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The effect of washing on the shelf-life parameters of spinach (*Spinacia oleracea* L.)

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SUMMARY

Spinach is a very popular green leafy vegetable because of its versatile usage and beneficial for the health. However, spinach may contain several pathogen bacteria: Escherichia coli, Klebsiella spp., Salmonella spp., Enterobacter spp., Citrobacter spp., Shigella spp. and Listeria monocytogenes, which can cause several serious health problems. This study investigates the effects of washing with citric acid for the shelf-life parameters of spinach in comparison to the effect of washing with water and control. Washing of spinach with 0.5% citric acid solution decreased the elasticity of the spinach leaves, as well as the chlorophyll content. On the other hand, the total plate count, as well as the yeast and mold count could be decreased with this treatment, but difference was not detectable at the forth storage day. The fecal indicator E. coli did not change, indicating washing was not effective in this case. Further optimisation of treatment and storage conditions may decrease microbial risk of fresh spinach consumption without decreasing its sensory quality.

Keywords: *spinach; shelf-life; washing; citric acid*

INTRODUCTION

Spinach (*Spinacia oleracea* L.) is a green leafy vegetable, which is popular because of its versatile usage. It can be eaten raw as a side dish, in vegetable smoothies, on pizza or in sandwiches, or we may cook it and add to soups, stews and sauces. According to the latest data from 2021, the largest acreage spinach was harvested is China (732,399 ha), on the second place is Indonesia (44,049 ha) and the third one is the United States of America with 24,403 ha (FAO, 2021). Spinach contains 91.4% water, 2.9% protein, 3.6% carbohydrate, 0.4% fat and 2.2 g fiber per 100 g (Roberts and Moreau, 2016). It is an excellent source of vitamin K, folic acid and magnesium. It contains vitamin B6, calcium, iron, zinc and high amount of oxalic acid too. Regarding phytochemicals in spinach, there are lots of carotenoid insomuch that Spanish people intake beta-carotene by eating spinach, while average European people by eating carrot (O'Neill et al., 2001).

However, spinach has food safety risk. Between 2014 and 2021, 2028 illnesses, 477 hospitalizations and 18 deaths related to consuming leafy green vegetables were reported by Centers for Disease Control and Prevention (CDC) in the USA (CDC, 2023). The most common pathogen bacteria in fresh products are pathogenic *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp., *Enterobacter* spp., *Citrobacter* spp., *Shigella* spp. and *Listeria monocytogenes* (Yeni et al., 2016).

Because of the composition, spinach is a perishable food, so it needs some preservation technologies to increase its shelf lives. One of the most frequent used food additives was citric acid (E330) in the product of French market from 2012 to 2018. More than 10.000 products contain it (Chazelas et al., 2020). Citric acid is bactericide, because it can change the pH of the bacterium cells, which is an important factor to bacterial growth (Li et al., 2023). In a 2018 experiment, Al-Rousan et al. (2018) investigated the effectiveness of citric acid and acetic acid against *Salmonella* Typhimurium, *E. coli* O157:H7 and *Staphylococcus aureus* bacteria in the traditional Arabic salad, Tabbouleh that contains raw vegetables. The salad was treated with 1–1.4% citric acid, 0.3–1.4% acetic acid alone and in two combinations: 1% citric acid 0.4% acetic acid or 1.4% citric acid and 0.3% acetic acid. The media were stored at 4, 10 and 21 °C for 7 days. At room temperature, acetic acid showed greater inhibitory activity against the bacterial growth, but at refrigeration temperatures (4 °C and 10 °C) citric acid proved to be more effective against *Salmonella* Thyphimurium and *E. coli*. The combination of the two acids proved to be more effective against *Salmonella* Typhimurium and *E. coli* O157:H7, but was not effective against *S. aureus*. Finten et al. (2017) investigated the disinfectant activity of a 0.5% citric acid solution on spinach leaves as an alternative to 200 mg L^{-1} Na-hypochlorite. The discs cut from the leaves were inoculated with non-pathogenic model bacteria, i.e. non-pathogenic strains of *E. coli* and *Listeria innocua*. Citric acid proved to be a better inhibitor against bacterial growth and sensor organ could not show any difference (Finten et al., 2017).

