% ethanol		Control (20% ethanol)***	
	Suspension* (CFU/ml)	Swabs** (number of CFU)	Suspension (CFU/ml)	Swabs (number of CFU)	Suspension (CFU/ml)	Swabs (number of CFU)	Suspension (CFU/ml)	Swabs (number of CFU)
0	5100	>300	5100	>300	5100	>300	5100	>300
2	75	5	>300	11	>300	27	>300	>300
4	0	3	75	4	0	2	>300	>300
6	0	1	100	0	0	1	>300	>300
8	0	0	0	0	0	0	>300	>300
10	0	0	0	0	0	0	>300	>300

***** count in suspension – eggs were rinsed in saline solution after the treatment and such suspension was tested for the number of easily detachable *Salmonella* cells;

****** swabs of the surface of the egg were taken and plated on XLD agar to determine the number of viable cells still attached to eggshell surface;

******* *Salmonella* was not detected in sterility control.

All three tested treatment solutions showed potent bactericidal activity. Complete elimination of *Salmonella* was achieved after 8 minutes of exposure. Thus, a reduction of a minimum of 3.71 log units was achieved during the 8 minutes of treatment. The 25% decoction solution exhibited the same efficiency as the pure decoction without affecting the color of the eggshells.

Potential for multiple use of treatment solutions

We tested the possibility of multiple applications of used treatment solutions by monitoring the count of bacteria and *Salmonella* in solutions after their first use. The highest bacterial count was found in the ethanol extract (210 CFU/ml) whereas a 25% decoction and pure decoction produced on average only 30 and 20 CFU/ml, respectively. After transferring colonies onto *Salmonella* selective XLD agar, it was determined none of the colonies belonged to the *Salmonella* genus. Thus, the decoction potentially enables multiple applications. After prolonged storage of the treatment solution for 60 days at room temperature, the pure decoction had visible mold contamination, while 25% decoctions in 10% and 20% ethanol remained clear and were not contaminated by fungal growth.

DISCUSSION

It has been shown that egg washing can significantly reduce the number of microorganisms on the egg surface, but it can also cause damage to the eggshell and allow the entrance of microorganisms inside the egg (BAIN, 2005). Adequate washing can reduce bacterial counts by 2-3 log CFU/ml (ZEIDLER, 2002). Ideal antimicrobial additive to wash water should have the ability to efficiently reduce large numbers of bacteria in a short time, even in the presence of organic matter and should be safe for workers and the environment and be economically viable (SCOTT and SWETNAM, 1993). Chlorine and chlorine-based compounds are the most often used disinfectants for egg washing (CAO ET AL., 2009). Egg washing with water containing chlorine and iodine-based disinfectants resulted in 2 to 3 log reduction, however, disinfectants were far less effective in higher organic load (KNAPE *et al*., 1999). Washing with ozonized water in a concentration of 3 mg/kg for 30, 60 and 90 s reduced *Salmonella* Enteritidis count to 2.10, 2.34 and 2.47 log CFU/ml (KOIDIS *et al*., 2020).

Even though chlorine disinfection is highly effective, a significant drawback of its use is that in reaction with organic matter it can produce trihalomethane and other potentially carcinogenic organochlorine compounds. For that reason, it is highly important to find alternative antimicrobial agents for egg washing (KNAPE *et al*., 2002; UPADHYAYA *et al*., 2013). UPADHYAYA *et al*. (2013) tested pure plant-derived compounds as egg sanitizers in the presence of organic matter. The results indicate that *Salmonella* count was lowered to undetectable levels even in such conditions, proving that plant antimicrobial agents, including our extracts, could be more efficient in egg decontamination than conventional disinfectants such as chlorine in the presence of organic matter. Plant extracts are an excellent candidate for egg decontamination since they are environmentally friendly, safe, recyclable and already proven to be efficient (TAYEL *et al*., 2014). Excessive use of conventional antimicrobial agents in the food industry is leading to the development of highly antibiotic-resistant *Salmonella* strains (ANGULO *et al*., 2000). To avoid the formation of strains resistant to antibiotics, it is crucial to use alternative antimicrobial agents (e.g., plant extracts) for decontamination in the food industry (XU and LEE, 2001).

