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# Evaluation of Grapefruit Juice (*Citrus paradisi*) as an Alternative Disinfectant for Hatching Eggs

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**MG and JB designed the study. KIAA and JB collected the data. LW and BND did the lab work. JB and KD made statistical analyses and wrote the manuscript.**

**Key words**
**Coturnix coturnix japonica, Grapefruit juice, Hatching eggs, Disinfection, Hatchability, Japanese quail.**

## ABSTRACT

The aim of the study was to evaluate hatching results and quality of Japanese quail chicks (*Coturnix coturnix japonica*) after applying red grapefruit juice (*Citrus paradisi*) for hatching eggs disinfection. 420 eggs were randomly divided into 3 groups (150 pcs each). 1st group was not disinfected (NC – negative control), the 2nd one was disinfected with formaldehyde and $KMnO_4$ fumigation (PC - positive control). In 3rd group fresh juice of red grapefruit was used as disinfectant (GT). Eggs were incubated under standard condition. On 14th day eggs were candled to determine infertile eggs or dead embryos, as well as samples of eggs to microbial analysis were. After 17.5 days of incubation its results were evaluated. Chicks were reared for 14 days and their livability and body weight gains were registered. The effectiveness of grapefruit juice as a disinfectant for hatching eggs was noticed in terms of reducing the development of microflora on the eggshell. Its use did not directly affect the hatching results and chick quality, however, they were similar to control groups, what may confirm possible use of GT juice as an alternative disinfection method.

## INTRODUCTION

Grapefruit (*Citrus paradisi*) is an evergreen tree belonging to the Rutaceae family. It is generally believed that it is a hybrid of pomelo and sweet orange. Originally grapefruit appeared on Barbados Island, but in the 19th century first trees were planted in Florida, where its cultivation continues up to this day (Uysal *et al*., 2011; Okunowo *et al*., 2013). Several color varieties significantly differ from each other in chemical composition and taste were bred. Special interest is aroused by red variety of grapefruit, which, due to a high content of bioactive substances, is used also for non-food purposes. Grapefruit juice is used in traditional medicine as a natural antibacterial, antifungal and antiviral preparation. Besides, because of lycopene and wide range of carotenoids the presence in juice of this fruit effectively neutralizes free radicals delaying, among others, aging processes. Due to large amounts of organic acids, flavonoids and furanocoumarins (Krajewska-Kułak *et al*., 2001) grapefruit extract has found its use as an effective, environmentally friendly, cleaning and antiseptic agent.

There are several reports on the essential oil and juce of citrus peels, some of which includes; D-limonene, β-myrcene, α-pinene, β-pinene, γ-terpinene, α-terpinolene, α-caryophyllene, copaene, β-phellandrene (Fuselli *et al*., 2008). Plants are a complex mixture of some compounds such as hydrocarbons, alcohols, esters, aldehydes and have been reported to exhibit inhibitory activities against a wide spectrum food spoilage microorganism (Liaqat *et al*., 2017). Also the antimicrobial roles of flavonoids are well documented (Cushnie and Lamb, 2005). Biostatic or biocidal properties of grapefruit juice were proved against many groups of microbials like *Staphylococcus aureus* and *M. luteus* and *E. coli* (Malinowska-Pańczyk *et al*., 2010). The juice of these fruits also shows antifungal activity, particularly in relation to the yeast-like fungi such as *Candida albicans* or *C. tropicalis*. Studies showed clearly a strong inhibition of fungi colonies growth by relatively low concentrations of grapefruit extract (Jaworska-

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Zaremba *et al*., 2012).

The knowledge about biocidal and biostatic grapefruit properties allows to take an attempt its practical use in poultry production, one of whose direction is obtaining hatching eggs. Their proper disinfection is a crucial factor for obtain good quality of newly-hatched chicks (Sacco *et al*., 1989). This issue is also important from the point of view of hatchery workers' safety, who, through the use of traditional disinfection methods, are exposed to potentially carcinogenic formaldehyde fumes (Kupczewska-Dobecka, 2007; Hayretda and Kolankaya, 2008).

