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Complete microbiological analysis of citrus fruits and the effect of heat on microbial load & antimicrobial activity

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Citrus fruits are very popular both for raw consumption as well as juices, jam and jelly. Besides the nutritional properties, citrus fruits exhibit some antimicrobial properties by containing polymethoxylated flavones, flavonoids, steroids, saponins, alkaloids, reducing sugars, terpenoids etc. But sometimes such fruits can be contaminated with bacteria which find their ways in the consumers causing different disease conditions. The current study revealed the microbial load of Lemon, Lotkone, Orange, Malta and Amoloki and the study showed complete absence of *Klebsiella* spp. and *Escherichia coli*. The highest total viable bacterial and fungal count was 4.2×10^4 cfu/g and 2.0×10^5 cfu/g respectively. *Pseudomonas* spp. was the highest predominating bacteria with lower degree of contamination by *Listeria* spp. and *Staphylococcus* spp. Applying heat at 60 ^oC for 30 minutes, 1 hour and 2 hours proved the reduction of bacteria over time. Antibacterial activity after heat treatment for all cases (after 30 minutes, 1 hour and 2 hours). Antibacterial activity was lowest after 2 hours of heat treatment for amoloki and there was no such activity at all for Amra after 2 hours. Other citrus fruits surprisingly showed no antibacterial activity after heat treatment.

Key words: Citrus fruits, Microbial load, Antibacterial activity, Heat treatment

Citrus fruits possess microbiological contamination and contribute to an increase in the rate of infectious disease (1, 2). Common contaminants include *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Aeromonas* spp., *Staphylococcus* spp., *Streptococcus* spp., *Vibrio* spp., *Pseudomonas* spp., all of which serve as a threat to public health (2-4). Citrus fruits consists of various beneficial nutrient content such as minerals, energy, fiber content, ascorbic acid, folate, potassium, phytochemicals etc. (5-10) Different reports have verified the antimicrobial activity of fruits to be efficient against various infections including enteritis, arthritis, cardiac complications, etc. (3, 9-12).

Bacterial & Fungus may be transmitted through citrus fruits (13). Increase in popularity can also be attributed to government campaigns promoting the consumption of fruits and vegetables (14). Fruits play important roles against cardiovascular & cancerous disease (4). Fruits contain antioxidants which help protect cells and tissues from free radicals, while contributing to the proper functioning of DNA repair mechanisms, digestion, cell metabolism etc. (15). Countries such as Iran has seen an increase in the purchase of fresh fruits (16). Various European countries have also realized the same trend in consumption (3). The nutritional benefits account for majority of consumers relying on fruits as a source of vitamins (2). Even with all the benefits, factors such as mishandling during storage, transportation and insufficient preparation propagated the spread of food-borne diseases (16, 17). Use of non-performed medicines, inefficiencies in waste management, lack of studies in antibacterial treatment of foods and relatively poor management of healthcare system have contributed to the development of a major health care issue in the food chain (13-15, 17). Researchers and scientists have also applied several sterilization methods to reduce the microbial load such as pressure and heat treatments, which include hot water, vapor heat or hot air treatments (13).

Current study attempted to identify the microbiological loads of citrus fruits, collected from Dhaka City, Bangladesh and to elucidate the effect of heat on the microorganisms, in an attempt to set up heat as an operative, low cost and easy to use method of sterilization .The current study also describe the antimicrobial activity of the citrus fruits and whether heat will affect its actions.

MATERIALS AND METHODS

Samples. Six different raw citrus fruits (Lemon, Amoloki, Amra, Orange, Lotkone and Malta) were collected from public roadside markets early in the morning. All samples were instantly shifted to the Microbiological Laboratory at Stamford University, Bangladesh, Dhaka and subjected to microbiological investigation.

