

Microbial Status of Fresh Cut Cooking Banana Variety *Alukesel* (*Musa acuminata* × *Musa balbisiana*, ABB Group) as Affected by Pre-Treatments

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Received : 30th July 2015 / Accepted : 05th November 2015

ABSTRACT

Fresh cut (minimally processed) cooking banana variety *Alukesel* was subjected to different pre-treatments, packed in polystyrene packages and stored at 5-7 °C for a week. Effect of several identified pre-treatments on Total Plate Count (TPC) and Total Yeast and Mould counts (TYM) were evaluated. Bacteria isolated from plates were identified using biochemical tests and molecular tools. In pre-treated samples, microbial counts were within safe-to-consume limits. *Bacillus cereus*, *Enterobacter ludwigii* and *Bacillus thuringiensis* were identified from fresh cut samples using molecular tools. Citric acid at 3% w/v effectively controlled bacteria, yeasts and moulds and this observation was significantly different from the control ($p < 0.05$). The present study has shown that 3% citric acid was the most effective pretreatment for minimal processing of *Alukesel* which controlled bacteria, yeasts & moulds completely. *Alukesel* pretreated with 3% citric acid was free from food borne pathogens such as *Salmonella*, *Clostridium*, *Yersinia*, and *Listeria*.

Keywords: Minimal processing, *Alukesel* banana, pre-treatments, microbiological quality

INTRODUCTION

Minimally processed products (fresh cuts) are fruits or vegetables subjected to a process of physical alteration such as cutting, trimming, slicing while ensuring that they retain the fresh state after processing (Moretti *et al.*, 2000). The value of fresh cuts lies in the primary characteristics of freshness and convenience. Food safety, nutrition and sensory quality in fresh cuts have to be maintained at a high level, while providing extended shelf-life and freshness (Aguilar *et al.*, 2004).

Fresh cuts deteriorate faster than intact produce. Physical damage during preparation of fresh cuts cause an increase in respiration rates, biochemical changes and microbial spoilage, which may result in deterioration of colour, texture and flavour of the commodity of concern (Aguilar *et al.*, 2004).

Microbiological safety is one of the key factors

to be considered during the preservation of fresh cuts. The number of microorganisms found on produce can vary according to product quality, product characteristics, resident micro-flora of the product, handling, processing, storage, distribution and retail display. Further, microbial growth is influenced by the physiology of the fresh cut product, pre- and post-harvest processing treatments and packaging (Watada, 1997).

Microorganisms found in the fresh cuts can be categorized mainly into two types namely, psychotropic and mesophilic. Some pectinolytic bacteria that can proliferate at low temperature are *Bacillus* spp., *Xanthomonas compestris*, *Erwinia carotovora* and *Pseudomonas*. *Erwinia* and *Pseudomonas* have a competitive advantage over other microorganisms that could potentially be harmful to humans (Hui *et al.*, 2006). In terms of microbial safety, psychotropic pathogens,

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such as *Listeria monocytogenes*, *Aeromonas hydrophila* and *Yersinia enterocolitica* and mesophiles such as *Salmonella*, *Escherichia coli* and *Clostridium botulinum* are of particular concern (Goularte *et al.*, 2004). With sufficient time, several types of yeast and moulds also may grow to levels that cause food spoilage in fresh cuts (Hui *et al.*, 2006).

During preparation of fresh cuts, produce are immersed in pre-treatment solutions at the final stage of handling operations. Compounds such as ascorbic acid, citric acid, sodium metabisulphite, sodium chloride and calcium chloride have been utilized throughout the world as suitable preservatives to manage microbial problems and reduce browning for maintaining quality of fresh cuts such as potato, apple (Ahvenainen, 1996), bell pepper (Ediriweera *et al.*, 2012), Pineapple plus amberella (Daranagama *et al.*, 2012), Ela Batu (*Solanum surattense*) (Dharmabandu *et al.*, 2007) and Jak fruit (Latifah *et al.*, 2007).