The aim of the study was the evaluation of hatching results and quality of Japanese quail chicks (*Coturnix coturnix japonica*) after applying red grapefruit (*Citrus paradisi*) juice for hatching eggs disinfection. Before the main experiment, preliminary studies were conducted to evaluate the effect of juice from various grapefruit varieties on chosen microorganisms.

## MATERIALS AND METHODS

The preliminary study was conducted by agar diffusion method. Environmental micro-organisms have been selected which are typical pathogenic flora witch may contribute to deterioration of hatching results and they are the most commonly used in antimicrobial properties model. Also they differed in resistance to disinfectants. An inoculum with an optical density of 0.5 in the McFalrlanad's scale was prepared from the 24 h culture of *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa* and then plated on a surface of Muller Milton agar (MH II) on a Petri dish (96 mm). For the *C. albicans* fungus YNB medium (Yeast Nitrogen Base) was applied to which the inoculum was prepared from a 24 h culture of fungi on Sabouard's solid medium. 8-10 mm holes (wells) were cut in the substrate and 100 µl of the tested substance was applied to them. Samples were incubated at 37 °C for 24 h, at this time the grapefruit juice diffused into the MH or YNB agar medium from its place of application. A standard disc with penicillin antibiotic (10 µg) was used as a positive control for bacterial strains and a disc with amphotericin B (20 µg) was used for the *C. albicans*. Negative control was sterile 0.75% NaCl saline. The microbial growth inhibition zone was measured after 24 and 48 h.

In the second stage of experiment the freshly squeezed juice with red grapefruit was used. By HPLC method (PN-EN 12630:2002) sugar content in juice was evaluated. The analysis showed 25.9±0.7 $gl^{-1}$ of saccharose (≈0.026%), 12.00.3 $gl^{-1}$ glucose (0.012%) and 14.5±0.3 $gl^{-1}$ fructose (0.015%).

The material consisted of 420 Japanese quail hatching eggs. Before being placed in hatching apparatus, eggs from 1st group were not subjected to any disinfectant (NC, negative control), 2nd group was disinfected by fumigation of formalin with potassium permanganate mixture (PC, positive control). In the 3rd group freshly squeezed juice of red grapefruit (GT) was used, wherein the eggs were dipped individually. The eggs were hatched artificially using a BIOS hatching apparatus under standard conditions of incubation: temperature was 37.6-38.0 °C with 50-65% relative humidity in the setting compartment, and 37.0–37.5 °C with 75–80% relative humidity in the hatching compartment. The eggs were turned 8 times a day during the incubation period. After 14 days of incubation the eggs were candled to determine the number of infertile eggs and dead embryos, then eggs were moved from the setter to the hatching compartment.

Also samples for microbial analysis were collected on 14th day of experiment. 3 eggs per each group were placed in sterile boxes containing 50ml of sterile buffer salt solution (PBS) with 3 drops of TWEN 80. Containers with eggs were left on the stirrer for 1 h. Samples were serially diluted in PBS and plated on sterile medium in order to obtain the total number of aerobic mesophilic bacteria, the total number of bacteria, coliform bacteria, hemolytic bacteria, *Salmonella* spp., *Staphylococcus* spp. (Gentry and Quarles, 1972; Jones *et al*., 2002; PN-EN ISO 4833:2004; PN-ISO 21528-1:2005). After incubation colonies were counted and presented as cfu/1 ml of liquid from the egg. To identify the bacterial colonies microscopic examination was performed as well as Gram's staining method and API biochemical tests (bioMérieuxPolska®). The medium used were agar for aerobic mesophilic bacteria and total number of bacteria, agar + 5% sheep blood for hemolytic bacteria, MacConkey's for coliform bacteria, Baird Parker agar (supplemented with 5% egg yolk-tellurite) for *Staphylococcus* spp. and agar *Salmonella-Shigella* for *Salmonella* spp.

