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Occurrence and Antimicrobial Susceptibility of *Salmonella* sp. and *Escherichia coli* in Minimally-processed and Frozen Fruit Pulps

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ABSTRACT

Fruits and fruit pulps are widely consumed worldwide due to their nutrients, flavors and varieties. However, these products become contaminated with pathogens during harvest or production, which are risks to consumers. This study analyzed the microbiological quality of frozen fruit pulps and minimally processed fruits sold in supermarkets and the presence *Salmonella* sp. and pathogenic Escherichia coli. Almost all frozen fruit pulps samples were adequate to consume, only one samples of unpasteurized mango pulp was positive for *E. coli* carrier of est1b gene that codify thermostable toxin of Enterotoxigenic E. coli. Minimally processed fruits presented high yeast and mold counts in 36.25% (29/80) of the samples and 27.6% (22/80) had thermotolerant coliforms. In addition, one sample of grated coconut had *E. coli* and one sample of melon honeydew had *Salmonella* sp. *E. coli* O157:H7 was absent in all samples of minimally processed fruits. *E. coli* showed greater resistance to ampicillin and chloramphenicol. Multidrug resistance was observed in 14.3% (2/14) of *E. coli* isolates. Only one strain of *Salmonella* sp. was resistant to antibiotic sulfamethoxazole/trimethoprim. Therefore, the enforcement of pasteurization in the fruit pulp processing, as well hygienic-sanitary control in lead up of minimally-processed fruits and temperature control in storage are recommended to minimize the risk of foodborne disease.

Keywords: mango, melon, multidrug resistance, pathogenic *E. coli.*

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Introduction

Due to their healthy characteristics, fruit pulps are consumed daily by many people. They are often used to replace industrialized beverages in bars, restaurants and at homes. The production processes of fruit pulps include pasteurization and freezing[1,2]. However, in case of production problem, equipment failure or inadequate storage temperature, the microbiological standard of the final product may change.

Fresh fruit consumption has increased in the last years because the people search for healthy diets and due to the convenience of consuming ready-to-eat foods with very similar characteristics to those of natural products. However, there is a risk of contamination by pathogenic microorganisms during handling practices and several of them are resistant to fruit acids and freezing may be present in this foods.

Fresh fruits can often be contaminated by pathogenic or deteriorating microorganisms as a result of cultivation, handling and processing practices[3].

Production of minimally processed fruits should be performed carefully to avoid contamination of final products and obtain superior quality and long shelf life of the products. Raw material is selected, sanitized, sliced and stored at a suitable temperature to limit the growth of deteriorating and pathogenic microorganisms[4]. During slicing, utensils and poor hygiene conditions of handling the fruits may also contaminate the product. Sliced fresh products present a high level of moisture and nutrients that support growth of microorganisms[5].

Raw fruits and vegetables are carriers of pathogens, and foodborne outbreaks associated with fruit consumption have increased, causing concern to industries and regulatory agencies[6].

Salmonella and *Escherichia coli*, responsible for the transmission of diseases via consumption of different foods, may be present in fruits and fruit products[4], as they can develop and survive in environments of low temperature and high acidity[7].

From 2001 to 2009, four outbreaks of *Salmonella* occurred in Canada associated with the consumption of several fruits (melon, watermelon, blueberry, pineapple and kiwi), melons (cantaloupe) and fruit salads[8]. Other fruits such as papaya[9], frozen grated coconut[10], mango[11], and frozen mamey pulp[12] have been considered carriers of salmonellosis.

In the United States, fruits and fruit products were recalled by US Food and Drug Administration from 2003 to 2011 for presenting mainly *Salmonella, Listeria monocytogenes, Clostridium botulinum* and *E. coli* O157:H7, with 71% of taken products contaminated with *Salmonella* and 18% with *L. monocytogenes*[13].

Beuchat reports close relation between consumption of raw vegetables and foodborne diseases and some of the reasons are: adaptation of pathogenic microorganisms to environmental stress conditions, improper processing practices, increased consumption of ready-to-eat foods and globalization[7].

In Brazil, few studies have been carried out on the prevalence of pathogens in fruits and pulps whereas the increased consumption of these products, this study aims to evaluate the microbiological quality of frozen fruit pulp and minimally processed fresh fruit commonly marketed, and verify the presence of *Salmonella sp.* and pathogenic *E. coli*. In addition, the antimicrobial resistance profile of these pathogens was evaluated.