Total bacterial and fungal analysis. Total bacterial and fungal analysis was carried out as mentioned in Feroz et al. (18). Ten grams of the sample was homogenized for 1 min in sterilized 90 ml of physiological saline using a

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stomacher (Model No. 061-21001; Atect Co. Ltd, Japan). From this homogenate, serial dilutions were arrayed and surface plated (0.1 ml, in duplicate) on Nutrient Agar (NA) and Sabaroud Dextrose Agar (SDA). NA plates were incubated at 37 ^{o}C for 48 hours, while the SDA plates were incubated at 25 ^{o}C for 5 days. After incubation, the bacterial counts and fungal counts per gram were considered from NA plates and SDA plates, gradually (18-20).

Identification of coliform bacteria. From the dilution 10^{-2} & 10^{-3}, the 0.1ml sample was spread onto MacConkey agar for the detection of total coliform. The plates were incubated at 37 ^{o}C for 24 hours.

Identification of *Pseudomonas* spp. *and Staphylococcus* spp. and *Listeria* spp. *Staphylococcus* spp., *Pseudomonas* spp. and *Listeria* spp. were identified from the Mannitol Salt Agar (MSA), Pseudomonas agar and *Listeria* identification media respectively after spreading 0.1 ml of the diluted samples on these media. Results were observed after 24 hours incubation at 37 ^{o}C.

Determining Antimicrobial Activity. Determination of anti-bacterial activity was performed by using the agar well diffusion method as previously described (2, 3, 17, 21). Culture suspensions of 9 laboratory bacterial strains (*Bacillus* spp., *Pseudomonas* spp., *Vibrio* spp., *Escherichia coli*, *Klebsiella* spp., *Staphylococcus* spp., *Listeria* spp., *Salmonella* spp., and *Aeromonas* spp.) were prepared in normal saline equivalent with the turbidity of the McFarland standard. Each of the test bacterial lawn was made by separately spreading evenly over the separate Muller-Hinton agar (MHA). Wells with volumes of 8 mm^3 were made through the MHA (17, 21). Each of the crashed fruit blends (100 µl) was added to the wells along with the disc of gentamicin 10 µg as the positive control and an aliquot of 100µ l normal saline was used as the negative control. After drying the plates were then incubated at 37 °C for 12-18 hours.

Effect of Heat on microbial and fungal growth. Samples were transferred into the sterilizer at 60 ^{o}C for 3 intervals via 30 minutes, 1 hour and 2 hours (18). After treatment, total bacterial counts and total fungal counts, as well as antimicrobial activity was determined by the methods mentioned above (2, 3, 17, 21).

RESULTS AND DISCUSSION

Five different citrus fruits (Lotkone, Lemon, Orange, Amoloki, Amra and Malta) were subjected for microbiological analysis in this current study. Malta showed the highest load of total viable bacterial count (4.2×10^4 cfu/g) whereas other citrus samples showed nearly similar results ranging from 1.7×10^3cfu/g to 6.6×10^3 cfu/g. Total fungal count was found to be highest in orange (2.0×10^5 cfu/gm) and lowest in amra (1.3×10^3 cfu/g). Coliform bacteria *Escherichia coli* and *Klebsiella* spp. were absent in all of these five selected samples. *Pseudomonas* spp. was also found in all the samples ranging from 7.5×10^4 cfu/g in Amra to 1.1×10^2 cfu/g in bv malta. *Staphylococcus* spp. and *Listeria* spp. were found in less quantity compared to other bacteria. Lowest *Staphyloccus* spp. count was observed in orange (1.6×10^1 cfu/g) and two citrus fruits were free from *Listeria* spp. (lemon and orange). All of the citrus fruit samples showed nearly similar results except orange which showed greater degree of fungal growth than other samples. Overall, fungal growth might be responsible for the presence of mycotoxin if not washed thoroughly before consumption or using for making juices (22, 23). Orange provides the most acidic condition among the other fruits which encouraged the growth of fungus. No growth of coliform bacteria indicates that the fruits were out of reach of the faecally contaminated water. Being an indigenous bacteria, *Pseudomonas* spp. can be found in every types of products and so the samples are also reflecting the same condition. But the actual growth of *Pseudomonas* spp. is higher than any other bacteria. *Pseudomonas* spp. and *Listeria* spp. are able to spread enteric disease amonth the people who consumes these fruits without maintenance of proper hygiene (22, 23).

