International Journal of
Enteric Pathogens

Int J Enteric Pathog. 2020 February;8(1):8-14 Original Article

http://enterpathog.abzums.ac.ir doi 10.34172/ijep.2020.03

Effects of Citrus sinensis Essential Oil and Intrinsic and Extrinsic Factors on the Growth and Toxin-Producing Ability of *Clostridium botulinum* Type A

Adel Rezayi[1], Vadood Razavilar[1*], Zohreh Mashak[2], Amirali Anvar[1]

[1]Department of Food Hygiene, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2]Department of Food Hygiene, Karaj Branch, Islamic Azad University, Karaj, Iran

***Corresponding Author:**
Professor Vadood Razavilar, Department of Food Hygiene, Science and Research Branch, Islamic Azad University, Tehran, Iran, Tel: +989121026502
Email: V.razavi@ut.ac.ir

Published Online February 28, 2020

Keywords: *Citrus sinensis*, Nitrite, Nisin, NaCl, *Clostridium botulinum*

Abstract

Background: Considering the high fatality of botulism, the control of *Clostridium botulinum* and its neurotoxins has clinical importance. In this regard, using chemical preservatives, natural essential oils (Eos), and changes in the growth predisposing factors of bacteria are suitable methods to control the growth and toxin producing of *C. botulinum* in foods.

Objective: The current survey was done to assess the effects of *Citrus sinensis* EO and intrinsic and extrinsic factors on the growth and toxin producing of *C. botulinum* type A.

Materials and Methods: In this experiment with a factorial design, *C. sinensis* EO (0.0%, 0.015%, 0.03%, and 0.045%), nisin (0, 500, and 1500 IU/mL), nitrite (0, 20, and 60 ppm), pH (5.5 and 6.5), storage temperature (25 and 35°C), and sodium chloride (NaCl, 0.5% and 3%) were used to assess bacterial growth in the brain heart infusion medium. Finally, the mouse bioassay method was also used to assess toxicity.

Results: *Clostridium sinensis* EO with a concentration of 0.045%, as well as the reduction of pH and temperature could significantly delay the growth of bacteria ($P \leq 0.05$) in contrast to the use of NaCl and nisin alone. However, all concentrations of sodium chloride (NaCl), nisin, and *C. sinensis* EO (< 0.045%) in interaction with each other, especially in combination with nitrite, showed good synergistic effects.

Conclusion: These results suggested that using certain concentrations of *C. sinensis* EO and nisin, along with other suboptimal factors caused a significant decrease in the nitrite contents of foods with a significant reduction in the growth and toxin-producing ability of *C. botulinum*.

Received October 17, 2019; **Revised** November 23, 2019; **Accepted** November 28, 2019

Background

Botulinum toxin is recognized as a serious cause of food-borne botulism produced by the *Clostridium botulinum*.[1,2] This disease has high clinical and microbial importance due to the high mortality rate.[1,2] Thus, it is essential to decrease the risk of this toxin in diverse kinds of food samples.

Using high temperature, along with the boost concentrations of sodium chloride (NaCl) and chemical preservatives such as nitrite is a traditional method for decreasing the risk of *C. botulinum* in foodstuffs.[3] Nitrite and its precursor, nitrate, are known to positively affect the flavor, appearance, quality, and safety of cured meat. In particular, nitrite can guarantee the safety of food products through inhibiting the growth of microorganisms, especially *C. botulinum*.[4]

Nevertheless, concerns are growing with respect to the risks of nitrite application in meat products, including the formation of nitrosamines (i.e., mutagenic and carcinogenic compounds).[5] The use of Hurdle technology is a possible approach for decreasing the concentrations of nitrite in foodstuffs, along with other processing technologies and compounds.[6]

In addition, nisin is a small and stable peptide with antimicrobial effects on different Gram-positive bacteria and the only authorized bacteriocin used as a food preservative.[7] In Hurdle technology, this peptide can be used effectively as a natural preservative.[6] Further, natural essential oils (EOs) are the mixtures of different extracted chemicals from the plants. Their relatively low cost, antimicrobial activities, low toxicity, and natural origin make them as a suitable option as food preservatives.[8]

Citrus sinensis, also known as orange, is an ancient medicinal plant with highly beneficial and therapeutic activities.[9,10] Citrus EOs have potential antimicrobial properties against spore-forming and food poisoning bacteria and fungi. Moreover, *C. sinensis* EOs and their major components are generally recognized as safe in

the food industry. Several studies have also reviewed the antioxidant and antimutagenic properties of orange Eos.[9,10]

Considering the above-mentioned explanation, this study aimed to determine and compare the effects of nitrite, nisin, and *C. sinensis* (sweet orange) EO alone and in combination with each other, affected by different levels of pH, NaCl concentration, and temperature on growth and toxin-producing ability of *C. botulinum* type A.

