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Effect of antimicrobials on decontamination efficiency and growth potential of enteric pathogens on fresh-cut vegetables

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Abstract
The study describes the inherent difference among fresh cut (minimally processed) vegetables in acquiring the enteric pathogen population after an *in vitro* challenge test, decontamination efficiency of different sanitizers and the growth potential (δ) of survivor populations of enteric pathogens on minimally processed of forms four different vegetables viz. spear mint leaves, cilantro leaves, cucumber and capsicum after a sanitization process. The sanitizers used were chlorine (100 mg/L free available chlorine), Neutral Electrolysed water (NEW, 100 mg/L Free available chlorine), nisin (20 mg/L), lactic acid (2500 mg/L), citric acid (5000 mg/L), the dose of which were selected based on visual observation on phytotoxic effects due to higher levels of usage. The study revealed a significant difference among minimally processed vegetables to harbour the enteric pathogens even at the same inoculum exposure. After in vitro challenge with equal amount of inoculum load, spearmint leaves harboured significantly low levels of pathogenic bacteria viz., *Listeria monocytogenes*, *Salmonella enterica and Escherichia coli*, and did not show positive growth potential during storage at low temperature. Among the sanitizers tested, NEW (100 mg/L) was most efficient in decontamination of enteric pathogens and to prevent the further growth of the pathogens during storage of the vegetables.

Keywords: *Listeria monocytogenes*, sanitizer, fresh-cut, *Salmonella*, *E. coli*

1. Introduction
Minimally processed or fresh cut vegetables (FCVs) business sector has gained importance in the past decade due to increased demand arising from urban lifestyle, improved cold storage facilities and logistics. Besides control of spoilage organisms, the control of pathogens is also very important in FCVs. A number of disease outbreaks are linked to the consumption of FCVs, most of which are linked to contamination with *Salmonella*, *Escherichia coli*, and *Listeria monocytogenes* (Machado-Moreira *et al*., 2019) [9]. Decontamination of FCVs is a critical unit operation to reduce the microbial risk to improve the microbiological safety of FCVs (Joshi *et al*, 2013; Roseberg *et al*., 2021) [7]. Sodium hypochlorite with @ 50-200 ppm free available chlorine (FAC) is most widely used for fresh and fresh-cut produce sanitization. However, chlorine-based compounds are corrosive, cause respiratory tract and skin irritation and react with organic matter present in the water. Reaction of chlorine with organic matter results in formation of carcinogenic compounds called trihalomethanes, due to which, it's use in FC vegetable sanitization is prohibited in European Union countries (Gil *et al*., 2009) [16]. As an alternative, chemicals with no proven toxicity and zero-residue status are tested constantly for their use as sanitizers. Among such compounds, organic acids, bacteriocins, electrolysed water etc., showed encouraging results on decontamination efficiency in various studies. Yoon & Lee (2018) [14] have reviewed the decontamination efficiency of various antimicrobial agents on fresh fruits and vegetables; and concluded that a total surface bacterial population in them may be reduced by a level of 1.12, 1.74, 1.87 and 3.01 log CFU/g using dip treatment with sodium hypochlorite, organic acids, hydrogen peroxide and neutral electrolysed water respectively. The limitation to achieve a higher decontamination level is the phytotoxic symptoms and/ or regulatory issues associated with higher doses of the above disinfection agents.
Most of the previous studies on the use of sanitizers have not investigated the potential risk of microbial survival and proliferation during storage of the FCVs after their treatment with the sanitizers. The knowledge on regrowth or decline of survivor population on food products are an important consideration in the microbiological risk assessment.

Thus, it becomes important to look for decontamination strategies that prevent the resuscitation and re-growth of the pathogen during storage of the fresh-cut produce. This study was aimed at addressing this issue by evaluating the decontamination efficiency of sanitizers against three major food pathogens *viz*. *E. coli*, *Salmonella and Listeria monocytogenes* and their resuscitation in different FC salad vegetables to result in population increase during storage.

2. Materials and Methods

2.1 Vegetables

Freshly harvested green capsicum, cucumber, cilantro leaves and mint leaves were procured from the local market and cleaned with potable water. The vegetables were then minimally processed to suit their use in cuisines. Capsicum was cut into 2 cm × 2 cm sized pieces, cucumber to 3-4 mm sized discs and the compound leaves of the leafy greens (Cilantro and mint leaves) were separated from the stem. These prepared vegetables were used as matrices for carrying out spiking studies with the pathogens and their response to sanitization and population changes during storage with a modification of a method given by Sant'Ana *et al.* (2012) [12], as detailed below.