Amoloki, Lotkone, Amra, Lemon, Malta and Orange, all showed similar results after heat treatment at 60 ^{o}C. Heat exposure was done for 30 minutes, 1 hour and 2 hours for all samples and then microbiological analysis was done again to determine the degree of reduction of bacteria which we found during the first microbiological test procedure of this study (Tables 2-7). In general, a decrease in microbial and fungal growth was observed as a result of heat treatment. Increased time of treatment corresponded with increased microbial and fungal growth reduction. Heat application did not result in regrowth of eliminated microorganisms or serve as a initiator of growth for originally absent microorganisms. For Amoloki, we found complete reduction of *Listeria* spp. and total fungal count after 2 hours of heat treatment (Table 2). In case of Lotkone, total fungal count, *Listeria* spp. and *Staphylococcus* spp. were killed by 100% after 2 hours of treatment (Table 3). Same results have been found for Orange, Malta and Amra (Tables 4, 6, 7). In case of Amra, total fungal count was reduced complete after 30 minutes of heat exposure unlike other samples

TABLE 1. Microbial load of different citrus fruits samples.

Sample	Microbial load (cfu/g)						
	TVB	TF	*E. coli*	*Klebsiella* spp.	*Pseudomonas* spp.	*Staphylocccus* spp.	*Listeria* spp.
Lotkone	4.8×10^3	7.3×10^3	0	0	2.4×10^4	6.8×10^1	6.7×10^2
Lemon	1.6×10^3	2.8×10^3	0	0	2.7×10^4	2.3×10^1	0
Orange	1.7×10^3	2.0×10^5	0	0	4.4×10^3	1.6×10^1	0
Amloki	6.6×10^3	8.1×10^3	0	0	2.1×10^3	3.2×10^1	6.8×10^2
Amra	3.2×10^3	1.3×10^3	0	0	7.5×10^4	6.5×10^3	1.0×10^2
Malta	4.2×10^4	8.0×10^4	0	0	1.1×10^2	1.2×10^3	1.3×10^2

TVB = Total viable bacteria; TF = Total fungi

TABLE 2. Effect of heat on Amloki.

Temperature Used	Microbial load (cfu/g)				
	TVB	TF	*Pseudomonas* spp.	*Staphylococcus* spp.	*Listeria* spp.
30 Minutes	4.3×10^3	6.1×10^2	8.0×10^2	5.2×10^2	4.2×10^2
1 Hour	3.2×10^2	3.6×10^3	7.2×10^2	8.5×10^1	3.1×10^1
2 Hours	2.8×10^2	0	5.6×10^2	3.1×10^1	0

TVB = Total viable bacteria; TF = Total fungi

TABLE 3. Effect of heat on Lotkone.

Temperature Used	Microbial load (cfu/g)				
	TVB	TF	*Pseudomonas* spp.	*Staphylococcus* spp.	*Listeria* spp.
30 Minutes	3.2×10^3	6.9×10^3	7.6×10^2	5.8×10^2	5.5×10^1
1 Hour	2.8×10^3	5.4×10^1	6.4×10^1	4.7×10^1	4.3×10^1
2 Hours	2.1×10^2	0	5.7×10^1	0	0

TVB = Total viable bacteria; TF = Total fungi

TABLE 4. Effect of heat on Amra.

Temperature Used	Microbial load (cfu/g)				
	TVB	TF	*Pseudomonas* spp.	*Staphylococcus* spp.	*Listeria* spp.
30 Minutes	2.4×10^3	0	3.3×10^2	1.8×10^2	2.6×10^1
1 Hour	1.6×10^3	0	2.6×10^2	1.6×10^1	1.7×10^1
2 Hours	1.2×10^2	0	1.5×10^2	0	0

TVB = Total viable bacteria; TF = Total fungi

TABLE 5. Effect of heat on Lemon.

