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Isolation of pathogenic microorganisms from fresh fruits and screening the efficacy of different disinfectant solution against the pathogens

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ABSTRACT

Fruits are important parts of our daily diet to maintain a healthy life as they have potential nutritional values. But unfortunately, a number of foodborne diseases have been noticed due to the consumption of raw and contaminated fruits. The present study was carried out to isolate the pathogenic microorganisms from fruits sample and to determine the effects of some household cleaning methods including washing and various common chemical treatments for the removal of bacterial load. A conventional spread plate technique was performed for the detection of bacteria. Total heterotrophic bacteria, *Escherichia coli*, *Staphylococcus* spp. *Salmonella* spp. and *Listeria* spp. were found in 30 samples of 6 categories including Java apple (*Syzygium cumini*), Carambola (*Averrhoa carambola*), Indian gooseberry (*Phyllanthus emblica*), Olive (*Olea europaea*) Koromcha (*Carissa carandas*) and Pear (*Pyrus*). Tap water, hot water (50°C), 100 mg/L sodium hypochlorite (NaOCl), 50 mg/L calcium lactate, 4% acetic acid and 2 ml/L CleanAva were used as decontaminating agents. All samples were soaked in tested cleaning agents for 20 min at room temperature. All the tested solutions were found to be effective and reduced bacterial loads in fruits compared to the unwashed fruits samples ($p<0.01$). It was revealed, NaOCl, calcium lactate, acetic acid and CleanAva were more effective cleaning agents than water wash. Two to three log of bacterial load was reduced when samples were subjected to treat with decontaminating agents. Potable water, the types and concentration of the disinfectant solutions are important parameters for effective washing.

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1. Introduction

Fresh fruits and vegetables are essential parts of healty diet because fresh products are a prominent source of nutrients, different types of vitamins and minerals (1-3). Phytochemical and other nutrients may lost during cooking or other processes, but consumption of raw fruits can retain the complete phytonutrient for instances phenolic, flavonoid, carotenoid, glucosinolates, terpenoid and triterpenes (4). Phytochemical and phytohormones of fruits haveP strong antimicrobial activity. Additionally, fruits have been regarded as microbiologically safer than other unprocessed food items.

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However, to prevent the acute foodborne illness associated with pathogen and chemical pesticide, appropriate washing procedure and hygiene practice are still required before consuming fruits (5-6).

Earlier study showed that fresh produce can be contaminated by microorganisms anywhere from agricultural land to table. Good agriculture practice (GAP) is crucial as microbiological quality and safety of fresh produce largely depend on cultivation and harvesting methods (7,8). Contamination can take place during pre-harvest, at the time of harvesting through faecal material, human handling, harvesting tools and at the post-harvest stage by peeling, trimming, slicing, packaging, transport containers vehicles, ice and water. Improper storage methods (e.g., temperature, atmosphere, and pressure) and ice or water etc. and distribution system are also responsible for contamination (4-6,13). Plant cell has cell wall which acts as a protective barrier against microorganisms. However, any kind of injury of cell wall allow pathogen to grow inside plant cell and the availability of nutrients enhance the spoilage rate (12). Generally, foodborne infection occurs after consuming fruits containing pathogens such as *Escherichia coli*, *Salmonella* spp. *Shigella* spp, *Clotridium perfringens, Vibrio* spp., *Listeria monocytogens*, *Bacillus cereus* etc. (14-16). Viruses are eminent source of spoilage of fruits and vegetables. Norovirus and hepatitis A virus are the most common viral food contaminants (17).