2.2 Inoculum preparation

Log phase cultures of the enteric pathogens *viz*., *Salmonella enterica* (MTCC 3225, 3218 and 3224), *Listeria monocytogenes* (MTCC 657, 839 and 657) and *Escherichia coli* (MTCC 9537, 1610 and NCIM 43888) were raised in Brain Heart Infusion broth. The cells were harvested by centrifugation at 9500 rpm for five minutes, and resuspended in 0.85% saline solution. The microbial population in each case was adjusted to approximately 2 $\times 10^{12}$ CFU/mL by optical measurement method using a DensiCHEK™ Plus (Biomereiux®) based on manufacturer's instructions. These cultures were then stored at 4 ℃ for three days further use in spiking studies. Meanwhile, the population of the pathogens in the saline stock culture was validated through plating on Trypicase soya agar. A measured quantity of inoculum from each strain were transferred to a single to obtain a cocktail of strains of the single species and then mixed well. The inoculum cocktail was transferred to 500 mL sterile water in a beaker to obtain a population level of $1X10^9$ CFU/mL and was used for spiking the vegetables.

2.3. *In vitro* challenge (spiking) of FC vegetables with enteric pathogens

The plant materials prepared as mentioned under Section 2.1. were dipped for 15 minutes in the inoculum prepared as per the procedure mentioned under section 2.2. The vegetables after spiking with the mixed inoculum were surface dried. All the operations related to spiking and surface drying were carried out in class II type B2 Biological safety cabinet (Model: Esco Lab culture).

2.4 Sanitization of *in vitro* challenged vegetables

Vegetables spiked with enteric pathogens prepared under Section 2.3, were sanitized using different sanitizers. The sanitizers used were as follows: sodium hypochlorite (100 mg/L Free available chlorine), Neutral electrolysed water (100 mg/L free available chlorine), lactic acid (2500 mg/L), citric acid (5000 g/L), hydrogen peroxide (2500 mg/L) and nisin (20 mg/L). These concentrations were selected based on their maximum permitted levels and observations from a preliminary study on FCV decay due to the phytotoxicity effects at higher doses. Shortly, during the preliminary studies, it was observed that treatment with >100 mg/L sodium hypochlorite imparted off odour to the vegetables. The GMP chemicals *viz*. lactic acid and citric acid at higher concentrations than mentioned above caused faster spoilage in the cut vegetables by causing sogginess, compared to untreated vegetables. Treatment with Neutral electrolysed water at concentrations higher than 100 mg/L caused browning of tissues

The sanitizer solutions were prepared in sterile beakers and the spiked vegetables were dipped in the solutions for five minutes. The spiked vegetables were then surface dried in a biological safety cabinet. A similar dip treatment in water served as control. Fresh- cut vegetables were then packed @ 25 g/pack in 25µm thick polypropylene bags with 10 pinholes. Pinholes were made to prevent build-up of in pack carbon dioxide as a result of the respiration by vegetable tissues. All samples were stored at 8 ℃ and six replications of each treatment were maintained.

2.5. Enumeration of enteric pathogen population present on vegetables after spiking and sanitization

Quantification of the pathogens from the same sample was done thrice *viz.*, immediately after spiking (*in vitro* challenging) with pathogens, immediately after decontamination step, and on the fifth day of storage. The population estimation was done by serial dilution and plating method on selective agar medium. For this, the contents of the packages were transferred to sterile stomacher bags and blended at 300 rpm for 30 seconds in a stomacher. Serial dilution was followed, and plating was done in two different selective media for each organism. Quantification of *Salmonella* was done on Brilliant green agar and Xylose Lysine Deoxycholate agar in replicates and the mean values of the number of typical colonies were estimated. Population of *Listeria monocytogenes* was analysed using PALCALM agar and MOX agar and the average colony count was taken. *Escherichia coli* population was estimated using Eosin Methylene Blue agar and Tryptone Bile Glucuronic agar, and the average of the typical colonies were calculated.