Microbiological qualities of certain fresh cuts have been previously reported. According to Kang and Lee (1997), total aerobic plate count in fresh cut green pepper ranged from 4.6 to 7.2 log₁₀ CFU/g during a 6 day storage period at 5 °C. As reported by Nur Aida *et al.*, (2007), in processed mung bean sprouts stored at 2 °C, Total plate count (TPC) ranged between 7.05-7.46 log₁₀ CFU/g, while Total Yeast and Mould counts (TYM) varied between 2.05-4.15 log₁₀ CFU/g. According to Dharmabandu *et al.*, (2007) mean aerobic plate counts of fresh cuts of *Solanum surattense* (Ela Batu) ranged between 66 to 133 CFU/g. Microbial counts should be under the required legal limits to be considered safe to consume in any fresh cut meant for human consumption. Therefore, microbial quality is of dire significance in the preparation and storage of fresh cuts as value added products meant for consumers.

Cooking banana variety *Alukesel* belonging to family Musaceae is a hybrid derived from *Musa*

acuminata and *M. balbisiana*, which contains a considerable amount of fiber, carbohydrate, vitamins and potassium (Simmonds, 1966). In Sri Lanka, variety *Alukesel* is commonly used in the preparation of savoury curries and fried chips. This commodity could be stored for longer periods intact, however undergoes browning soon after slicing. Banana thus stains the hands of the handler due to melanin formed on the cut surface. A recent investigation was carried out in our laboratory on the effect of selected pre-treatments on physicochemical / sensory properties and degree of browning of fresh cut *Alukesel* and the major findings have been published (Siriwardana *et al.*, 2015). However, no research data are currently available on microbiological aspects of fresh cut *Alukesel*.

The objectives of current study were (1) to evaluate the microbial status of fresh cut *Alukesel* banana subjected to individual or combinations of pre-treatments of sodium chloride, calcium chloride, citric acid, ascorbic acid, and sodium metabisulphite stored at 5-7 °C for a week and (2) to identify bacteria associated with pre-treated *Alukesel* using molecular tools. If *Alukesel* banana is relatively free of hazardous food-borne pathogens and browning is managed, this type of value-added fresh cuts could be made available to local consumers for preparation of chips and curries.

MATERIALS AND METHODS

Preparation of Alukesel banana

Cooking banana variety *Alukesel* (*Musa acuminata* × *Musa balbisiana*, ABB Group) of 90 day maturity was purchased from supermarkets in Kiribathgoda, Sri Lanka and transported to the laboratory at the Department of Botany, University of Kelaniya. Banana were peeled and washed with distilled water, dipped in chilled water for 2 min. Subsequently, the produce were cut into slices (8-10 g) using

a sharp stainless steel knife under aseptic conditions. The resulting slices were separately dipped in selected pre-treatment solutions, singly or in combination, such as 3% (w/v) ascorbic acid (T₁), 3% (w/v) citric acid (T₂), 2% (w/v) ascorbic acid (T₃), 2% (w/v) citric acid (T₄), 1.5% (w/v) citric acid + 1.5% (w/v) ascorbic acid (T₅), 2% (w/v) sodium metabisulphite (T₆), 2% (w/v) calcium chloride (T₇), 2% (w/v) sodium chloride (T₈) or distilled water (control) (T₉) for 5 min. Samples were drained and air dried for 15 min and slices (8-10) were packed in polystyrene packages of 100 g capacity and sealed by placing clip-on lids (Latifah *et al.*, 2000). Packages were labeled and placed on plastic trays (30 cm × 40 cm) and stored in a cold room at 5-7 °C and 80-85% relative humidity. Four replicate packages were used per treatment.

Assessment of microbiological properties

Alukesel samples were subjected to the following microbiological assessments on day 0 (initial day) and after 7 days.

Total aerobic Plate Count (TPC): Twenty (20.0) g of *Alukesel* was homogenized with 180 mL of sterile 0.9% NaCl (Merck Specialties Private Limited, India) in a blender for 2 min and a dilution series was prepared up to 10⁻⁵. From 10⁻³, 10⁻⁴ and 10⁻⁵ dilutions, 1 mL was plated to 12 mL of molten plate count agar (PCA) (Oxoid Ltd., England). Plating was done in duplicate. Plates were incubated at 28 ± 2 °C for 72 hours and colony forming units (CFU) were determined using the equation described by SLS Part 1 (1991). Four replicate samples were used per treatment.