Materials and Methods

Plant Materials

The *C. sinensis* cv. *Valencia* fruits were obtained from Ramsar (Mazandaran, Iran) in May 2017 and identified at Citrus Research Institute of Iran. A Clevenger apparatus was used to expose fresh orange peels to hydro-distillation for 3 hours.[11] Then, gas chromatography (Agilent Technologies, USA) was carried out to determine EO composition. The procedure was done on an ionization energy of 70 eV, followed by performing mass spectrometry in the electron ionization mode.

Nisin

Nisin was purchased from Sigma Aldrich (Sigma, USA) Company (containing 2.5% active nisin). To prepare a nisin concentration of 10 000 IU/mL, nisin (1 g) was added to a 100-mL volumetric flask, followed by adding 0.02 M hydrochloric acid to reach 100 mL. Additionally, to separate insoluble whey proteins, the solution was centrifuged for 15 minutes at 5000×g. Afterward, it was sterilized via filtration through a 0.22-µm disposal and nonpyrogenic syringe filter (Millipore, Bedford, MA). The stock solution was diluted in sterilized 0.02 N HCl during the experiments to obtain nisin working solutions of 500 and 1500 IU/mL, respectively.[12,13]

Nitrite

Sodium nitrite was purchased from Merck Company (Millipore, Germany).

Test Organism

In this study, *C. botulinum* type A (RS5) was provided by the Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

Experimental Design

The experiment with a factorial design included different *C. sinensis* EO concentrations (i.e., 0.0, 0.015%, 0.03%, and 0.045%), nisin contents (0, 500, and 1500 IU/mL), nitrite contents (0, 20, and 60 ppm), pH values (5.5 and 6.5), storage temperatures (25 and 35°C), and NaCl concentrations (0.5% and 3%). It was carried out to study the growth of *C. botulinum* type A in the brain heart infusion (BHI) medium over 32 days (time to detection).

Inoculum Preparation

After three consecutive passages of lyophilized bacteria in the BHI broth (Merck Millipore, Germany), *C. botulinum* type A spores were cultured in the egg yolk agar (HiMedia, India) and BHI agar (Merck Millipore, Germany) via surface plating in an anaerobic jar (HiMedia, India) containing gas packs and an indicator strip (Merck Millipore, Germany) and then incubated at 35°C for 3 weeks. Next, the colonies were washed and gathered with the phosphate buffer at a pH of seven using the sweeping method. After the centrifugation of spores containing suspensions for 15 minutes at 5000×g, they were washed with sterile water and suspended in 50% ethanol (Merck Company, Germany). Finally, the spores were determined via plating on the BHI agar and stored at 4°C until further use.[14,15]

Broth Substrate Preparation

BHI powder was dissolved in distilled water inside a screw-capped flask (250 mL) through mild heating. Considering the experimental design, salt (NaCl, Merck Millipore, Germany) and sodium nitrite were added in different quantities. During the study (32 days), dimethyl sulfoxide (DMSO 5% (v/v), Merck Millipore, Germany) and 0.05% (w/v) agar-agar (Merck Millipore, Germany) were added to the broth substrate as the emulsifier and stabilizer, respectively. Furthermore, DMSO and agar-agar were added to combinations without EO (0.0%) at the same quantities to consider possible effects on the growth of the bacteria.[16]

Then, HCl (1N, Merck, Germany) was used to adjust the pH in the combinations (6.5 and 5.5) and a pH meter (PHS, 3e, China) was used for pH measurements. After adding the flask content into screw-capped tubes (9 mL per tube), it was autoclaved for 15 minutes at 121°C. The aliquots of nisin concentrations (stock solution), as well as sterilized EOs were added to the tubes after cooling in order to prepare final concentrations (active nisin concentrations). No changes were reported after the re-measurement of the pH in the final combinations (broth).