After 17.5 days of incubation the hatching results were evaluated (fertility, hatchability, periodical embryonic mortality). Chicks were reared for 14 days, the mortality and individual body weight on 7th and 14th day of rearing were registered.

Eggs were weighted before being placed in setter and then at compartment changing (alive, dead embryos and infertile) and at hatching (unhatched). On this basis, shell conductance was calculated (mg $H_2O$/d/mmHg) according to Christensen *et al*. (2001).

The data were analyzed with the use of statistical package SPSS 20.0PL (IBM, 2011). The one-way ANOVA with Tukey's post-hoc test was carried out. The number of bacteria colony forming units were verified using nonparametrical $\chi^2$ test.

## RESULTS

Results of preliminary studies were presented in Table I. Grapefruit juice from both yellow and red varieties showed bactericidal properties. Slightly higher values were observed for red grapefruit. The analysed grapefruit juice showed the strongest effect against *P. aeruginosa* strain. The size of growth inhibition zone for the red grapefruit variety amounted 16 mm and 14 mm for yellow one. Slightly lower bactericidal values were observed against *Staphylococcus aureus* strain, 14 mm for red and 12 mm for yellow variety. The weakest bactericidal action was observed against Gram-negative *E. coli* isolate, 12 and 8 mm depending on grapefruit variety, red and yellow, respectively. For the isolate *C. albicans* fungi used in the experiment, no inhibitory effects were observed.

**Table I.- Growth inhibition zones (in mm) of selected microorganisms under the influence of grapefruit juice and control substances.**

| Bacteria species | Grapefruit variety | | Control | |
|---|---|---|---|---|
| | Yellow | Red | + | - |
| *Staphylococcus aureus* | 12 | 14 | 24 | 0 |
| *Escherichia coli* | 8 | 12 | 19 | 0 |
| *Pseudomonas aeruginosa* | 14 | 16 | 11 | 0 |
| *Candida albicans* | 0 | 0 | 15 | 0 |

For further analysis the red variety grapefruit juice was chosen because, based on the results from the first part of experiment, it was characterised by stronger antimicrobial properties. The results of quail eggs microbial analyse in 14th day of their incubation are presented in Table II. A significantly lower number of fungal colonies was found in both, traditionally disinfected (PC) and with grapefruit juice (GT) treated group. Similar relationships were stated in case of the total number of bacterial colonies, in the GT group value of this parameter was 11.5% lower than in the control group.

In the next step of analyse, the microflora species composition on hatching eggs shell was analysed (Table II). In case of *Staphylococcus* spp. it was noted that grapefruit juice was more effective than formaldehyde in number of bacteria reduction, although both disinfection methods gave worse results than in non-disinfected group. In the case of *Staphylococcus sciuri*, complete elimination of bacteria was observed after the use of both disinfection methods at relatively high abundance in the negative sample. High efficiency of grapefruit juice against *Streptococcus* spp. is especially noteworthy, as it made it possible to limit their development by more than four times in relation to formaldehyde. A slightly higher *Salmonella* spp. value and *Staphylococcus* spp. was observed in GT group, as well as *Streptococcus* spp. in PC. Differences were not statistically significant. The resulting discrepancies may resulted from various viscosity of egg shells after juice using in the GT group. It could facilitate the adhesion of microorganisms present in flowing air in the incubator. The bigger proportion of *Streptococcus* spp. in PC group shows low effectiveness of formaldehyde against *Streptococcus* bacteria.