2.6. Calculation of decontamination efficiency and growth potential

Decontamination efficiency (log 10 CFU/g) = Population after spiking (log CFU/g) – Population after sanitizer treatment log CFU/g

Growth potential (δ) = log (Survivor population on Day 1)– log (Survivor population Day 5)

3. Results and Discussion

3.1. Population of enteric pathogens after *in vitro* challenge test on various vegetables

Spiking resulted in the adherence of pathogens on the FC vegetables. The population observed after spiking through dip treatment was lowest in spear mint leaves (1.11 to 3.43 log CFU/g), and was highest in capsicum (3.64-6.2 log CFU/g) (Fig 1). This shows the preferential attachment of the pathogens on certain vegetables. The leafy greens (Cilantro and spear mint) used in the study harboured less population compared to the fruit vegetables (capsicum and cucumber). The differences among minimally processed vegetables in

terms of their ability to support the enteric pathogens on different plants surfaces has been reported (Beuchat, 2002; Deering *et al*, 2012, Cui *et al*, 2017) [3, 5, 4]. Relatively low level of the enteric pathogen population on mint and Cilantro leaves may be partly explained by lower level of tissue damage in them during minimal processing compared to the extensive tissue injury in capsicum and cucumber. The fruit vegetables used in this study have undergone higher extent of tissue damage due to peeling and cutting unit operations in their preparation steps, and the wounding damage in leafy greens were limited to the tender stem. Spear mint (*Mentha spicata*) and cilantro leaves possess high antibacterial activity due to the presence of essential oil components (Takeuchi *et al*., 2000, Foudah *et al*., 2021) [13, 6]. These factors also would have contributed additionally to the less adherence of viable bacteria in mint leaves after *in vitro* challenge test. The results obtained in this study clearly indicate the differential vulnerability of the vegetables to become carriers of human enteric pathogens and thus the importance of requirement in commodity-based risk assessment strategies in fresh-cut produce. It can also be inferred from the study that, mint leaves showed highest resilience among the vegetables tested, in preventing the adhesion of enteric pathogens.

Among the three pathogens tested, the population of *E. coli* was highest in all FCVs spiked, while the lowest population was observed in case of *L. monocytogenes* (Fig. 1). This indicates the higher risk of transmission of *E. coli* than Salmonella and *L monocytogenes* through vegetables. It has been earlier reported that ability of pathogens to attach to fresh produce depends on intrinsic and extrinsic factors including motility of the pathogens, their interaction with other organisms and nutrient molecules leaching out from the plant tissue (Aruscavage *et al*., 2006, Lim *et al*, 2014) [1, 8].

3.2. Decontamination efficiency of sanitizers

3.2.1 *Listeria monocytogenes*

The decontamination efficiency measured in terms of reduction in log CFU/g and survival of *Listeria monocytogenes* in the FC vegetables is given in Fig 2 a. Sanitizers selected in this study evidently improved the decontamination efficiency against *L. monocytogenes*. Highest decontamination efficiency was noticed in the treatment involving NEW treatment, which ranged from 3.85 log CFU/g to 3.08 log CFU/g, followed by nisin treatment (1.24 – 2.6 log CFU/g). Organic acids were far less effective than even sodium hypochlorite. Survival and growth of *L. monocytogens* during storage of these vegetables is given in Table 1. It was observed in this study that, FC vegetables treated with nisin showed highest negative growth potential (δ) of the organisms in treated samples, ranging from -1.29 to - 2.99 in different vegetables. Thus, nisin treatment prevented the proliferation of *L. monocytogenes* which resulted in maintaining the population as low as that of the NEW treatment by end of the storage period. Thus, nisin is found to be the best sanitizer among those tested in this study to prevent the proliferation of *L. monocytogenes* in fresh- cut vegetables.

3.2.2 *Salmonella*

The data on decontamination efficiency and survival of *Salmonella enterica* in the FC vegetables is presented in Fig 2b and Table 2, respectively. Dip treatment with 100 ppm NEW eliminated the population completely in all the three vegetables throughout their storage period, showing the strong negative growth potential as due to NEW treatment.

3.2.3. *E. coli*

Spiking with *E. coli* resulted in a population ranging from 3.42 -6.01 log CFU/g in various vegetables (Table 3). The effect of sanitizers was less on *E. coli* compared to the other two pathogens used in this study (Fig 2c). Highest reduction in population was obtained by using 100 ppm NEW, followed by 100 ppm chlorine. Reduction in population was 1.7-1.85 log due to NEW treatment, which was identified as the best treatment in different vegetables in the study.