Inoculation and Storage of Broth Substrate

The spore suspension (1 mL) was inoculated into the substrate, which was collected in the previous stage. The final concentration of the spores of the substrate was equal to 4×10^4 CFU/mL. To prepare anaerobic conditions, 1 mL of sterilized paraffin was added to each tube and the inoculated tubes were finally stored at 25°C and 35°C for 32 days. Visible growth (turbidity) was monitored at 5 intervals (i.e., 2, 4, 8, 16, and 32) and time was recorded during the experiments.

Toxin Detection by Mouse Bioassay

Mouse bioassay was used for toxin detection according to the Food and Drug Administration protocol. This method is considered the gold standard due to high sensitivity in toxin detection (0.01 ng/mL).[17,18] After removing the turbid tubes from the incubators, cold centrifugation was

carried out for 15 minutes at 7000×g. Next, supernatant liquids were frozen at -20°C until injection into 6-8 weeks old mice weighing 18-25 g. The frozen samples were placed at room temperature for half an hour before use. Then, 2 mL of each tube sample was transferred to another tube and heated at 100°C in a water bath for 10 minutes. One mouse from each pair was used for intraperitoneal injection of 0.5 mL of an unheated fluid while the other mouse received 0.5 mL of the heated fluid. Eventually, the mice were monitored for the signs of botulism for 48 hours.[14,18]

Statistical Analysis

The present study evaluated the main and interactive effects of EO, nitrite, and nisin, with respect to salt concentration, pH, and temperature, on the growth and toxin-producing ability of *C. botulinum* using the analysis of variance test in SPSS for Windows (version 21.0, SPSS Inc.) and $P< 0.05$ was considered as a significant level.

Results

Table 1 signifies the chemical composition of EO (Gas chromatography-mass spectrometry results). Limonene is recognized as the main EO component (92.85%).

Table 2 presents data related to the effects of nitrite, nisin, and *C. sinensis* EO (separately or in combination), with respect to salt concentration and temperature, on growth time and turbidity. Based on the results, the growth of bacteria was strongly affected by pH reduction. More precisely, no turbidity was observed in the tubes within 32 days, and growth was completely inhibited with a pH reduction from 6.5 to 5.5. Decreasing temperature from 35 to 25°C also delayed the growth time significantly ($P \leq 0.05$). On the other hand, increasing salt concentration from 0.5% to 3% alone did not cause a major difference in the time of turbidity ($P \leq 0.05$). However, when 3% salt concentration was associated with other preservatives, especially nitrite, it could delay the growth of bacteria for a longer time.

According to the results (Table 2), the evaluated concentrations of nisin (500 and 1500 IU/mL) alone had no significant effects on the prevention of the growth of *C. botulinum* type A ($P \leq 0.05$) although a good synergistic effect was found in interaction with nitrite and EO. Then, the effect of increasing concentration from 500 to 1500 IU/mL was determined when nisin was associated with certain concentrations of EO and nitrite.

Figure 1 displays the turbidity time of *C. botulinum* type A with the inoculum level of 4×10^4 CFU/mL during 32 days storage in the BHI broth as affected by salt, temperature, and minimum preservative values at pH 6.5.

Figure 2 depicts the turbidity time (day) of *C. botulinum*

Table 1. Essential Oil Composition of *Citrus. sinensis* cv. *Valencia* Identified by GC-MS

Compound	Percentage
α-pinene	0.93
Sabinene	0.42
β-myrcene	2.32
Limonene	92.85
Deconol	0.4
Linalol	0.28
Sum	97.2

Note. GC-MS: Gas chromatography-mass spectrometry.

Table 2. Turbidity Time (Day) of *C. botulinum* Type A With the Inoculum Level of 4×10^4 CFU/mL During 32 Days Storage in the BHI Broth as Affected by Temperature and Preservatives at pH Value of 6.5