**Table II.- Total number of microbials ($Log_{10}$ CFU $1ml^{-1}$ liquid from egg) and more common bacteria isolated from egg shells depending on hatching eggs disinfection method .**

| Total number of microbials | NC | PC | GT | Total | SEM |
|---|---|---|---|---|---|
| Fungi | 0.667[a] | 0.460[b] | 0.390[b] | 0.491 | 0.156 |
| Bacteria | 1.465[a] | 1.240[ab] | 1.183[b] | 1.296 | 0.089 |
| **Identification of bacteria species (% of total isolates)** | | | | | **$\chi^2$ (p-value)** |
| *Escherichia coli* | 8.97 | 6.82 | 8.00 | 8.16 | 0.928 |
| *Salmonella* spp. | 8.97 | 9.09 | 24.00 | 11.56 | 0.042 |
| *Staphylococcus sciuri* | 52.56 | 0.00 | 0.00 | 27.89 | 0.000 |
| *Staphylococcus* spp. | 6.41 | 22.73 | 48.00 | 18.37 | 0.001 |
| *Streptococcus* spp. | 20.51 | 54.55 | 12.00 | 29.25 | 0.006 |
| Non indentified | 2.56 | 6.82 | 8.00 | 4.76 | 0.439 |
| Total | 100 | 100 | 100 | 100 | - |

[a, b], differences between mean values for treatments are significant at p≤0.05. Groups: NC (negative control), not disinfected; PC (positive control), disinfected traditionally (fumigated with formaldehyde and KMnO4); GT, disinfected with fresh juice of red grapefruit.

**Table III.- The hatching results depending on hatching eggs disinfection method.**

| Trait | Treatment | | | $\chi^2$ (p-value) |
|---|---|---|---|---|
| | NC | PC | GT | |
| Eggs fertility (%) | 83.20 | 81.25 | 82.99 | 0.990 |
| Hatchability of fertile eggs (%) | 75.96 | 75.00 | 70.49 | 0.926 |
| Hatchability of set eggs (%) | 63.20 | 60.94 | 58.50 | 0.926 |
| Mortality of fertile eggs (%) | 24.04 | 25.00 | 27.05 | 0.919 |
| Mortality of set eggs (%) | 20.00 | 20.31 | 22.45 | 0.908 |
| | | | | **SEM** |
| Proportion of chick in egg weight (%) | 68.83 | 68.04 | 66.47 | 0.669 |

For abbreviations, see Table II.

After eggs incubation the analysis of hatching was performed. Its results are shown in Table III. There were no differences in the hatching indices in relation to the disinfection method used. Slightly smaller hatchability of fertile eggs and bigger embryos mortality were found in the GT group, but these differences, in comparison to NC and PC groups, were not statistically significant. Also in the group treated with grapefruit juice, chick proportion in egg was about 2% smaller than in other groups. However, the best results, were obtained in the negative group indicating the presence of the biological defence mechanism in quail eggs.

The results of newly-hatched chicks' body weight measurements of 14 days of rearing are shown in Figure 1. The biggest initial body weight was found in chicks derived from group of eggs treated with grapefruit juice as disinfectant. It is also worth to note that these birds obtained the biggest body weight gain during 7 days of rearing. After 14 days the heaviest were birds from group where eggs were incubated without disinfection. In any term of the study the differences between groups were not statistically confirmed.

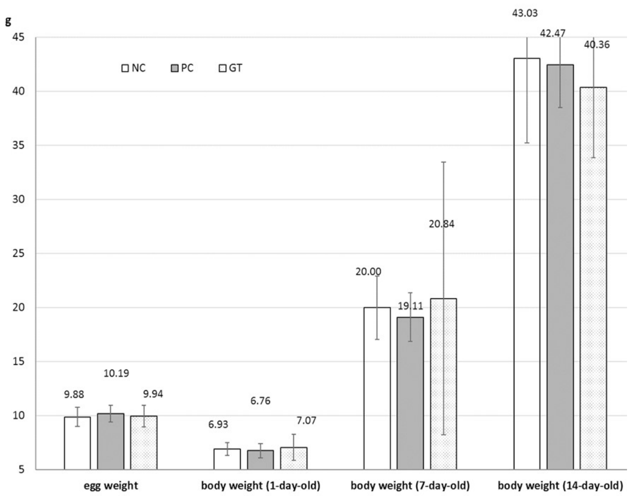


Fig. 1. Mean values (±SD) of eggs weight and body weight of quail chicks in 1, 7 and in 14 day of rearing depending on hatching eggs disinfection method.
For abbreviations, see Table II