Temperature Used	Microbial load (cfu/g)				
	TVB	TF	*Pseudomonas* spp.	*Staphylococcus* spp.	*Listeria* spp.
30 Minutes	3.6×10^3	3.1×10^2	1.5×10^1	1.2×10^1	2.2×10^1
1 Hour	3.2×10^2	0	0	0	0
2 Hours	2.8×10^2	0	0	0	0

TVB = Total viable bacteria; TF = Total fungi

(Table 4). *Pseudomonas* spp. was completely eradicated only in lemon (Table 5). Heat treatment at 60 ^{o}C was effective for fungi, *Staphylococcus* spp. and *Listeria* spp. after 2 hours of heat treatment. But total viable bacterial count was reduced but the reduction was not as much as other bacteria and fungi. This indicates the presence of thermophilic bacteria in these citrus fruits which might be difficult to reduce by further heat treatment. In this study study we used 60 ^{o}C temperature, a condition at which many bacteria can not be killed, instead they occupy resistance because of such selective pressure rendering them to withstand such condition. Exposure to higher temperature might reduce these bacteria more than using 60 ^{o}C.

Lotkon, Malta, Lemon and Orange showed no antimicrobial activity against *Staphylococcus* spp.,

TABLE 6. Effect of heat on Malta.

Temperature Used	Microbial load (cfu/g)				
	TVB	**TF**	***Pseudomonas* spp.**	***Staphylococcus* spp.**	***Listeria* spp.**
30 Minutes	4.6×10^3	2.6×10^2	2.2×10^2	2.6×10^1	2.8×10^2
1 Hour	3.8×10^3	0	1.6×10^1	1.8×10^1	0
2 Hours	2.9×10^2	0	1.1×10^1	0	0

TVB = Total viable bacteria; TF = Total fungi

TABLE 7. Effect of heat on Orange.

Temperature Used	Microbial load (cfu/g)				
	TVB	**TF**	***Pseudomonas* spp.**	***Staphylococcus* spp.**	***Listeria* spp.**
30 Minutes	4.7×10^3	4.3×10^2	8×10^2	4.2×10^1	2.2×10^1
1 Hour	3.7×10^3	3.7×10^1	5×10^2	1.1×10^1	1.1×10^1
2 Hours	2.9×10^2	0	2×10^1	0	0

TVB = Total viable bacteria; TF = Total fungi

TABLE 8. Antimicrobial activity of Amra.

Temperature	***Staphylococcus* spp.**	**Fungal count**	***Klebsiella* spp.**	***Pseudomonas* spp.**	***Listeria* spp.**
30 minutes	30 mm	0	17 mm	0	0
1 hour	30 mm	0	17 mm	0	0
2 hours	0	0	0	0	0

TABLE 9: Antimicrobial activity of Amloki.

Temperature	***Staphylococcus* spp.**	**Fungal count**	***Klebsiella* spp.**	***Pseudomonas* spp.**	***Listeria* spp.**
30 minutes	26 mm	23 mm	24 mm	27 mm	24 mm
1 hour	24 mm	22 mm	23 mm	26 mm	23 mm
2 hours	22 mm	20 mm	21 mm	24 mm	21 mm

Klebsiella spp., *Pseudomonas* spp., *Listeria* spp. and fungi after heat treatment at different exposure times like 30 minutes, 1 hour and 2 hours (Tables 8 & 9). The antimicrobial activity might be destroyed or inactivated after heat treatment. Use of heat imparted no adverse effects on the antimicrobial activity of Amra and Amoloki asserting its effectiveness in use as a disinfecting method without affecting food quality. Additionally, many citrus fruits, traditionally thought to possess antimicrobial activity, failed to express adequate activity. Rising rate of food borne illnesses make studies into adequate methods of decontaminating foods imperative, in order to retain the health of the society. The use of heat on foods would protect the quality of the foods without affecting nutritional benefits or affecting the profit margins of the food production and/or agricultural companies. Its application will greatly improve the quality of the foods, particularly as it is a non-chemical method of sterilization.

CONCLUSION

Overall, the findings of this study clearly indicate a complete bacteriological profile of local market citrus fruits, which is of public health significance. Further studies with some advanced molecular settings for the

better detection of the pathogens as well as some good solvent extraction methods need to be established to use citrus fruits as a potential therapeutic agent which may reduce the use of conventional drugs for the remedy of various diseases.

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