EO (%)	Nitrite (ppm)	Nisin (IU)	Salt 0.5%		Salt 3%	
			25 °C	35 °C	25 °C	35 °C
0	0	0	4	2	4	2
		500	4	2	4	2
		1500	4	2	4	2
	20	0	4	2	4	2
		500	4	2	4	4
		1500	8	4	8	4
	60	0	8	4	16	8
		500	8	4	16	16
		1500	16	8	32	16
0.015	0	0	4	2	4	2
		500	4	2	4	2
		1500	4	2	8	4
	20	0	4	2	8	4
		500	8	2	16	4
		1500	8	4	16	8
	60	0	16	8	16	16
		500	16	8	32	16
		1500	32	8	32	16
0.03	0	0	4	2	4	2
		500	4	2	8	4
		1500	8	4	8	8
	20	0	8	4	16	8
		500	16	8	16	16
		1500	32	16	32<	32
	60	0	32	16	32<	32
		500	32<	32	32<	32<
		1500	32<	32<	32<	32<
0.045	0	0	8	4	8	4
		500	8	4	8	8
		1500	16	8	16	8
	20	0	16	16	16	16
		500	16	16	32<	32
		1500	32<	32	32<	32<
	60	0	32<	32	32<	32<
		500	32<	32<	32<	32<
		1500	32<	32<	32<	32<

Note. BHI: Brain heart infusion; EO: Essential oil; *C. botulinum: Clostridium botulinum*. *There was no growth in the pH of 5.5 in all tubes until after day 32 (32<) thus its information is not presented in the table.

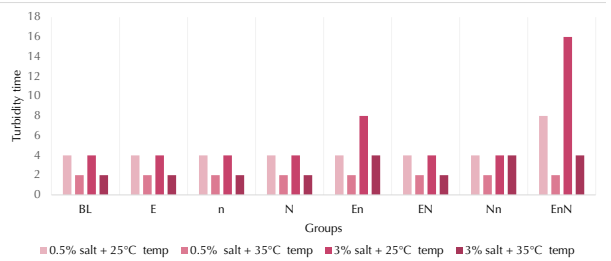

Figure 1. Turbidity Time (day) of *C. botulinum* Type A With the Inoculum Level of 4×10⁴ CFU/mL During 32 Days Storage in the BHI Broth as Affected by Salt, Temperature, and Minimum Preservative Values at pH 6.5.
Note. BL: Blank; E: Essential oil 0.015%; n: Sodium nitrite 20 ppm; N: Nisin 500 IU; En: Essential oil and nitrite; EN: Essential oil and Nisin; EnN: Essential oil, nitrite, and Nisin.

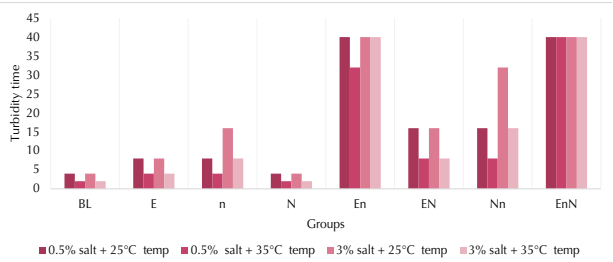

Figure 2. Turbidity Time (day) of *C. botulinum* Type A With the Inoculum Level of 4×10⁴ CFU/mL During 32 Days Storage in the BHI Broth as Affected by Salt, Temperature, and Maximum Preservative Values at pH 6.5.
Note. BL: Blank; E: Essential oil 0.045%; n: Sodium nitrite 60 ppm; N: Nisin 1500 IU; En: Essential oil and nitrite; EN: Essential oil and Nisin; EnN: Essential oil, nitrite, and Nisin.

type A with the inoculum level of 4×10⁴ CFU/mL during 32 days storage in the BHI broth as affected by salt, temperature, and maximum preservative values at pH 6.5. The present results indicated that 20 ppm of sodium nitrite alone could not prevent the germination and growth of bacteria, but the growth of bacteria was obviously postponed by increasing the concentration of nitrite to 60 ppm. However, the synergistic effect of nitrite with salt, nisin, and *C. sinensis* EO was also significant ($P \leq 0.05$). According to the results, concentrations ≤ 0.03% of *C. sinensis* EO did not influence the growth time of *C. botulinum* type A. By increasing EO concentration to 0.045%, bacterial growth occurred with a significant delay compared to lower concentrations ($P \leq 0.05$). Nonetheless, the subinhibitory concentrations of *C. sinensis* EO, especially 0.03% concentration, in combination with other inhibitory agents, showed good synergistic effects.

In this study, despite the use of moderate concentrations of preservatives and synergistic effects, the growth of *C. botulinum* type A was completely inhibited during 32 days of the study in certain combinations.