In Table IV water conductivity of egg shells during incubation was presented. The used disinfection methods did not affect this parameter. Slightly higher values were noted in the PC and GT groups than in the non-disinfection one (NC). This means that the mixture of sugars present in the grapefruit juice did not contribute to the reduction of water loss.

The obtained results, apart from the differences due to the disinfection method used, indicated also the specificity of quail eggs. These birds, in spite of domestication, exhibit many natural features and adaptations, which may be confirmed by relatively low efficiency of any sanitation of hatching eggs before their incubation. Unlike in hens' eggs, there was no significant improvement in hatching or elimination of microorganisms from the surface of shell, which may be related to different structure of this element.

**Table IV.- Water conductivity of egg shells depending on hatching eggs disinfection method (mg$H_2O$/day/g of egg weight).**

| Time (days) | Eggs | Treatment | | | SEM |
|---|---|---|---|---|---|
| | | NC | PC | GT | |
| 15 | Fertile | 1.67 | 1.93 | 2.00 | 0.100 |
| | Infertile | 2.21 | 1.97 | 2.72 | 0.254 |
| | Dead embryos | 1.25 | 2.18 | 2.32 | 0.273 |
| 17.5 | Unhatched | 2.09 | 2.28 | 2.21 | 0.138 |

For abbreviations, see Table II.

## DISCUSSION

The amount of microorganisms on the egg shell may be modified by many factors such as laying conditions, hens maintenance system, permanent and transient microflora effects, microbial contamination of feed and production facilities, handling of eggs immediately after laying and type of egg packages (Adesiyun *et al*., 2006; Jones *et al*., 2004; Wall *et al*., 2008; Węsierska, 2006; Batkowska *et al*., 2014). In case of proper disinfection, the absence microorganisms penetrate the shell and enter egg content and embryo, causing its death. It is critical to obtain such a reduction of microflora to provide a relatively sterile incubation and to obtain good quality chicks (Sacco *et al*., 1989).

Cvetnić and Vladimir-Knežević (2004) evaluating the effect of grapefruit extract by agar diffusion method, confirmed that this extract causes the growth inhibition zone of 20 bacterial strains and 10 yeast strains among others in case of *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *C. albicans*.

Also, the inhibitory effect of grapefruit extract on *S. aureus* has been demonstrated in tests using an agar diffusion method. In this case the growth inhibition zone was much more differentiated than in the present work, ranging from 11 to 53 mm (Uysal *et al*., 2011). Also, studies conducted by Sharma and Sharma (2010) confirmed the fungicidal properties of 5% grapefruit juice extract, which allowed for 15 mm *C. albicans* inhibition zone, but there was also a significant inhibitory effect on *S. aureus* and *E.*

*coli*, where the inhibition zone amounted to 11 mm and was very consistent with own results.

Studies involving the practical use of red grapefruit juice are compatible with the available references for microbiological evaluation. The antimicrobial properties of grapefruit juice probably result from its ability to cell walls and cytoplasmic membranes destruction (Kędzia, 2000), however, reports of the effectiveness of microbial population growth inhibition using *Citrus paradisi* juice show a considerable variability. Grapefruit preparations are characterized by a high content of flavones, flavonols and flavonones (mainly naringin). In addition, they contain monoterpenes and sesquiterpenes, carotenoids, pectins, fiber, vitamins B1, C, PP and mineral salts (Peterson *et al*., 2006). The mechanism of action of these extracts consists in damage of bacterial cell membrane, inhibiting amino acid synthesis and cell respiration processes, as well as outside leakage of cytoplasmic components (Maćkiw *et al*., 2012).