Total Yeast and Mould count (TYM): One mL from 10⁻³, 10⁻⁴ and 10⁻⁵ dilutions prepared under total aerobic plate count, were separately plated along with 12 mL molten yeast and mould agar (YMA). Plating was done in duplicate. Plates were incubated at 28 ± 2 °C for 72 hours and CFUs were determined (Nur Aida *et al.*, 2007). Four replicate samples were used per treatment.

Identification of bacteria isolated from total plate count

Pure cultures of bacteria were prepared by isolating bacteria from plates prepared for TPC which were grown on nutrient agar (NA) (Oxoid Ltd., England). The plates were incubated at 28 ± 2 °C for 72 hours. Three sets of pure bacterial cultures were prepared and isolated bacteria were labeled as strain 1 (S1), strain 2 (S2), strain 3 (S3), strain 4 (S4) and strain 6 (S6). One set each of bacterial cultures was used to identify physiological and biochemical characters of bacteria through Gram's staining, endospore staining, motility determination, growth in oxygen environment, growth in anaerobic environment, catalase activity, acid and gas production from glucose, citrate utilization, methyl red test, indole production and lactose fermentation (Benson, 2002). Based on the results of biochemical tests, bacteria were identified up to the genus level with the aid of Bergey's Manual (Holt, 2000).

Another set of bacterial cultures was deposited in the Department of Botany, Culture collection (University of Kelaniya); a similar set of 2-day old cultures were subjected to PCR amplification and gene sequencing at Genetech Molecular Diagnostic & School of Gene Technology, Colombo 8, Sri Lanka.

DNA Extraction and PCR amplification: Total genomic DNA was isolated from colonies of S1, S2, S3, S4 and S6 (on nutrient agar) using the Invitrogen PureLink Genomic DNA mini Kit (Cat No: K1820-01) and extracted DNA was stored in 100 µL of TrisEDTA pH=8 buffer at -20 °C. DNA was then used as a template for the amplification of the 16S rRNA region by using universal primers, 27F/800R and 518F/1492R (Lane *et al.*, 1991, Turner *et al.*, 1999).

Agarose gel Electrophoresis and PCR product purification: The amplified PCR product was visualized on a 2% agarose gel in 0.5 X TBE containing ethidium bromide using a UV transilluminator (E-Gel imager,

Life Technologies Israel). The band size was observed using an appropriate DNA molecular weight marker. PCR products were purified using the PureLink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen USA Cat No: K2200-01).

DNA Sequencing: Purified PCR products were subjected to DNA sequencing using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, USA, Part No. 4337455) and sequenced by programming the ABI Prism® 310 Genetic Analyzer (Applied Biosystem, USA). The 16s rRNA region sequence of the bacteria obtained, was compared with the available sequences in the National Center for Biotechnology Information (NCBI) database, using the BLAST program in order to identify bacteria to species/strain level.

Statistical analysis

The experimental arrangements of TPC and TYM were of completely randomized designs (CRD). Data obtained for microbiological properties were subjected to General Linear Model of ANOVA using MINITAB software. When significant effects were observed, treatment means were compared using Tukey's multiple comparison test at $p = 0.05$ significance level.

RESULTS

Assessment of microbiological properties

Total aerobic Plate Count (TPC): The TPC enumerated from samples of *Alukesel* on day 0 ranged from 2.78 – 4.42 \log_{10} CFU/g whereas, it ranged from zero to 4.64 \log_{10} CFU/g by day 7. The TPC decreased by day 7, when compared to day 0, in most treatments except in 3% ascorbic acid, 2% ascorbic acid, 1.5% citric acid + 1.5% ascorbic acid, 2% sodium metabisulphite and

control. On 7th day after storage, the highest TPC of 4.64 \log_{10} CFU/g was observed in the 2% ascorbic acid pre-treated samples. Control also indicated a high total plate count value of 4.50 \log_{10} CFU/g by day 7. The lowest plate count of zero \log_{10} CFU/g was observed in 3% citric acid treated samples (Figure 01). Statistically significant difference ($p < 0.05$) was observed between day 0 and 7 for TPC of fresh cut *Alukesel* samples and the control when the effect of pre-treatment, time and interaction of pre-treatment x time was considered (Table 01).