The present observations on decontamination efficiency of FC vegetables emphasizes the usefulness of NEW as a sanitizer for the fresh cut industry. Electrolyzed water, also known as electrochemically activated solution, is one class of emerging disinfectants that functions as a broad-spectrum, aqueous chemical oxidant. Electrolyzed water has been evaluated for several food safety industrial applications, including use as a fresh produce wash as well as for cleaning and disinfection in dairy manufacturing clean-in-place systems. The antimicrobial efficacy of electrolyzed water is attributed to pH, chlorine and oxidation-reduction potential (ORP). Manipulating the pH-dependent aqueous chemistry of electrolyzed water to a near neutral pH ensures that the $HOCl^{-}$ molecule predominates. Neutral electrolyzed water (NEW; pH 7) has been shown to be effective in reducing or eliminating bacterial pathogens and cultivable human NoV surrogates, e.g., murine norovirus (Moorman *et al*., 2017) [10]. Current study too proves the effectiveness of NEW as a sanitizer for fresh- cut vegetables.

3.3 Growth potential of pathogens on minimally processed vegetables

Growth potential (δ) is defined as the difference between the population of a microorganism at the end of shelf-life of specific food and its initial population. The growth potential determination of *Salmonella*, *Listeria monocytogenes* and *E. coli* in ready- to-eat vegetables can be very useful to envisage likely threats to food safety. Values of ≥ 0.5 $\log_{10}$ is considered as positive growth potential, while a value of ≤ -0.5 $\log_{10}$ is considered as a negative growth potential (Sant'Ana *et al*., 2012, Noor *et al*., 2015) [12, 2]. This study reported a clear cut positive growth potential for *Listeria*, *Salmonella* and *E. coli* in capsicum in undipped control and water dipped samples, where no antimicrobial sanitizers were used. Growth potential of *Salmonella* spp. and *Listeria monocytogenes* in nine types of ready-to-eat vegetables stored at variable temperatures during shelf-life was assessed by Sant'Ana *et al*., (2012) [12] who reported that *L. monocytogenes* was able to grow ($\delta \geq 0.5$ log 10), had higher growth potential than *Salmonella* during storage of collard green, arugula, cabbage, spinach, water cress etc.

Though the decontamination efficiency of nisin treatment on *Salmonella a*nd *E coli* was less in comparison with NEW treatment, the growth potential of these two organisms was lowest in nisin treated samples, resulting in reduction of the these pathogens to non-detectable levels at the end of storage in capsicum and cilantro leaves. In cucumber too, a very high negative growth potential was observed due to nisin treatment. Considering the ability of NEW and nisin in decontamination and to suppress the proliferation of survivor population after decontamination process, these two antimicrobials may be considered as futuristic antimicrobials for FC vegetable sanitization process.

Table 1: Change in population and growth potential of *Listeria monocytogenes* on FC vegetables after sanitization

Treatments	Capsicum			Cucumber			Cilantro leaves			Spear mint leaves		
	Day 1	Day 5	Growth potential (δ)	Day 1	Day 5	Growth potential (δ)	Day 1	Day 5	Growth potential (δ)	Day 1	Day 5	Growth potential (δ)
Undipped (Control)	6.20±0.34	6.8±0.02	0.6	5.98±0.81	5.32±0.24	-0.66	4.23±0.13	4.04±0.35	-0.23	2.1	ND	>-2.1
Water	5.91±0.34	5.98±0.38	0.07	5.36±0.12	4.90±0.21	-0.46	4.00±0.98	3.68±0.29	-0.38	1.2	ND	>-1.2
Nisin (20 mg/L)	4.23±0.10	2.34±0.22	-1.89	3.38±0.71	2.08±0.32	-1.30	2.99±0.01	N.D	>2.99	ND	ND	NA
(Chlorine (100 mg/L)	5.37±0.40	2.65±0.31	-2.72	5.19±0.12	4.07±0.33	-1.12	4.69±1.62	3.69±0.23	-1.00	ND	ND	NA
NEW (100 mg/L)	2.79±0.21	1.57±0.04	-1.22	2.13±0.36	0.22±0.00	-1.91	1.15±0.14	N D	>1.15	ND	ND	NA
Lactic acid (2500 mg/L)	5.98±0.81	6.01±0.23	-0.33	4.01±0.41	3.97±0.61	-0.03	3.53±0.22	3.12±0.54	-0.32	ND	ND	NA
Citric acid 5000 mg/L)	5.93±0.23	5.88±0.21	0.05	4.98±0.38	4.45±0.24	-0.53	3.92±0.31	3.82±0.33	-0.10	ND	ND	NA

ND: Not detectable, NA: Not applicable (Since the Day 1 population was not detectable after the sanitizer treatment)

Table 2: Change in population and growth potential of *Salmonella enterica* on FC vegetables after sanitization