The mice receiving non-heated samples died within 48 hours after injection, showing botulism symptoms such as muscle weakness, fuzzy hair, and respiratory failure (wasp-like narrowed waist) although all those mice, which had received heated specimens, survived, therefore, factors used in this study prevented toxin formation by inhibiting bacterial growth.

Discussion

The properties of EOs, including flavor and antimicrobial activities, are directly related to their chemical constituents. Limonene is the main component of *C. sinensis* EO, and myrcene, alpha and beta pinene, and sabinene are among the other major constituents.[19-22] In our study, β-myrcene and limonene were recognized as the main components of *C. sinensis* EO, which is consistent with the results of the mentioned studies. Many factors such as genetic variations, climate, postharvest factors (e.g., storage conditions), and extraction methods can affect the chemistry of Eos.[23]

Previous studies examined the effects of *Citrus* EOs on food spoilage organisms and food-borne pathogens. For instance, Settanni et al evaluated the antimicrobial activity of some *Citrus* EOs via hydro-distillation against *Staphylococcus aureus, Salmonella enteric,* and *Listeria monocytogenes*. In their study, greater effectiveness against Gram-positive bacteria was found in most EOs compared to *Salmonella*. This finding can be attributed to the resistant outer membrane of Gram-negative bacteria.[24] In this regard, Randazzo et al found that the incorporation of lemon EOs in chitosan films could be effective in controlling *L. monocytogenes*, particularly under refrigerated applied conditions.[25]

However, few studies examined the effects of EOs, especially *C. sinensis* EO, on *Clostridium botulinum*. For example, Ismaiel and Pierson evaluated the effects of thyme, clove, pimenta, black pepper, Origanum, onion, garlic, and cinnamon oil on the germination and growth of *C. botulinum* types A, B, and E. At 150 and 200 ppm, all oils completely prevented germination and the spores of type A were more sensitive than those of types B and E.[26]

In another study, Nevas et al examined the antibacterial effects of 13 Finnish EOs against 12 bacterial strains. *C. botulinum* types B and E, besides *Clostridium perfringens,* were the most sensitive bacteria. They concluded that due to the sensitivity of *C. botulinum* to EOs, it can be used to reduce the concentration of nitrite in meat products.[27] Similarly, Chaibi et al studied the antibacterial activity of 9 EOs, prepared by hydro-distillation, on the vegetative cells and spores of *Bacillus cereus* and *C. botulinum* 62A. The spores showed less resistance compared to vegetative cells. *Bacillus* spores were more sensitive than that of clostridium. Orange EO had bacteriostatic effects and did not show bacteriocidal properties at evaluated concentrations.[28]

In our study, *C. sinensis* EO with a concentration of 0.045% caused a major delay in the growth of *C. botulinum*

type A at both temperatures and salt concentrations. However, the EO could not prevent bacterial growth for more than eight days. Considering the effect of salt concentration and pH on *C. botuli*num, Lalitha and Gopakumar found that the spores of *C. botulinum* types A and B could grow in cooked meat at 30°C and produce toxins up to 8% salt. They further reported that the lag phase and time to toxicity increased by decreasing the storage temperature. Overall, salt tolerance decreased by decreasing pH and storage temperature.[29]

Likewise, Jensen et al investigated the growth probability of proteolytic *C. botulinum* at different temperatures. The log percent probability of growth was 2 at 20°C and 30°C and 2.7 at 37 °C [30]. In addition, Baker et al concluded that in vacuum packaging at 30, 20, 16, and 8°C, toxin-producing ability occurred on days 0.5, 1, 2, and 9 in *C. botulinum* type E, respectively. Contrarily, under MA packaging, no toxicity was observed at 4°C even after storage for 60 days.[31]

In the present study, decreasing temperature from 35 to 25°C led to a delay in growth and toxin-producing ability. Further, the pH reduction from 6.5 to 5.5 in all studied conditions completely prevented *C. botulinum* growth and toxin-producing ability, which indicates the higher importance of pH in controlling food-borne botulism compared to the other factors. However, many studies showed that pH less than 5 and even less than 4.8 is needed for the effective prevention of *C. botulinum* growth. This difference in pH between our study and some other studies can be related to differences in the inoculum dose and bacterial strain.