Our aims were to study the citric acid washing on viable microbial number following washing of spinach leaves and the effect of this washing on sensory parameters of leaves during storage.

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MATERIALS AND METHODS

Preparation of spinach sample

Fresh, washed, ready-to-eat spinach leaves were purchased from the local hypermarket the night before the testing day and stored in their original packaging in the refrigerator until use. On the starting day of the experiment, samples were prepared for microbiological analysis, and larger leaves were selected for sensory analysis. The measurements from one package were performed on 10 leaves regarding sensory analysis and with 10 g regarding microbiological analysis.

Washing procedure and storage conditions

In the experiment, two distinct washing processes were employed and one batch was left as control (unwashed). The initial process involved using citric acid, where in the leaves were immersed in a sterile 0.5% solution of anhydrous citric acid (sourced from VWR, Hungary) for 2 minutes. Subsequently, the second washing process utilized sterile water, with the leaves immersed in distilled water. The washed leaves were dried at room temperature, in open air for one hour, and subsequently stored in sterile plastic bags in darkness at a controlled temperature of 12 °C for a period of up to 4 days until required for further use.

Detecting microbial contamination

We used Dichloran Rose Bengal Chloramphenicol Agar (DRBC) medium (VWR, Hungary) for the enumeration of yeast and molds, and Tryptone Bile X-glucuronide Agar (TBX, BIOLAB, Hungary) for the detection of *E. coli* and coliforms. Plate Count Agar (PCA, BIOLAB, Hungary) was used for the determination of total plate count. Media were prepared as described by the producer with distilled water, under constant stirring, then sterilized at 121 °C for 15 min. Peptone water (VWR, Hungary) was prepared as described and sterilized at 121 °C for 30 minutes and used for dilution and homogenization.

Samples of packaged spinach were randomly chosen from the refrigerated incubator for microbiological assessment on days 0, 1, and 4 of the storage period. Samples (10 g batch) were placed into sterile stomacher bags containing 90 ml of 0.001 g ml^{-1} bacteriological peptone (VWR, Hungary) and homogenized for 2 minutes with a Star BlenderTM LB400 (VWR, Hungary). The dilution series was prepared from the supernatant following homogenization, with 100 µl applied to the surface of DRBC, and 1 ml used for poured plates of PCA and TBX agar media. Each dilution was replicated four times. Inoculated media were then incubated in the absence of light at 44 °C and 30 °C for 3 days for TBX agar and PCA agar, respectively, and at 25 °C for DRBC agar over 5 days. Colony-forming units (cfu) were enumerated following the incubation period. To determine the colony-forming units (cfu) from the total count of bacterial cells we used the next general formula:

$$\mathrm{cfu} = \frac{\sum \mathrm{c}}{(\mathrm{n}_1 + 0{,}1\ \mathrm{n}_2 + 0{,}01\ \mathrm{n}_3)\mathrm{d}}$$

Where $\sum c$ is the amount of total disk colonies counted, n_1 is the number of countable plates in the first dilution, n_2 is the number of plates in the second dilution, n_3 is the number of plates in the third dilution. Dilution factor is mark by d, which applies to the first dilution.

Sensory studies

To determine the relative chlorophyll content, SPAD-502 chlorophyll meter (Minolta, Japan) was used (Frommer et al., 2019). Ten leaves were measured from each treatment. Average value was calculated per leaf from six measurements.

TX plus Texture Analyzer for determining spinach leaves tensile strength, what correlates with freshness. We made a specific ball shaped 6 mm ball probe to penetrate the leaf at a speed of 1 mm s^{-1}, and a post-test speed of 10 ms^{-1}. Each leaf was placed between two clamped plastic plates with coinciding holes to keep the leaf flat. The probe moved a standard distance of 8 mm. From this test a force–displacement graph for each spinach leaf was generated and the maximum force to puncture the leaf was recorded. We tested 10 leaves from each treatment.