The grapefruit extract is considered as one of the most effective natural remedies against gastrointestinal infections caused by *C. albicans* (Krajewska-Kułak *et al*., 2001). Grapefruit extracts had also an inhibitory effect on such bacteria as *Staphylococcus aureus*, *Staphylococcus epidermis*, *Enterococcus faecalis*, *E. coli*, *S. typhimurium* (Uysal *et al*., 2011). Opposite results were obtained by Adukwu *et al*. (2012), which pointed the lack of antibacterial properties of grapefruit juice against *S. aureus*.

Except the juice also grapefruit extract is used for disinfection, but the efficacy often results from its concentration. Inhibitory effects in relation to *E. coli* and *K. pneumoniae* have already been achieved with 5% grapefruit extract (Sharma and Sharma, 2010). Different results were presented by Cvetnić and Vladimir-Knezević (2004), who used 33% grapefruit extract and showed no antibacterial activity against *E. coli* strain and *P. aeruginosae*. The combination of grapefruit and fig extracts inhibits the growth of *K. pneuoniae* (Gupta *et al*., 2011). This study has confirmed its efficacy in terms of reducing the total number of colonies of fungi and bacteria as well as *Staphylococcus sciuri* and *Streptococcus* Spp. Attention should be paid on a generally small number of bacterial colonies found on the egg shells. It is several times smaller than reported by other authors (Nowaczewski *et al*., 2013) and may indicate high microbiological purity of eggs and stock from which the eggs were obtained.

There is no available data relating to hatching results of eggs disinfected with juice or grapefruit extracts. Therefore, its effect compared to other natural antimicrobial substances should be verified. One of the herbal preparations with disinfection properties is thyme extract (*Thymus vulgaris*). Research on this species has demonstrated that it has strong antimicrobial, antiviral and antioxidant properties (Stahl-Biskup and Laakso, 1990; Nevas *et al*., 2004). However, Shahein and Seedeek (2014) indicated an increase in the total number of bacteria and the number of coliform colonies in the group disinfected with thyme oil. Also the results of hatching were worse than in the control group. Oregano oil (*Origanum vulgare*) also manifests antibacterial, antioxidant, antifungal, cytotoxic, insecticidal and nematicidal properties. They result from the presence of such compounds as carvacrol, thymol, terpinen and p-cymene (Koščova *et al*., 2006). Practical application was confirmed by spraying hatching eggs with oregano oil. A significant reduction in the number of bacteria, yeast and mold colonies on the egg shell was obtained, what was comparable with formaldehyde disinfection (Copur *et al*., 2010). Also the use of other plant extracts is promising (oregano, garlic, cinnamon). They contributed to significant limitation or even eliminating the total number of microorganisms (Yildrim *et al*., 2003), while improving (Copur *et al*., 2010; Shahein and Seedeek, 2014) or without negative effect on hatching results or chicks quality (Ulucay and Yildirim, 2010; Copur *et al*., 2011).

## CONCLUSIONS

Studies have documented the effectiveness of grapefruit juice (red variety) as a disinfectant for hatching eggs in terms of inhibition of eggshell microflora development. It has been shown that grapefruit juice does not create preferential conditions for any particular species of microorganisms. However, its use did not directly affect hatching results and quality of obtained chicks. Since the laboratory-proven properties of red grapefruit juice are not always confirmed by practical use, it may be necessary to assess the interaction of disinfection with other environmental factors such as those that could modify juice performance. However, the use of grapefruit juice is not without any justification because this alternative disinfection method does not deteriorate hatching results, while increasing the safety of hatchery workers due to the lack of carcinogenic factors present in traditional methods.

*Statement of conflict of interest*

The authors declares that there is no conflict of interests regarding the publication of this article.

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