Total Yeast and Mould count (TYM): The TYM count enumerated from samples on day 0 ranged from zero to 4.43, which was somewhat similar to results seen on day 7 (zero to 4.10 \log_{10} CFU/g). On day 0, yeasts and moulds were not observed in distilled water samples while on day 7 no colonies were observed in 3% ascorbic acid, 3% citric acid, 2% ascorbic acid, 1.5% citric acid + 1.5% ascorbic acid and 2% sodium chloride treated *Alukesel*. In most pre-treated samples, yeast and mould counts decreased by day 7, compared to day zero, except in 2% sodium metabisulphite indicating the antimicrobial activity of certain pre-treatments such as ascorbic acid, citric acid and sodium chloride (Figure 02). Control, by day 7 indicated a TYM count of 3.27 \log_{10} CFU/g. There was a significant difference ($p < 0.05$) of TYM counts of samples compared to control when the effect of pre-treatment, time and interaction effect of pre-treatment x time were considered (Table 01).

Identification of bacteria isolated from total plate count

Based on biochemical tests conducted, five strains of bacteria were identified from TPC experiment (Table 02). Strains 1, 2, 3 and 6 were identified as *Bacillus sp.* and stain 4 as *Enterobacter spp.*

During molecular identification based results obtained from NCBI BLAST tool, the identity of S1, S2, S3, S4 and S6 bacterial cultures were confirmed as *Bacillus cereus*, *Bacillus cereus*, *Bacillus cereus*, *Enterobacter ludwigii*

and *Bacillus thuringiensis*. These results were deposited in Genebank under the accession numbers KT970720, KT970721, KT970722, KT970723 and KT970724.

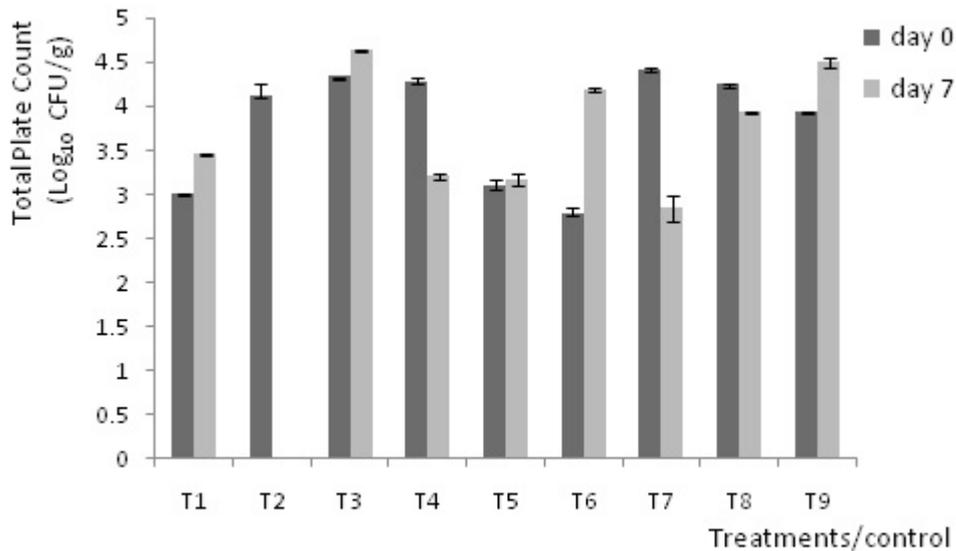


Figure 01: Effect of selected pre-treatments on TPC of fresh cut Alukesel banana, in comparison to control, 0 and 7 days after storage at 5-7 °C. (T₁ - 3% ascorbic acid, T₂ - 3% citric acid, T₃ - 2% ascorbic acid, T₄ - 2% citric acid, T₅ - 1.5% citric acid + 1.5% ascorbic acid, T₆ - 2% sodium metabisulphite, T₇ - calcium chloride, T₈ - sodium chloride, T₉ - distilled water (control)). Data points of TPC represent the mean of eight replicates ± standard error.