Worldwide, decontamination of fresh fruits is a matter of concern. New methods and safe disinfecting agents are introducing for the elimination of pathogenic microorganisms and pesticides from fresh produce (18). The most common household processes such as washing with tap water are not proven to be enough for the complete removal of microorganisms. Washing fresh produces with water removes sand, soil, and other debris but unable to remove microorganisms completely. Consumers generally choose natural preservatives for lessening the growth of foodborne pathogens in food (19). Any antimicrobial agent needs to be passed toxicological issue and approved before use. A variety of techniques have been used to decontaminate fresh produce from muds, insects, microorganisms and chemical fertilizer (20). Application The application of effective antimicrobial treatments can help to prevent the transfer of contamination further. A wide number of sodium and calciumbased decontaminating agents are popular for their antimicrobial activity. Chlorine containing water is a common treatment method for fresh produce at ranges from 50 to 200 ppm (21). At pH 6 to 7.5, optimum concentration of hypochlorous acid and hypochlorite provides the highest antimicrobial activity. Plasmolysis of the microbial cell occurs when NaOCl react with the cellular enzymes of bacteria and thus leading to the death of the microbes (22). Organic acids are available and easy to use but sometimes cause discoloration because of their acidic properties. Vinegar contains strong acetic acid which passes through the cell membrane causes acidification of cell organelles resulting in bacterial cell death (23-25). Calcium based sanitizers have proved their efficiency against microorganisms (26).

Nowadays, some commercial decontaminating agents are available in super shops and they can thoroughly eliminate microorganisms from fruits. Hence, this present study was designed to point out the presence of the pathogen in different types of raw fruits and to explore the effectiveness of sanitizer (CleanAva), acetic acid, tap water, hot water, NaOCl and calcium lactate as a cleaning agent.

2. Materials and Methods

2.1. Sample collection

For the isolation of microorganisms, total 30 fruits samples of 6 categories including Java apple (*Syzygium cumini*); Carambola (*Averrhoa carambola*), Indian gooseberry (*Phyllanthus emblica*) Olive (*Olea europaea*), Koromcha (*Carissa carandas*) and Pear (*Pyrus*) were collected from the super shops in Dhaka city between December 2020 to February 2021. Samples were collected aseptically in a sterile bag and transported to the laboratory immediately for microbiological analysis (27).

2.2. Sample processing

All tested samples were chopped and 20 g sample was added to sterile conical flask containing 180 mL distilled water. After ten-fold serial dilution with normal saline, samples spread on agar plates for enumeration of bacterial colonies. Then the same samples were soaked with different agents such as tap water, hot water (50°C), 100 mg/L sodium hypochlorite (NaOCl), 50 mg/L calcium-lactate ($C_6H_{10}CaO_6$), 4% acetic acid and 2 ml/L CleanAva composed of alkyl polysaccharide) for 20 min at room) temperature (28). After soaking samples were subjected to microbiological analysis.

2.3. Enumeration of total viable bacteria

For the enumeration of total viable bacteria (TVB) 0.1 mL of each sample were spread onto Nutrient agar (NA) and plates were incubated at 37°C for 24 h (29).

2.4. Isolation of pathogenic bacterial load

For the isolation and identification of Coliforms and fecal Coliforms, 0.1 mL suspension were spread over MacConkey agar and Membrane fecal Coliform (MFC) agar. For Coliform, plates were incubated at 37° C for 18- 24 h. The presence of *E. coli* was further confirmed by the green metallic sheen colonies on eosin-methylene blue (EMB) agar. For fecal Coliforms, plates were incubated at 44.5°C for 24 h (30). On the other hand, for the isolation of *Salmonella* spp., *Shigella* spp., 0.1 mL samples were inoculated on Xylose Lysine Deoxycholate (XLD) agar while the presence of *Vibrio* spp., *Staphylococcus* spp., and *Listeria* spp. was calculated by spreading 0.1 mL of dilute sample onto Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar, Mannitol salt agar (MSA) and *Listeria* agar, respectively. All the plates were incubated at 37°C for 24 h. After incubation characteristic colonies were counted (31-33).

2.5. Statistical Analysis

For performing statistical analysis, http://www.socscistatistics.com online software was used. Using T-test calculator for two independent means samples were analyzed by two tailed hypothesis at significance level 0.01.

3. Results

3.1. Enumeration of bacterial load without wash

Table 1 shows the total bacterial cell count in unwashed fruits samples. The highest and lowest counts of total heterotrophic bacteria in unwashed fruits were 7.84 ±0.47 and 7.11±0.31 log 10 cfu/g in Java apple and Olive, respectively. The highest and lowest count of total *Escherichia coli* was recorded 6.91±0.28 and 6.21 ±0.71 log 10 cfu/g in Indian gooseberry and Pear, constitutively. *Staphylococcus* spp., *Salmonella* spp. and *Listeria* spp. were also found in all untreated fruits and their range was between 6.57±0.66 to 3.13±0.83 log 10 cfu/g. *Klebsiella* spp., *Vibrio* spp., *Shigella* spp. and fecal coliform were absent in all tested samples (Table 1).