Statistical analysis

Before comparisons the homogeneity of variances was tested with Levene-test and the normal distributions in the groups were tested with histograms and Shapiro-Wilk tests. In case of homogenous variances and normal distributions parametric tests (one-way ANOVA and t-tests) were used. For pairwise comparison LSD post-hoc test was used following ANOVA. If the data did not meet the conditions of the parametric tests, nonparametric Kruskall-Wallis test was used for multiple and Mann-Whitney U test for pairwise comparison.

RESULTS AND DISCUSSION

Citric acid treatment reduced fungal colony counts compared to both control and water-washed samples ($p<0.01$) on day 0 (without storage). Following one day storage, water-washed treatment reduced yeast and mold number on the spinach surface, than citric acid-wash ($p<0.01$), while this result was reversed on the fourth storage day (*Figure 1*). None of the samples were unsatisfactory (>log 4 cfu g^{-1}), but only washing could reduce the yeast and mold number to satisfactory level (<log 3 cfu g^{-1}) (EüM decree 4/1998 (XI.11.)).

The initial total yeast and mold content was log 3.37 ± 1.9 cfu g^{-1}. This result is similar to previously reported contamination of spinach from wholesale market (Zhou et al., 2023), although higher (>log 6 cfu g^{-1}) was also mentioned (Yossa et al., 2013). Washing with chlorine and peroxyacetic acid could reduce the initial cfu, but contamination increased during storage in previous study (Zhou et al., 2023).

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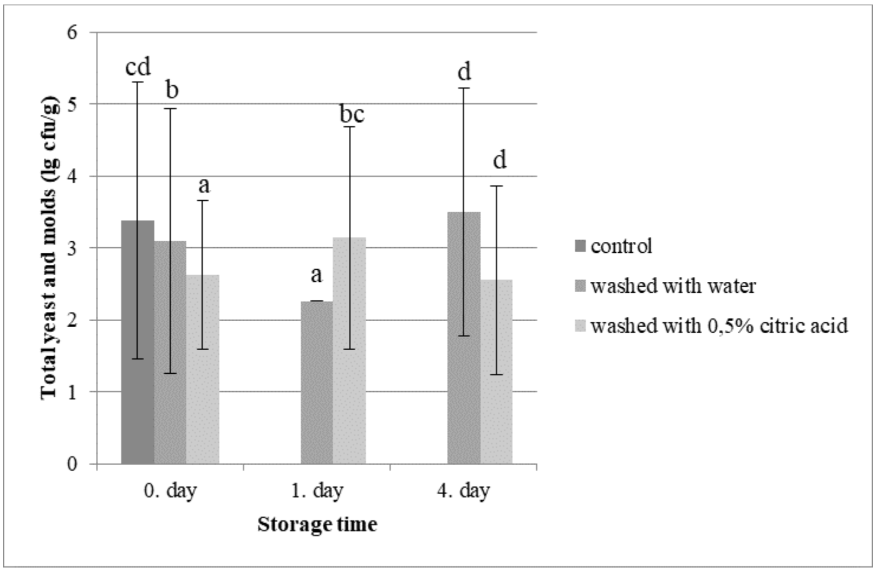
Figure 1. **Number of total yeast and mold of spinach following washing and storage at 12 °C**