Treatments	Capsicum			Cucumber			Cilantro leaves			Spearmint leaves		
	Day 1	Day 5	Growth potential (δ)	Day 1	Day 5	Growth potential (δ)	Day 1	Day 5	Growth potential (δ)	Day 1	Day 5	Growth potential ((δ)
Undipped (Control)	3.64±0.06	4.12±0.4	0.58	3.40±0.19	1.23±0.23	-2.17	3.22±0.38	1.72±0.25	-1.5	1.1	ND	NA
water	3.34±0.02	3.61±0.10	0.27	3.21±0.04	1.39±0.05	-1.82	2.69±0.28	1.52±0.38	-1.17	ND	ND	NA
Nisin (20 mg/L)	2.69±0.12	ND	>-2.69	2.84±0.25	0.39±0.00	-2.45	2.50±0.10	ND	>-2.5	ND	ND	NA
Chlorine (100 mg/L)	2.60±0.10	2.25±0.20	-0.35	3.29±0.01	0.87±0.48	-2.32	0.53±0.00	ND	>0.53	ND	ND	NA
N.E.W (100 mg/L)	N.D.	ND	NA	ND	ND	NA	N.D.	ND	NA	ND	ND	NA
Citric acid (5000 mg/L	3.2±0.08	3.1±0.04	-0.1	2.57±0.33	1.52±0.09	-1.05	1.88±0.22	1.01±0.13	-0.87	ND	ND	NA
Lactic acid (2500 mg/L)	3.8±0.2	3.62 ±0. 2	-0.2	2.99±0.28	1.68±0.16	-1.21	2.36±0.15	1.95±0.25	-0.35	ND	ND	NA

ND: Not detectable; NA: Not applicable (Since the Day 1 population was not detectable after the sanitizer treatment)

Table 3: Change in population and growth potential of *Escherichia coli* on FC vegetables after sanitization

Treatments	Capsicum			Cucumber			Cilantro leaves			Spear mint leaves		
	Day 1	Day 5	Growth potential (δ)	Day 1	Day 5	Growth potential (δ)	Day 1	Day 5	Growth potential (δ)	Day 1	Day 5	Growth potential (δ)
Undipped (Control)	5.83±0.21	6.1±0.04	0.27	6.01±0.22	6.11±0.22	0.11	5.17±0.12	5.27±0.10	0.10	3.42±0.09	1.5	-1.92
Water	5.24±0.01	5.27±0.03	0.03	5.81±0.22	5.87±0.22	0.06	5.08±0.22	5.29±0.21	0.21	2.93±0.10	0.54	-2.39
Nisin 20 mg/L	4.75±0.21	5.18±0.09	-0.43	4.62±0.40	4.87±0.45	0.25	4.20±0.29	3.13±0.19	-1.07	1.96±0.04	ND	>-1.96
Chlorine 100 mg/L	4.65±0.06	5.09±0.12	0.44	3.91±0.39	3.85±0.54	-0.06	4.88±0.25	4.19±0.15	-0.69	1.73±0.17	ND	>-1.73
N.E.W 100 mg/L	4.23±0.06	3.87±0.04	-0.36	4.67±0.49	4.01±0.21	-0.66	3.32±0.12	2.80±0.02	-0.52	1.28±0.06	ND	>-1.28
Lactic acid 2500 mg/L	5.09±0.56	5.29±0.09	0.2	5.23±0.40	5.19±0.41	0.04	4.20±0.29	3.13±0.19	-0.27	2.51±0.04	ND	>-2.51
Citric acid 5000 mg/L	5.30±0.09	5.18±0.15	-0.12	5.26±0.40	5.07±0.40	-0.19	4.20±0.29	3.13±0.19	-0.29	2.59±0.21	ND	>-2.59

ND: Not detectable

4. Conclusion

The study proved the inherent difference among the vegetables in acquiring and supporting pathogen growth at the same level of inoculum exposure. Spear mint leaves acquired very limited population of the pathogens, and did not support the pathogen growth at all during storage. Present study showed that NEW (100 ppm) can be efficiently used for decontamination of the food borne pathogens as well as to curb the growth of enteric pathogens in FC vegetables during low temperature storage. Nisin was effective in preventing the further growth of the pathogens during the storage of minimally processed vegetables. Among the three pathogens tested, *E. coli* was found to be more capable of surviving on the vegetable surface after *in vitro* challenge test, and the growth potential of this pathogen was affected at a lesser magnitude due to the various disinfectants tested. It was also found that the commonly used sanitizer, sodium hypochlorite also appreciably reduces the growth potential of the pathogens, while the organic acids like citric acid and lactic acid showed weak disinfection property, though they were able to reduce the growth potential of the pathogens during storage of the vegetables.

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