3.2. Effect of cleaning agents on total viable bacteria

After washing with hot water (HW) and NaOCl, total viable bacteria exhibited 2 log reduction (p<0.01) compared to the unwashed sample. One log reduction was noted after washing with tap water (TW). On the contrary, 2 to 4 log bacterial reduction was observed when the samples were treated with calcium lactate, acetic acid and commercial cleaning agent CleanAva, and bacterial count was ranged between 4.50±0.35 to 2.55±0.60 log10 cfu/g (p<0.01). Acetic acid and CleanAva showed comparatively better efficacy by reducing a significant bacterial load (Table 2). All the experiments have been done three times and one representative data have been shown.

3.3. Effects of cleaning agent on *Escherichia coli* populations

When fruits were washed with tap water the highest and lowest load of *E.coli* was found in Carambola (5.47 ±0.28 log 10 cfu/g) and Koromcha (5.16±0.45 log 10 cfu/g) respectively. After treated with HW (50°C) and NaOCl the lowest count was recorded in Olive (4.10 ±0.40) and Carambola (2.75±0.28) log 10 cfu/g, respectively (p<0.01). A remarkable bacterial load reduction was noticed when fruits samples were cleaned with calcium lactate, acetic acid and CleanAva. In case of calcium lactate treatment, the lowest count was documented in Koromcha (2.23±0.37 log 10 cfu/g). After treatment with acetic acid and CleanAva, no growth was observed in java apple, Indian gooseberry, Olive, Koromcha and Pear (Table 3).

Table 1. Bacteriological analysis of fresh fruits without wash (WW)

Fruits (n=30)	Bacterial load (log_{10} cfu/g ± SD)				
	TVC	***Escherichia coli***	***Staphylococcus* spp.**	***Salmonella* spp.**	***Listeria* spp.**
Java apple	7.84±0.47	6.86±0.46	6.20±0.13	6.38±0.78	3.84±0.86
Carambola	7.44±0.37	6.31±0.68	6.57±0.66	5.27±0.59	3.57±1.37
Indian gooseberry	7.18±0.45	6.91±0.28	6.55±0.61	5.94±0.41	3.88±0.52
Olive	7.11±0.31	6.28±0.23	5.95±0.47	6.11±0.59	3.13±0.83
Koromcha	7.14±0.37	6.33±0.65	6.52±0.66	5.17±0.69	3.45±1.39
Pear	7.75±0.58	6.21±0.71	6.23±0.47	6.19±0.16	3.74±0.58

TVC: Total viable bacteria
All the experiments have been done three times and one representative data have been shown

Table 2. Numbers of total heterotrophic bacteria in fruits after washing with water and disinfectant solutions

	(Bacterial load (log_{10} cfu/g ± SD					
Fruits	**TW**	**HW**	**NaOCl**	**Calcium lactate**	**Acetic acid**	**CleanAva**
Java apple	6.33±0.55	4.68±0.62	4.89±0.55	2.79±0.55	3.88±0.52	2.81±0.97
Carambola	6.48±0.37	4.39±1.30	4.36±1.02	3.54±1.01	3.60±0.53	2.90±0.71
Indian gooseberry	6.34±0.51	4.00±0.85	3.13±0.83	3.32±1.55	3.41±0.99	2.75±0.50
Olive	6.01±0.67	3.98±0.82	3.99±0.63	4.50±0.35	3.98±0.82	2.55±0.60
koromcha	6.14±0.54	4.10±0.89	3.16±0.81	3.12±1.16	3.71±0.91	2.65±0.16
Pear	6.84±0.47	4.00±0.85	4.91±0.42	3.31±0.82	3.11±0.86	2.99±0.81

*TW:Tap water; HW:Hot water

Table 3. Counts of *E.coli* in fruits after washing with water and disinfectant solutions