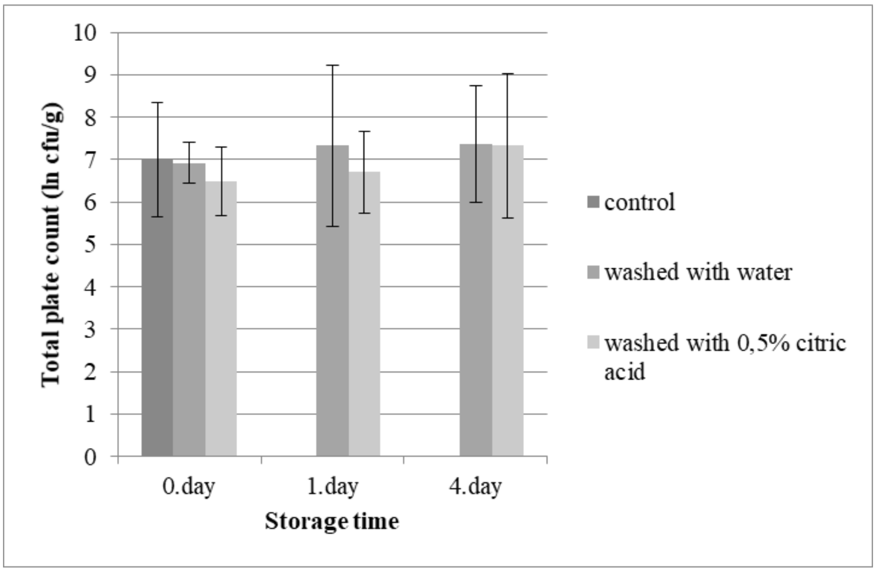


Different letters indicate significant differences (p<0.05) with Mann-Whitney U test

Samples were unsatisfactory (>log 5 cfu g^{-1}) (EüM decree 4/1998 (XI.11.)). Similarly to the previous results, citric acid wash could reduce the microbial contamination, which was also detectable following one day storage, but the washing was not sufficiently decreased the total plate count (<1 log reduction). At the fourth storage day, colony counts were increased, and become as high in citric acid-treated samples as on water washed samples (*Figure 2*).

The total aerobic mesophilic count was approximately 7 log cfu g^{-1} (Abadias et al., 2008; Yossa et al., 2012), similarly to our log 7.0 ± 1.3 cfu g^{-1} result before washing. Spinach from stores had high microbial number, and microbial contamination increased during storage, similarly to previous reports (Yossa et al., 2012; Zhou et al., 2023). The 0.5–1% citric acid was previously reported to reduce the total plate count in naturally infected samples and following contamination with bacterial solutions (log 6–7 cfu g^{-1}) (Jeon and Ha, 2020).

Figure 2. **Total plate count of spinach following washing and storage at 12 °C**



Different letters indicate significant differences (p<0.05) with Mann-Whitney U test

E. coli was detected in the samples. Both washing could reduce the *E. coli* count of spinach, and this number increased only at the fourth storage day, but the change was not proven statistically (p>0.3). The *E. coli*

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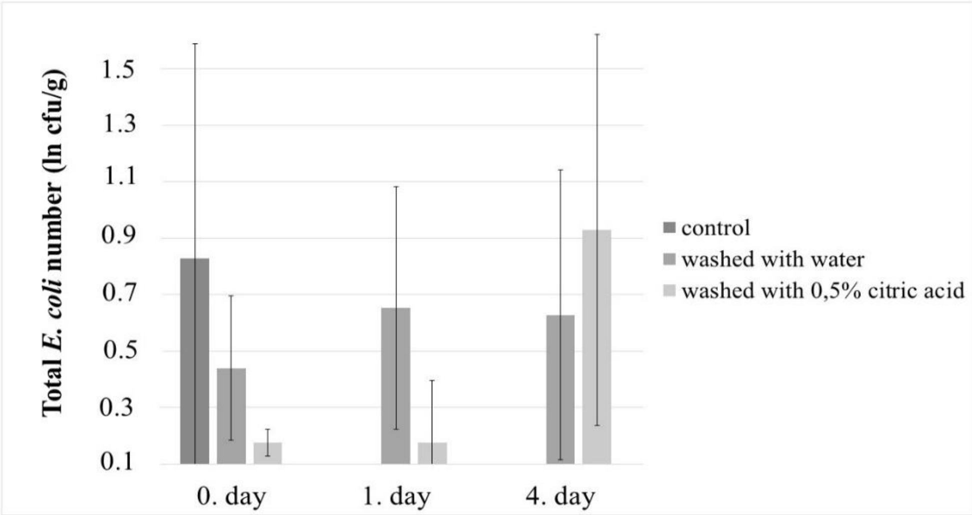
contamination did not changed following the first day in the water washed samples (*Figure 3*). *E. coli* number was reduced less than 1 log with up to 11 min treatment with 1% citric acid (Jeon and Ha,, 2020; Cho and Ha, 2021). However Finten et al. (2017) detected reduction (>1 log CFU cfu g^{-1}) following washing with 0.5% citric acid, up to 9 days storage.