TAYEL *et al*. (2018) examined the antimicrobial activity of *Quercus infectoria* ethanol extract against bacteria *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella* Typhimurium, as well as fungus *Candida albicans*. After 1h of egg submer-

sion exposure to *Q. infectoria* extract, count reduction of all tested strains was in the range of 30.2-42.6%, while after 5h of exposure, count reduction was between 95.9 and 98.8%, indicating that used oak extract is an efficient agent for egg decontamination. In this case, MIC for *Salmonella* Typhimurium was determined to be 1.25 mg/ml, which is in the range of MIC values for *Salmonella* strains obtained in our experiment with *Allium cepa* extracts. Tayel *et al*. (2014) also examined the antimicrobial activity of a series of ethanol extracts obtained from camphor (*Cinnamomum camphora*), garlic (*Allium sativum*), licorice (*Glycyrrhiza glabra*), olive (*Olea europaea*), oak (*Q. infectoria*), pomegranate (*Punica granatum*), senna (*Senna alexandrina*) and ziziphus (*Zizyphus spina christi*) against *Salmonella* Enteritidis and *Salmonella* Typhimurium. The least efficient extracts were from olive and ziziphus, with MIC values over 1 mg/ml for both salmonella types. The most efficient extracts were those obtained from oak, pomegranate and senna, with MIC values between 0.15 and 0.2 mg/ml for both types of salmonella, which is in the MIC range of 0.08-2.5 mg/ml determined for onion dry scale extracts in our study.

SOLJOUR *et al*. (2004) examined the bactericidal activity of three commercial compounds for cleaning and disinfection (sodium carbonate, sodium hypochlorite and potassium hydroxide) at 10^2, 10^4 and 10^6 CFU/ml *Salmonella* Enteritidis surface count. Compound applied at the recommended concentrations for their application (sodium carbonate 36 ppm; sodium hypochlorite and potassium hydroxide 200 ppm) were unable to completely remove higher salmonella contamination levels of 10^4 and 10^6 CFU/ml - it was necessary to apply 5-20 times higher concentrations than recommended by the manufacturer for complete bacteria removal.

UPADHYAYA *et al*. (2013) tested plant-derived antimicrobial pure compounds, namely *trans*-cinnamaldehyde (TC), carvacrol (CR), and eugenol (EUG), for their ability to decontaminate eggs with and without organic matter presence (fresh layer droppings). Eggs with starting count of ~6.4 log CFU/ml were treated with water solutions of plant-derived antimicrobials in concentrations of 0.0, 0.25, 0.50 and 0.75% at 30 sec, 3 and 5 minutes at 32 and 42°C. Washing with pure water without antimicrobials and with water with 200 mg/kg chlorine has led to 2 log reduction. Carvacrol eliminated salmonella after only 30 seconds of treatment to an undetectable level, eugenol at 0.25% removed ~5.0 log CFU/ml after 5 minutes, and at 0.50% and 0.75% EUG complete reduction was obtained after 30sec. TC at 0.75% reduced counts for 5 log CFU/ml after five minutes.

In comparison to our results, UPADHYAYA *et al*. (2013) obtained a much more efficient *Salmonella* reduction. Their study used pure plant derived compounds in high concentrations; however, this process would be highly expensive in practical application due to the high cost of such compounds. Also, in practical application there could be a problem to obtain uniform compound dissolution in water resulting in unequal effects.