	(Bacterial load (log_{10} cfu/g ± SD					
Fruits	**TW**	**HW**	**NaOCl**	**Calcium lactate**	**Acetic acid**	**CleanAva**
Java apple	5.33±0.37	4.34±0.85	3.84±0.86	3.40±0.89	1.13±0.85	NG
Carambola	5.47±0.28	4.83±0.49	2.75±0.50	2.47±0.48	1.16±0.87	NG
Indian gooseberry	5.26±0.30	4.85±0.57	2.99±0.81	2.39±0.71	NG	NG
Olive	5.39±0.45	4.10±0.40	2.89±1.00	2.54±0.54	NG	NG
koromcha	5.16±0.45	4.63±0.62	3.41±0.81	2.23±0.29	NG	NG
Pear	5.28±0.23	4.39±1.30	2.79±0.55	2.34±0.40	1.25±0.96	NG

*NG: No growth; TW: Tap water; HW: Hot water
All the experiments have been done three times and one representative data have been shown

3.4. Counts of *Staphylococcus* spp. in fruits after treatment

With tap water wash the lowest count of *Staphylococcus* spp was found in Koromcha (4.32±0.57 log 10 cfu/g). After treatment with HW (50°C) and NaOCl, the lowest bacterial load was recorded as 3.57 ±1.37 and 2.14±0.37 log10 cfu/g in olive and Koromcha, respectively (p<0.01). When these fruits samples were soaked in calcium lactate, highest count was noted in Carambola (2.84±0.36 log 10 cfu/g) while the lowest growth numberswas found as (2.17±0.12 log 10 cfu/g). Overall, 2 to 3 log reduction of Staphylococcal (p<0.01) count took place in all fruits. Acetic acid and CleanAva completely reduce *Staphylococcus* spp. from all samples (Table 4).

3.5. Enumeration of *Salmonella* spp. after treatment Primarily, with tap water wash, the highest load of *Salmonella* spp. was found in Olive (4.63±0.44 log 10 cfu/g). The lowest load was recorded as 2.12±0.55 and 2.11±1.89 log 10 cfu/g in Olive after washing with HW (50°C) and NaOCl, respectively (p<0.01) and 2 log deduction was exhibited in other samples. The effects of calcium lactate on *Salmonella* spp. was found to be quite similar to NaOCl. Two log population of *Salmonella* spp. was reduced after treating with calcium lactate and the lowest load was recorded in Carambola (2.14±0.54 log 10 cfu/g). Acetic acid was able to limit the growth of *Salmonella* spp. and no growth was observed in Indian gooseberry, Olive and Koromcha. However, no growth of *Salmonella* spp. was found when fruits were washed with CleanAva (Table 5).

Counts of *Listeria* spp. in fruits after treatment: The .3.6 highest load of *Listeria* spp. was observed in Indian gooseberry (3.88±0.52 log 10 cfu/g) and lowest load was recorded in Olive (3.13±0.83 log 10 cfu/g) before wash. After washing with TW and HW (50°C), the lowest bacterial load was documented in Olive (2.13 ±0.83) and Carambola (1.10±0.22) log 10 cfu/g, respectively (p<0.01). All the samples exhibited no growth on *Listeria* agar after treated with NaOCl, calcium lactate, acetic acid and CleanAva. Overall, 3 log reduction of *Listeria* count took place in all fruits samples (p<0.01) (Table 6).

4. Discussion

The Centers for Disease Control and Prevention (CDC) has reported the incidence of globally 250 foodborne diseases due to bacteria, virus, protozoa and toxic chemicals (CDC, 2018). Use of insecticides in agricultural fields resulting in the occurrence of biomagnification of hazardous chemical elements in soil, water, air, as well as on the surface of crops and vegetables (34).

Some pesticides are very good at pest control. However, continuous uses of insecticides accumulate in fresh produce and enter into our food chain (35,36). Fruits could be spoilage in several ways (Figure 1). Previous study demonstrated that contamination of fresh produce with *E. coli* and *Salmonella* spp. is commonly found in many samples (37).