Figure 3. **Total *E. coli* number of spinach following washing and storage at 12 °C**



Neither wash with water or with citric acid could eliminate the microbial contamination. The total plate count was higher, than the yeast and mold cfu.

The average SPAD value increased during the storage (Mann-Whitney U test, $p<0.05$) in both the control and the citric acid washed samples. It was higher in the fourth storage day ($p<0.01$) in the control unwashed, than in the washed samples. This parameter was the lowest in the water washed samples at the last storage day, indicating decreased relative chlorophyll content in the stored samples. The decrease was less following citric acid wash (*Table 1*). The SPAD measured chlorophyll content was significantly decreased at the third storage day in a previous study in lettuce and arugula (Cretescu et al., 2014), but the effect of washing was not reported previously.

Table 1. **Relative total chlorophyll content measured by SPAD**

Storage time (day)	SPAD value[1]		
	Control	Water washed	Washed with citric acid (0.5%)
0	37±7.0 ab		31±4.7 c
4	40±3.6 a	31±5.8 c	34±3.2 b

[1]Different letters indicate significant differences ($p<0.01$) with Mann-Whitney U test

The tensile strength, indicating the elasticity of the leaves decreased during the storage both in the non-washed sample and in the washed samples. The elasticity of the leaves was the lowest (LSD test, ANOVA $p<0.01$) at the water washed samples following 4-day storage, while less decrease of the citric acid treated samples was detected (*Table 2*). Previously washing with water or citric acid did not changed the hardness of spinach in combination with x-ray treatment without storage (Jeon and Ha, 2020).

Table 2. **Elasticity of leaves measured by Texture Analyzer**

Storage time (day)	Maximum puncture force (g)		
	Control	Water washed	Washed with citric acid (0.5%)
0	1251±241.8 a		
4	1090±335.5 ab	738±312.8 c	967±265.5 bc

[1]Different letters indicate significant differences with LSD test

CONCLUSIONS

Washing of spinach with 0.5% citric acid solution decreased the elasticity of the stored spinach leaves, and modified (decreased, $p<0.05$) the chlorophyll content. On the other hand, the total plate count and the yeast and mold count could be decreased with this treatment ($p<0.05$), but difference was not detectable at the forth storage day. The fecal indicator *E. coli* was detectable, and washing did not decreased their number significantly. Further optimization of treatment and storage conditions may decrease microbial risk of fresh spinach consumption without decreasing its sensory quality.

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REFERENCES

4/1998. (XI. 11.) EüM rendelet az élelmiszerekben előforduló mikrobiológiai szennyeződések megengedhető mértékéről.

Abadias, M.; Usall, J.; Anguera, M.; Solsona, C.; Viñas, I. (2008): Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. *Int. J. Food Microbiol.*, *123*, 121–129. doi:10.1016/j.ijfoodmicro.2007.12.013.

Al-Rousan, W.M.; Olaimat, A.N.; Osaili, T.M.; Al-Nabulsi, A.A.; Ayo, R.Y.; Holley, R.A. (2018): Use of acetic and citric acids to inhibit *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Staphylococcus aureus* in tabbouleh salad, Food *Microbiology, 73,* 61–66. https://doi.org/10.1016/j.fm.2018.01.001

CDC (2023): Centers for Disease Control and Prevention: Lettuce, other leafy greens and food safety. 2023. https://www.cdc.gov/foodsafety/communication/leafy-greens.html (Accessed: December, 2023)