Table 01: ANOVA of different pre-treatments and time on TPC and TYM count of fresh cut Alukesel.

Factor	d.f.	F value	P value
TPC			
A (pre-treatment)	8	237.78	0.00
B (time)	1	229.86	0.00
A × B	8	304.6	0.00
TYM			
A (pre-treatment)	8	10.43	0.00
B (time)	1	40.15	0.00
A × B	8	11.25	0.00

d.f. = degrees of freedom

P<0.05 indicates a significant difference among variables and control.

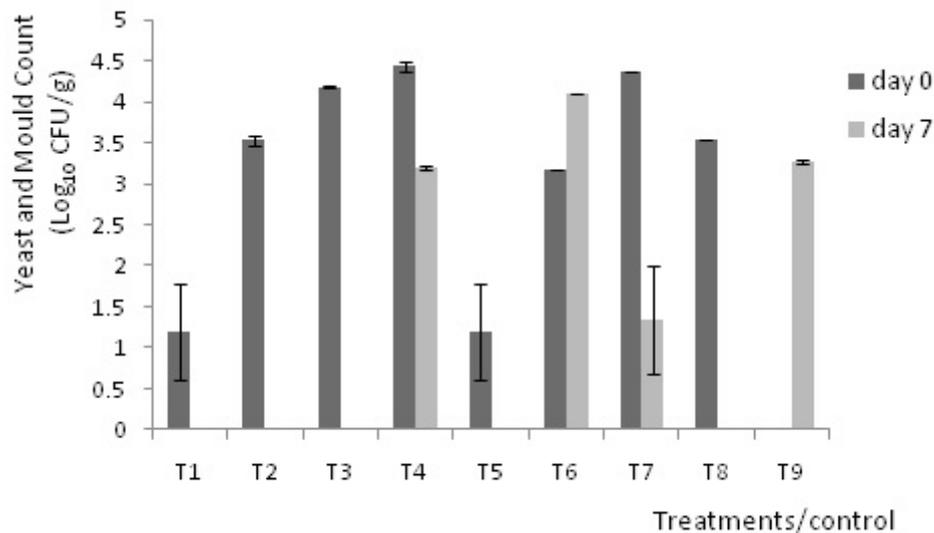


Figure 02: Effect of selected pre-treatments on TYM count of fresh cut Alukesel banana, in comparison to control, 0 and 7 days after storage at 5-7 °C. (T₁ - 3% ascorbic acid, T₂ - 3% citric acid, T₃ - 2% ascorbic acid, T₄ - 2% citric acid, T₅ - 1.5% citric acid + 1.5% ascorbic acid, T₆ - 2% sodium metabisulphite, T₇ - calcium chloride, T₈ - sodium chloride, T₉ - distilled water (control)). Data points of TYM count represent the mean of eight replicates ± standard error.

Table 02: Characteristics of bacteria associated with fresh cut Alukesel.

Characteristics	Strain 1	Strain 2	Strain 3	Strain 4	Strain 6
Colony colour	White	White cream	White	Light yellow	White
Colony shape	Round with irregular margins	Round with irregular margins	Irregular and spreading	Round with entire margins	Round with lobate margins
Cell shape	Rod	Rod	Rod	Rod	Rod
Gram's reaction	+	+	+	-	+
Endospore formation	+	+	+	-	+
Motility	+	+	+	+	+
Growth under Oxygen	+	+	+	+	+
Growth in Anaerobic Jar	+	+	+	+	+
Acid production from Glucose	-	-	-	-	-
Citrate Utilization	-	-	-	+	-
Catalase test	+	+	+	+	+
Indole Production	-	-	-	-	-
Methyl Red Test	-	-	-	-	-
Lactose Fermentation	-	-	-	+	-