Table 4. Numbers of *Staphylococcus* spp. in fruits after washing with water and disinfectant solutions bacterial load (log_{10} cfu/g ± SD)

Fruits	TW	HW	NaOCl	Calcium lactate	Acetic acid	CleanAva
Java apple	4.39±1.30	3.88±0.55	3.23±0.12	2.79±0.55	NG	NG
Carambola	4.74±0.58	3.84±0.86	2.88±0.39	2.84±0.36	NG	NG
Indian gooseberry	5.48±0.14	4.60±0.39	2.89±0.89	2.54±0.54	NG	NG
Olive	4.68±0.62	3.57±1.37	3.14±0.22	2.26±0.14	NG	NG
koromcha	4.32±0.57	3.88±0.86	2.14±0.37	2.17±0.12	NG	NG
Pear	4.49±0.36	3.13±1.26	3.64±0.29	2.18±0.17	NG	NG

*NG: No growth; TW: Tap water; HW: Hot water
All the experiments have been done three times and one representative data have been shown

Table 5. Counts of *Salmonella* spp. in fruits after washing with water and disinfectant solutions bacterial load (log_{10} cfu/g ± SD)

Fruits	TW	HW	NaOCl	Calcium lactate	Acetic acid	CleanAva
Java apple	4.34±0.85	3.63±0.21	2.63±0.21	2.27±0.28	1.25±0.96	NG
Carambola	4.36±1.02	2.76±0.57	2.90±0.22	2.14±0.54	1.80±1.61	NG
Indian gooseberry	4.15±0.44	3.33±0.12	2.34±0.51	2.64±0.55	NG	NG
Olive	4.63±0.46	2.12±0.55	2.11±1.89	2.21±0.11	NG	NG
koromcha	4.18±0.40	3.88±0.59	2.81±0.43	2.37±0.44	NG	NG
Pear	4.23±0.09	3.86±0.64	2.17±0.76	2.89±0.55	1.43±1.24	NG

*NG: No growth; TW: Tap water; HW: Hot water
All the experiments have been done three times and one representative data have been shown

Table 6. Counts of *Listeria* spp. in fruits after washing with water and disinfectant solutions

(Bacterial load (log_{10} cfu/g ± SD						
Fruits	**TW**	**HW**	**NaOCl**	**Calcium lactate**	**Acetic acid**	**CleanAva**
Java apple	2.84±0.45	1.73±0.21	NG	NG	NG	NG
Carambola	2.56±0.77	1.10±0.22	NG	NG	NG	NG
Indian gooseberry	2.81±0.52	2.17±0.51	NG	NG	NG	NG
Olive	2.13±0.83	1.71±1.89	NG	NG	NG	NG
koromcha	2.14±0.54	2.41±0.47	NG	NG	NG	NG
Pear	2.26±0.13	2.17±0.72	NG	NG	NG	NG

*NG: No growth; TW: Tap water; HW: Hot water
All the experiments have been done three times and one representative data have been shown

A variety of techniques have been used to decontaminate fresh produce from muds, insects, microorganisms and chemical fertilizer (38). In our study, we selected decontaminating agents of different pH range. Earlier studies reported that both alkaline and acidic pH are vulnerable for bacterial growth (21).

Hence, previous study supported our current study where it was observed that tap water wash is not effective to eliminate the load of microorganism and sometimes it could be the source of pathogenic microbes (17).

Hot water wash is less effective compared to sanitizer CleanAva significantly reduce the load of bacterial from all samples which indicates the importance of sanitizing agent for decontaminating fresh produce. EC Regulation 2073/2005 sets microbiological limits on different food categories (47). Moreover, the United States Food and Drug Administration (USFDA) provides guidelines for industry which concerning the microbiological quality of produce, namely, the "Guide to Minimize the Microbial Food Safety Hazards for Fresh-Cut Fruits and Vegetables" (48) and the "Guide to Minimize Microbial Food Safety Hazards of Leafy Greens" (49).

the different washing treatments, such as treated with NaOCl, calcium lactate, acetic acid and CleanAva. In the food processing area of different industry, 50 to 200 ppm concentrated sodium hypochlorite (NaOCl) solutions reported 2 to 3 log reductions of pathogenic bacteria for almost all samples when they were soaked with 100 mg/L NaOCl solutions (40).