Chazelas, E.; Deschasaux, M.; Srour, B.; Kesse-Guyot, E.; Julia, C.; Alles, B.; Druesne-Pecollo, N.; Galan, P.; Hercberg, S.; Latino-Martel, P.; Esseddik, Y.; Szabo, F.; Slamich, P.; Gigandet, S.; Touvier, M. (2020): Food additives: distribution and co-occurrence in 126,000 food products of the French market. *Scientific Reports, 10,* 3980. https://doi.org/10.1038/s41598-020-60948-w

Cho, G.-L.; Ha, J-W. (2021): Synergistic effect of citric acid and xenon light for inactivating foodborne pathogens on spinach leaves. *Food Res. International, 142*, 110210. https://doi.org/10.1016/j.foodres.2021.110210.

Cretescu, I.; Caprita, R.; Velicevici, G.; Camen, D.; Sirbu, C.; Buzamat, G.; Ropciuc, S. (2014): Researches regarding the influence of cold storage on the chlorophyll content in lettuce. *Scientific Papers Animal Science and Biotechnologies*, *47*, 162–162.

FAO (2023): https://www.fao.org/faostat/en/#data/QCL (Accessed: December, 2023)

Finten, G.; Agüero, M. V.; Jagus, R. J. (2017): Citric acid as alternative to sodium hypochlorite for washing and disinfection of experimentally-infected spinach leaves, *LWT- Food Science and Technology*, *82*, 318–325. http://dx.doi.org/10.1016/j.lwt.2017.04.047

Frommer, D.; Veres, Sz.; Radocz, L. (2019): Changes of relative chlorophyll content in sweet corn leaves of different ages infected by corn smut, *Agriculturae Conspectus Scientificus, 84* (2), 189–192.

Jeon, M-J.; Ha, J-W. (2020): Synergistic bactericidal effect and mechanism of X-ray irradiation and citric acid combination against food-borne pathogens on spinach leaves. *Food Microbiology*, *91*, 103543., https://doi.org/10.1016/j.fm.2020.103543.

Li, X.S.; Xue, J.Z.; Qi, Y.; Muhammad, I.; Wang, H.; Li, X.Y.; Luo, Y.J.; Zhu, D.M.; Gao, Y.H.; Kong, L.C.; Ma, H.X. (2023): Citric Acid Confers Broad Antibiotic Tolerance through Alteration of Bacterial Metabolism and Oxidative Stress. *International Journal of Molecular Sciences, 24,* 9089. https://doi.org/10.3390/ijms24109089

O'Neill, M.A.; York, W.S. (2001): *The Plant Cell Wall* (ed. J. K. C. Rose), Blackwell: Oxford. UK. pp. 1–54.

Premkumar, L. (2014): Fascinating Facts about Phytonutrients in Spices and Healthy Food. United States of America. Xlibris: Bloomington, Indiana, United States.

Roberts, J.L.; Moreau, R. (2016): Functional properties of spinach (*Spinacia oleracea* L.) phytochemicals and bioactives. *Food and Function*, 1–45.

USDA (2023): https://www.ers.usda.gov/webdocs/publications/106016/err-315.pdf?v=8253.6 (Accessed: December, 2023)

Yeni, F.; Yavas, S.; Alpas, H.; Soyer, Y. (2016): Most Common Foodborne Pathogens and Mycotoxins on Fresh Produce: A Review of Recent Outbreaks. *Food Science and Nutrition, 56,* 1532–1544.

Yossa, N.; Patel, J.; Millner, P.; Lo, Y.M. (2012): Essential oils reduce *Escherichia coli* O157:H7 and *Salmonella* on spinach leaves. *J Food Prot. 75,* 488–96. doi: 10.4315/0362-028X.JFP-11-344.

Zhou, B.; Luo, Y.; Nou, X.; Mwangi, E.; Poverenov, E.; Rodov, V.; Demokritou, P.; Fonseca, J.M. (2023): Effects of a novel combination of gallic acid, hydrogen peroxide and lactic acid on pathogen inactivation and shelf-life of baby spinach. *Food Control*, *143*, 109284. https://doi.org/10.1016/j.foodcont.2022.109284.