DISCUSSION

TPC provides information on the microorganisms (especially bacteria) growing aerobically at 30 °C, under the specified conditions (SLS Part 1, 1991). In *Alukesel*, there was a significant difference in the TPC between pre-treatments and the control, indicating the antimicrobial efficacy of pre-treatments used in this study. The most promising results in controlling TPC were displayed by *Alukesel* samples pre-treated with 3% citric acid, which was able to control the microbial growth completely, by day 7. It was also evident that the lower concentration of citric acid was less effective in controlling the microbial growth. According to a recent publication by our research group, it was reported that 3% citric acid pretreatment effectively controlled browning and maintained acceptable quality in fresh cut *Alukesel* (Siriwardana *et al.*, 2015). Similarly, Daranagama *et al.*, (2012) reported 4.93 log₁₀ CFU/g value for TPC for fresh cut ambarella (*Spondias dulcis*) and pineapple together as a mixed load, after pretreating with 1% citric acid and storing at 5-7 °C for seven days. Citric acid is one of the best known chelating agents, which can chelate Cu to control browning in minimally processed fruits and vegetables (Manolopoulou and Varzakas, 2011). Further, citric acid inhibits the growth of microorganisms by lowering the pH of processed commodity (Hui *et al.*, 2006).

According to Kumari (2009), the microbial level of all pre-treated fresh cut samples of *Lasia spinosa* treated with 0.5% ascorbic acid increased, during the storage period of 7 days at 5-7 °C. This is in agreement with the present data as a slight increase in TPC could be observed for samples pre-treated with 3% ascorbic acid, 2% ascorbic acid and 1.5% citric acid + 1.5% ascorbic acid by day 7, when compared to day 0. This may be possible as ascorbic acid can act as an intermediate in the metabolism and the growth rate of microorganisms, which can lead to slight increase in their proliferation. Sodium metabisulphite (at 2%) did not display

antimicrobial effect as TPC increased after 7 days of storage, although the same treatment was relatively effective in controlling browning of fresh cut *Alukesel* as reported by Siriwardana *et al.*, (2015).

The mesophilic aerobic bacterial counts on finished cut vegetables ranged depending on the commodities, season of the year and growing regions. According to Heard (2000), aerobic bacterial counts on bagged salads from the retail market ranged from 4.0-9.0 log₁₀ CFU/g. Allende *et al.*, (2004) reported that initial aerobic mesophilic bacterial load on fresh cut baby spinach leaves after washing with 2.0 mM chlorine was 7.2 log₁₀ CFU/g. However, it was reported that microbial populations of fresh cut baby spinach leaves increased up to 9.0 log₁₀ CFU/g level, during storage of 12 days at 5 °C. According to Francis *et al.*, (1999), the legal regulations on fresh cut vegetables established a maximum total limit of 7.7 log₁₀ CFU/g for TPC. In the present study, TPC ranged from zero to 4.64 log₁₀ CFU/g by day 7. It is important to highlight at this point that the TPC counts of test samples from all pre-treatments of *Alukesel* did not exceed the legal limit indicating that the samples processed this way are safe to consume.

The TYM counts provide a general guidance for the enumeration of viable yeasts and moulds in products at specified conditions (SLS Part 2, 1991). TYM were found to be slightly lower than TPC for *Alukesel* samples ranging from 0 - 4.10 log₁₀ CFU/g on day 7. These results are also in agreement with those of Nur Aida *et al.*, (2007) for minimally processed mung bean sprouts, where values ranged from 2.05 - 4.15 log₁₀ CFU/g. According to Nur Aida *et al.*, (2007) bacterial populations dominate over yeasts and moulds in vegetables. The yeasts and moulds were completely absent in the cooking banana samples pre-treated with 3% ascorbic acid, 3% citric acid, 2% ascorbic acid, 1.5% citric acid + 1.5% ascorbic acid and 2% sodium chloride. Both ascorbic and citric acid inhibit the growth of yeasts and moulds by lowering