Chlorine is relatively inexpensive and water soluble which increase its demand but formation of carcinogenic compounds like chloramines, trihalomethanes (THMs) and semicarbizides reduces its use (41). Another study on fresh-cut jackfruits proved that calcium lactate was effective in lowering bacterial and fungal populations by about 85% to 99.6% (40,42). Different studies showed the efficiency of vinegar (acetic acid) against pathogenic bacteria of fresh produce (43,44). *Salmonella* spp. and *E. coli* was commonly found in fresh produce and acetic acid has shown its activity against those bacteria. Two different studies reported that 4-5% acetic acid and lemon juice could effectively reduce the growth of bacteria (22,45). *Listeria monocytogenes* is able to grow at low temperature and can survive relatively low water activity (46). Previous report found 2.3 to 3 log reduction of *Listeria* spp. when samples were treated with 200 mg/L NaOCl and 1% acetic acid, constitutively. However, in our study complete reduction of *Listeria* spp. was noticed after washing with most of the sanitizers. The commercially available sanitizer CleanAva significantly reduce the load of bacterial from all samples which indicates the importance of sanitizing agent for decontaminating fresh produce. EC Regulation 2073/2005 sets microbiological limits on different food categories (47). Moreover, the United States Food and Drug Administration (USFDA) provides guidelines for industry which concerning

The microbiological quality of produce, namely, the "Guide to Minimize the Microbial Food Safety Hazards for Fresh-Cut Fruits and Vegetables" (48) and the "Guide to Minimize Microbial Food Safety Hazards of Leafy Greens" (49).

Proper cleansing of fresh produce depends on their composition, properties of pathogen, concentration of the antimicrobial agent, cleaning method and overall personnel hygiene. A large number of decontamination methods were developed and have been applied to improve the safety of fresh produce. However, in Bangladesh, most of the people still depends on only water wash which is not found to be effective. Additionally, if we consume carcinogenic agent and chemical residues along with the fruit it causes irreparable damage to our body. Nowadays people are more reliant on the organic solution rather than synthetic chemicals and sophisticated technology. Moreover, in developing countries like Bangladesh, where infrastructure and availability of resources are very weak, modern techniques might be difficult to establish by the government.

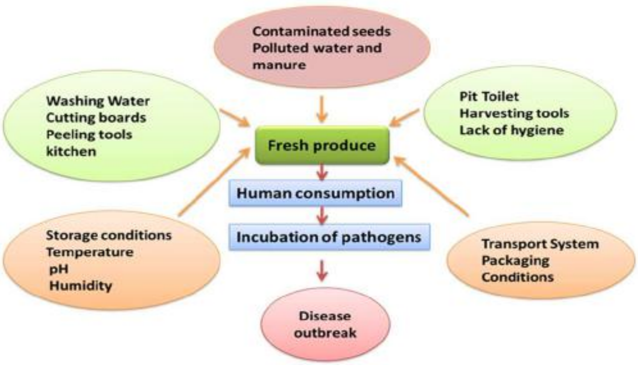

Figure 1. Microbial contamination of fresh produce in the farm-to-fork chain. This diagram represents potential contamination events in the different steps of the process. Contamination can take place during pre-harvesting, harvesting and post-harvesting period and which finally lead to an outbreak of disease.

5. Conclusion

Worldwide demand for fresh fruits is ever increasing for its beneficial role. However, contamination of fresh fruits is of great public health concern and measures must be taken to reduce such microbial proliferation. Only washing with tap water and hot water are not sufficient to reduce the bacterial load completely. Our study focused on some reliable and convenient cleaning agents for the decontamination of fruits items. Based on the results, it is concluded that sodium hypochlorite (NaOCl (100 mg/L), calcium lactate (50 mg/L), acetic acid (4%) and CleanAva (2 ml/L), especially the latter two were effective enough to inhibit foodborne pathogenic bacteria in fresh fruits over the conventional washing procedure.Therefore, it is suggested that the tested decontaminating agents have the potential to be used as a household washing solution that can assure the safety during consumption of fresh fruits.

Conflict of Interest

Author declare no conflict of interest.

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