Based on the results of our study, when salt (sodium chloride) was used alone, no significant difference was found between 0.5% and 3% salts ($P \leq 0.05$). It cannot be concluded that an increase in salt concentration does not have any effects on the growth of *C. botulinum*. However, it can be interpreted that both concentrations of the salt are inadequate to prevent growth.

The increase in salt concentration, when accompanied by an increase in other preservatives, especially nitrite, had a significant effect on postponing growth and toxin-producing ability. These findings are consistent with those of the study by Meng et al. They demonstrated that increasing the concentration of sodium chloride from 1% to 2%, accompanied by an increase in sodium lactate and a reduction in temperature, had beneficial effects on delaying toxin-producing ability.[32]

Regarding the effects of nisin on *C. botulinum*, Scott and Taylor stated that nisin could be a proper alternative to nitrite. However, more nisin is needed in processed meat probably owing to nisin binding to meat particles.[33] On the other hand, Okereke and Montville concluded that nisin alone cannot guarantee the health of food, but works well with other inhibitors such as sodium chloride.[34] According to a study by Khanipour et al, potassium sorbate, salt, and nisin exhibited major inhibitory effects on the growth of *C. sporogenes* (nontoxigenic surrogates of *C. botulinum*) at high pH (>4.5) and high moisture (>95%). The combinations of preservatives were found to be more effective than their independent applications.[35] Moreover, in a study by Zhang et al, the synergistic effects of D-limonene nano-emulsion with nisin were confirmed against four food microorganisms.[36]

In the present study, nisin was unable to prevent *C. botulinum* growth at the concentrations of 500 and 1500 IU/mL whereas, in combination with salt, *C. sinensis* EO, and nitrite, good synergistic effects were reported in this regard. The results also showed the synergistic effect of nitrite and EO on the test organisms. These findings are in line with those of the study by Cui et al, which used a combination of sodium nitrite and spice extracts, including sage, cloves, and nutmeg. The antibotulinal efficacy of this combination could reduce the amount of nitrite in the meat model system to approximately 10 ppm.[37]

Conclusion

In meat curing, nitrite is described as a multifunctional food additive. However, there is growing concern over the carcinogenic effects of nitrite in humans. One approach is to use low concentrations of sodium nitrite, along with other natural compounds which are acceptable to food organizations and consumers. The findings of this study showed that by using certain concentrations of *C. sinensis* EO and nisin, along with other suboptimal factors, the nitrite quantity needed to control the growth of *Clostridium botulinum* could be reduced to a satisfactory level. However, the efficacy of this complex in the food matrix should be considered in terms of antimicrobial properties and other nitrite functions.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

Ethical Approval

The experiment was conducted according to the protocol approved by the Islamic Azad University, Science and Research Branch, Tehran, Iran.

Authors' Contributions

All authors contributed equally in this article, especially in designing, laboratory analysis, statistical analysis, and manuscript writing.

Financial Support

This research was supported by the Islamic Azad University, Science and Research Branch, Tehran, Iran.

References

1. Johnson EA. Control of Clostridium botulinum in foods. In: Villa TG, Vinas M, eds. New Weapons to Control Bacterial Growth. Cham: Springer International Publishing; 2016:83-93. doi:10.1007/978-3-319-28368-5_4
2. Kull S, Schulz KM, Weisemann J, et al. Isolation and functional