the pH of processed commodity. Similarly, Ediriweera *et al.*, (2012) reported zero yeast and mould counts for fresh cut bell pepper, treated with 1% sodium chloride and stored at 5-7 °C by day 7. When sodium chloride is incorporated as a pre-treatment, high osmotic pressure results in plasmolysis of microbial cells causing dehydration and inhibition of growth of microorganisms. Further, chloride ion is toxic to most microorganisms (Hui *et al.*, 2006). According to Nur Aida *et al.*, (2007) the recommended limit for TYM of fresh cut produce is 5 log₁₀ CFU/g. Since the TYM in all the pretreated samples did not exceed the recommended limit, fresh cuts of *Alukesel* can be considered safe to consume. In most of pre-treated *Alukesel* samples, yeast and mould counts on day 7 were controlled at zero level. Therefore no attempt was taken to identify yeast and moulds to genus or species level during present research.

Using molecular tools, bacteria isolated from TPC were identified as *Bacillus cereus*, *Enterobacter ludwigii* and *Bacillus thuringiensis*. *B. cereus* can readily be isolated from various food products. This species is common in nature, and may survive different stresses during food production due to chemical and heat resistant endospores. Some strains of *B. cereus* are harmful to humans and cause foodborne illness, while other strains can be beneficial as probiotics for humans (Doyle *et al.*, 1989). *B. thuringiensis* is used worldwide as a biological insecticide due to its ability to produce crystal proteins with highly specific activity against certain insects. *B. cereus* and *B. thuringiensis* are closely related and genomic studies have proposed that they should be merged into a single species (Helgason *et al.*, 2000). After commercialization of *B. thuringiensis*-based insecticides, studies have shown that *B. thuringiensis* like *B. cereus* produces enterotoxins responsible for human diarrhoea. *B. thuringiensis* has however, only in one case been described to be implicated in food borne disease (Jackson *et al.*, 1995). One of the

bacteria isolated and identified from *Alukesel* – *E. ludwigii* belongs to *Enterobacter cloacae* complex consisting six bacterial species which are widely encountered in nature. Some stains of this species are part of the commensal enteric flora of the human gastro-intestinal tract, while some strains include human pathogens and have been previously isolated in human clinical specimens (Mezzatesta *et al.*, 2012). *E. ludwigii* has previously been isolated as an endophytic bacteria dwelling in strawberry fruit (De Melo Pereira *et al.*, 2012).

This is the first report on the evaluation of microbial levels of fresh cut *Alukesel*, using molecular tools although related work on physicochemical/sensory properties and degree of browning of fresh cut *Alukesel* have been reported recently (Siriwardana *et al.*, 2015). Although pre-treatments of ascorbic acid (3%) and citric acid + ascorbic acid (1.5% each) were better in controlling browning and sensory properties (Siriwardana *et al.*, 2015), microbial growth was not controlled to a significant level, using the above individual or combined treatments. However, 3% citric acid pre-treatment significantly reduced microbial growth by reducing TPC and TYM counts to zero level on day 7. As indicated by our previous research, *Alukesel* pre-treated with 3% citric acid retained acceptable sensory properties and also managed browning to a greater extent (Siriwardana *et al.*, 2015). Microorganisms such as Salmonella, Clostridium, Yersinia and Listeria are reported to be transmitted through contaminated food products, causing food borne diseases in humans and animals. Therefore, utmost care should be taken in identifying and quantifying microorganisms surviving on different commodities before any minimal processing technique is implemented on commercial scale in any country. However, no evidence of any of the above hazardous food-borne organisms were found during molecular identification of microorganisms from fresh cut *Alukesel*.

CONCLUSION

In conclusion, the present study has shown that 3% citric acid was the most effective pre-treatment for minimal processed *Alukesel*, which controlled bacteria, yeasts & moulds completely. *Alukesel* pre-treated with 3% citric acid was free from food borne pathogens such as *Salmonella*, *Clostridium*, *Yersinia* and *Listeria*.

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