Inedible onion parts exhibit significant antimicrobial properties. Still, less research has been conducted on them (e.g. BENKEBLIA 2004; ŠKERGET, MAJHENIČ, *et al*. 2009) compared to edible onion parts (e.g. ZOHRI *et al*., 1995; DOBRE *et al*., 2011). Onion dry scales contain a considerable amount of biologically active compounds, namely phenolic compounds such as flavonoids, phenolic acids and anthocyanins (BENKEBLIA, 2007). Dry scales have 3–5 times higher content of phenolic compounds and quercetin compared to edible onion parts, which could contribute to their higher antioxidative and antimicrobial activity (ŠKERGET *et al*. 2009). Namely, dry scale extracts with acetone, ethanol and mixtures of solvents with water, showed higher inhibition of bacteria and fungi compared to edible parts extracts (ŠKERGET *et al*. 2009). The inhibitory effect was noted for bacteria *Escherichia coli*, *Pseudomonas fluorescens*, *Bacillus cereus*, and fungi *Aspergillus niger*, *Trichoderma viride*, and *Penicillium cyclopium* with MICs in the range of 5–200 mg/ml. Results of another study also showed that phenolic compounds inhibited the growth of bacteria and yeast, and that the intensity of inhibition was dependent on the variety of onion and concentration (BENKEBLIA, 2004).

All used solvents in our study are polar (methanol, ethanol, acetone, and ethyl acetate). Polar solvents can be used to extract plant antimicrobial compounds, such as alkaloids, flavonoids, terpenoids, tannins, and saponins (SANTAS *et al*., 2010; PENECILLA and MAGNO, 2011; LANZOTTI *et al*., 2012). Our results based on MIC and MBC values indicate ethanol extract contained the most compounds with antibacterial effect. In future research focus should be on the examination of the composition of dry scale extracts, especially ethanol extract, as well as the extraction of compounds using non-polar solvents. Also, the effect on other important bacteria and fungi should be determined. Further testing of efficacy on bacteria in biofilm form should be performed, as this form is far more resistant to various environmental conditions including disinfection compared to planktonic form. The robustness and ruggedness of the disinfection method should be determined with the use of different batches of onion extracts, temperatures of solutions and other variables. Also, effectiveness with different levels of organic contamination should be determined.

Results of our study suggest that treatment solutions obtained from onion dry scales can be effectively used in egg production for the prevention of salmonellosis outbreaks by decontamination of an egg surface. Our treatments effectively reduced salmonella count for 1–2 log CFU/ml in as less as 2 minutes, while complete elimination (a minimum of 3.71 log units) was reached after 8 minutes. Having in mind natural levels of *Salmonella* eggshell contamination are far lower it can be implied that our treatment could be highly effective in practical application. To that, our treatment solutions are natural homogenous solutions, safe for use and cheap, because they are derived from plant waste material. Furthermore, solutions could be used multiple times as it was proven no bacterial salmonella contamination remained in treatment solutions after their initial use.

For these reasons, the solutions used in this experiment are highly promising as disinfecting agents, with special emphasis on decoction. Our small-scale simple submersion treatment can be easily and cheaply implemented in small farm production of eggs, especially in organic egg production where the use of conventional chemical disinfectants is restricted.

To conclude, ethanol, methanol, ethyl-acetate and acetone extracts and decoction made from onion (*Allium cepa*) dry scales exerted a significant antimicrobial potential against the S*almonellae*. Treatment solutions were effective both in microdilution assay, as well as in submersion treatment. According to the results of the microdilution assay, ethanol extract proved to be the most effective. To facilitate practical applications, we showed that a 25% decoction dissolved in 20% ethanol can also be effectively used for submersion treatment as an agent for superficial *Salmonella* decontamination of eggshells. It has all the necessary qualities for large-scale production and application, such as a strong bactericidal effect against a number of *Salmonella* strains, as well as a relatively short exposure time. To that, it can be used multiple times as there was no solution contamination after use. In addition, it is safe from a human health perspective and suitable for organic food production**.** Finally, it is cost-efficient and amenable to industrial-scale production as a natural product prepared from cheap waste material. Hence, tested onion scale treatment solutions (especially 25% decoction dissolved in 20% ethanol) have a large potential for practical application in *Salmonella* egg decontamination. To conclude, these harmless natural treatment solutions obtained from the recyclable onion waste material are a promising, efficient, cost-effective and eco-friendly alternative to conventional chemical egg disinfectants.

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