characterization of the novel *Clostridium botulinum* neurotoxin A8 subtype. PLoS One. 2015;10(2):e0116381. doi:10.1371/journal.pone.0116381
3. Glass KA, Johnson EA. Formulating low-acid foods for botulinal safety. New York: Marcel Dekker; 2001:323-350.
4. Bedale W, Sindelar JJ, Milkowski AL. Dietary nitrate and nitrite: benefits, risks, and evolving perceptions. Meat Sci. 2016;120:85-92. doi:10.1016/j.meatsci.2016.03.009
5. Dutra MP, Aleixo GC, Ramos ALS, et al. Use of gamma radiation on control of *Clostridium botulinum* in mortadella formulated with different nitrite levels. Radiat Phys Chem. 2016;119:125-129. doi:10.1016/j.radphyschem.2015.10.008
6. Alahakoon AU, Jayasena DD, Ramachandra S, Jo C. Alternatives to nitrite in processed meat: up to date. Trends Food Sci Technol. 2015;45(1):37-49. doi:10.1016/j.tifs.2015.05.008
7. Hatziioanou D, Gherghisan-Filip C, Saalbach G, et al. Discovery of a novel lantibiotic nisin O from *Blautia obeum* A2-162, isolated from the human gastrointestinal tract. Microbiology. 2017;163(9):1292-1305. doi:10.1099/mic.0.000515
8. Hąc-Wydro K, Flasiński M, Romańczuk K. Essential oils as food eco-preservatives: model system studies on the effect of temperature on limonene antibacterial activity. Food Chem. 2017;235:127-135. doi:10.1016/j.foodchem.2017.05.051
9. Fisher K, Phillips CA. The effect of lemon, orange and bergamot essential oils and their components on the survival of *Campylobacter jejuni, Escherichia coli* O157*, Listeria monocytogenes, Bacillus cereus and Staphylococcus aureus* in vitro and in food systems. J Appl Microbiol. 2006;101(6):1232-1240. doi:10.1111/j.1365-2672.2006.03035.x
10. Toscano-Garibay JD, Arriaga-Alba M, Sánchez-Navarrete J, et al. Antimutagenic and antioxidant activity of the essential oils of *Citrus sinensis* and *Citrus latifolia*. Sci Rep. 2017;7(1):11479. doi:10.1038/s41598-017-11818-5
11. Ferhat MA, Meklati BY, Chemat F. Comparison of different isolation methods of essential oil from Citrus fruits: cold pressing, hydrodistillation and microwave 'dry' distillation. Flavour Fragr J. 2007;22(6):494-504. doi:10.1002/ffj.1829
12. Neetoo H, Ye M, Chen H, Joerger RD, Hicks DT, Hoover DG. Use of nisin-coated plastic films to control *Listeria monocytogenes* on vacuum-packaged cold-smoked salmon. Int J Food Microbiol. 2008;122(1-2):8-15. doi:10.1016/j.ijfoodmicro.2007.11.043
13. Wan Norhana MN, Poole SE, Deeth HC, Dykes GA. Effects of nisin, EDTA and salts of organic acids on *Listeria monocytogenes*, *Salmonella* and native microflora on fresh vacuum packaged shrimps stored at 4 degrees C. Food Microbiol. 2012;31(1):43-50. doi:10.1016/j.fm.2012.01.007
14. Mashak Z, Razavilar V. Growth and toxicity of clostridium botulinum type A influenced by changes in pH, salt, heat and storage time in the broth model. Comp Pathobiol. 2005;2:33-44.
15. Whiting RC, Strobaugh TP. Expansion of the time-to-turbidity model for proteolytic *Clostridium botulinum* to include spore numbers. Food Microbiol. 1998;15(4):449-453. doi:10.1006/fmic.1998.0196
16. Misaghi A, Basti AA. Effects of Zataria multiflora Boiss. essential oil and nisin on *Bacillus cereus* ATCC 11778. Food Control. 2007;18(9):1043-1049. doi:10.1016/j.foodcont.2006.06.010
17. Bigalke H, Rummel A. Botulinum neurotoxins: qualitative and quantitative analysis using the mouse phrenic nerve hemidiaphragm assay (MPN). Toxins (Basel). 2015;7(12):4895-4905. doi:10.3390/toxins7124855
18. Sharma SK, Whiting RC. Methods for detection of *Clostridium botulinum* toxin in foods. J Food Prot. 2005;68(6):1256-1263. doi:10.4315/0362-028x-68.6.1256
19. Aberoomand Azar P, Nekoei M, Larijani K, Bahraminasab S. Chemical composition of the essential oils of *Citrus sinensis* cv. *Valencia* and a quantitative structure-retention relationship study for the prediction of retention indices by multiple linear regression. J Serb Chem Soc. 2011;76(12):1627-1637. doi:10.2298/jsc101218141a
20. Ferhat MA, Meklati BY, Smadja J, Chemat F. An improved microwave Clevenger apparatus for distillation of essential oils from orange peel. J Chromatogr A. 2006;1112(1-2):121-126. doi:10.1016/j.chroma.2005.12.030
21. Hosni K, Zahed N, Chrif R, et al. Composition of peel essential oils from four selected Tunisian *Citrus* species: evidence for the genotypic influence. Food Chem. 2010;123(4):1098-1104. doi:10.1016/j.foodchem.2010.05.068
22. Minh Tu NT, Thanh LX, Une A, Ukeda H, Sawamura M. Volatile constituents of *Vietnamese pummelo*, orange, tangerine and lime peel oils. Flavour Fragr J. 2002;17(3):169-174. doi:10.1002/ffj.1076
23. Raut JS, Karuppayil SM. A status review on the medicinal properties of essential oils. Ind Crops Prod. 2014;62:250-264. doi:10.1016/j.indcrop.2014.05.055
24. Settanni L, Palazzolo E, Guarrasi V, et al. Inhibition of foodborne pathogen bacteria by essential oils extracted from citrus fruits cultivated in Sicily. Food Control. 2012;26(2):326-330. doi:10.1016/j.foodcont.2012.01.050
25. Randazzo W, Jiménez-Belenguer A, Settanni L, et al. Antilisterial effect of citrus essential oils and their performance in edible film formulations. Food Control. 2016;59:750-758. doi:10.1016/j.foodcont.2015.06.057
26. Ismaiel AA, Pierson MD. Effect of sodium nitrite and origanum oil on growth and toxin production of *Clostridium botulinum* in TYG broth and ground pork. J Food Prot. 1990;53(11):958-960. doi:10.4315/0362-028x-53.11.958
27. Nevas M, Korhonen AR, Lindström M, Turkki P, Korkeala H. Antibacterial efficiency of Finnish spice essential oils against pathogenic and spoilage bacteria. J Food Prot. 2004;67(1):199-202. doi:10.4315/0362-028x-67.1.199
28. Chaibi A, Ababouch LH, Belasri K, Boucetta S, Busta FF. Inhibition of germination and vegetative growth of *Bacillus cereus* T and *Clostridium botulinum* 62A spores by essential oils. Food Microbiol. 1997;14(2):161-174. doi:10.1006/fmic.1996.0075
29. Lalitha KV, Gopakumar K. Combined effect of sodium chloride, pH and storage temperature on growth and toxin production by *Clostridium botulinum*. J Aquat Food Prod Technol. 2007;16(2):27-39. doi:10.1300/J030v16n02_04
30. Jensen MJ, Genigeorgis C, Lindroth S. Probability of growth of *Clostridium botulinum* as affected by strain, cell, and serologic type, inoculum size and temperature and time of incubation in a model broth system. J Food Saf. 1986;8(2):109-126. doi:10.1111/j.1745-4565.1986.tb00557.x
31. Baker DA, Genigeorgis C, Glover J, Razavilar V. Growth and toxigenesis of C. botulinum type E in fishes packaged under modified atmospheres. Int J Food Microbiol. 1990;10(3-4):269-289. doi:10.1016/0168-1605(90)90075-g
32. Meng J, Genigeorgis CA. Modeling lag phase of nonproteolytic *Clostridium botulinum* toxigenesis in cooked Turkey and chicken breast as affected by temperature, sodium lactate, sodium chloride and spore inoculum. Int J Food Microbiol.

1993;19(2):109-122. doi:10.1016/0168-1605(93)90177-i
33. Scott VN, Taylor SL. Temperature, pH, and spore load effects on the ability of nisin to prevent the outgrowth of *Clostridium botulinum* spores. J Food Sci. 1981;46(1):121-126. doi:10.1111/j.1365-2621.1981.tb14544.x
34. Okereke A, Montville TJ. Bacteriocin-mediated inhibition of *Clostridium botulinum* spores by lactic acid bacteria at refrigeration and abuse temperatures. Appl Environ Microbiol. 1991;57(12):3423-3428.
35. Khanipour E, Flint SH, McCarthy OJ, et al. Modelling the combined effects of salt, sorbic acid and nisin on the probability of growth of *Clostridium sporogenes* in a controlled environment (nutrient broth). Food Control. 2016;62:32-43. doi:10.1016/j.foodcont.2015.10.012
36. Zhang Z, Vriesekoop F, Yuan Q, Liang H. Effects of nisin on the antimicrobial activity of d-limonene and its nanoemulsion. Food Chem. 2014;150:307-312. doi:10.1016/j.foodchem.2013.10.160
37. Cui H, Gabriel AA, Nakano H. Antimicrobial efficacies of plant extracts and sodium nitrite against *Clostridium botulinum*. Food Control. 2010;21(7):1030-1036. doi:10.1016/j.foodcont.2009.12.023