Material and Methods

Sampling

One hundred samples of frozen fruit pulps of various flavors (açaí, mango, strawberry, cashew, guava, acerola and coconut) and 80 samples of minimally processed fruit were purchased at supermarkets in Campinas, São Paulo. The fruits analyzed were honeydew melon, crenshaw melon, piel del sapo melon, fruit salad, watermelon, pineapple, tangerine,

papaya and grated coconut. The samples were transported in isothermal boxes and analyzed at the Food Microbiology Laboratory I of the Department of Food Science from the University of Campinas.

Determination of pH

The pH of fruit pulps was measured according to the methodology of the Instituto Adolfo Lutz (IAL) [14]. The amount of 10 grams of the sample was diluted and homogenized in 100 ml of water for subsequent measurement in a pH meter (OHAUS, Starter 2100).

Yeast and Mold Count

Twenty five grams of the sample was weighed and homogenized with 225 ml of peptone (DIFCO) at 0.1%; after homogenization, 0.1 ml of each dilution (10^{-1}, 10^{-2} and 10^{-3}) was inoculated on the surface of plates with potato dextrose agar (OXOID) and incubated at 25°C for 3 to 5 days; then the colonies of molds and yeasts were counted and the results expressed in $CFU.g^{-1}$ [15].

Count of thermotolerant coliforms by the most probable number (MPN) technique

An aliquot of 1 ml of the dilutions prepared above was inoculated in a series of 3 tubes of the most probable number technique with lauryl tryptose broth (MERCK) for coliform count. The tubes are incubated at 35°C for 24-48 hours and the positive tubes with gas formation were transferred to the *Escherichia coli* broth (DIFCO) and incubated in a water bath at 45°C for 24-48 hours. Tubes with gas formation were confirmed for thermotolerant coliforms and was streaked on Levine eosin-methylene blue (L-EMB) agar (DIFCO) and incubated at 35°C for 24 hours [16] for isolation of *E. coli*. The characteristic colonies were confirmed through indole, methyl red, Voges Proskauer and Simmons citrate tests, and typical characteristics in triple sugar iron (TSI) agar (DIFCO) according to FENG, 2011[17].

Detection of pathogenic *E. coli*

Detection was performed by homogenizing 25 g of the sample with 225 ml of tryptone phosphate broth (DIFCO) and subsequent incubation at 44°C for 24 hours. A droplet was streaked on L-EMB and MacConkey agar and incubated at 35ºC for 24 hours[17]. Typical colonies were confirmed through indole, methyl red, Voges Proskauer and Simmons citrate tests, and typical characteristics in triple sugar iron agar (DIFCO) [17].

Detection of *E. coli* O157:H7

The detection of *E. coli* O157:H7 was performed only in samples of minimally processed fruits. For the identification of O157:H7, the amount of 25 g of the sample was homogenized with 225 ml of trypticase soy broth (TSB) (SIGMA) with acriflavine (0.05 mg/L), cefsulodine (10 mg/L) and vancomycin 8 mg/L). After incubation at 35°C for 24 hours, was streaked on MacConkey Sorbitol agar (MERCK) with tellurite and cefixime (SIGMA) [17].

DNA extraction

The DNA of strains was extracted according to FENG et al [17]. Briefly, 600 µl of the overnight culture were centrifuged at 12,000 x g for 10 minutes. After removing the supernatant, the cell mass was suspended in 100 µl of Tris EDTA buffer (10 mM Tris and 1 mM EDTA, pH 8.0), kept in a 100°C bath for 10 minutes, and cooled in ice bath. Then another centrifugation cycle was performed at 12,000 x g for 1 minute and the supernatant containing the DNA was transferred to another tube and kept at -20°C until it was used.

PCR for differentiation of pathogenic types of *E. coli*

The differentiation of ETEC (Enterotoxigenic *E. coli*), EPEC (Enteropathogenic *E. coli*), EIEC (enteroinvasive *E. coli*), EHEC (Enterohemorrhagic *E. coli*) and EAEC (Enteroaggregative *E. coli*) from *E. coli* strains was performed by detecting virulence genes *est1b* (thermostable toxin) for ETEC, *eae* (intimin) for EPEC, *ipaH* (invasion antigen) for EIEC, *stx1* (shiga toxin) for EHEC, and *agg* (aggregative adhesion fimbria) for EAEC,

according to Chandra et al [18] and Müller et al [19]. Table 1 shows the primers used in this study.

The strains used as positive controls for the virulence genes were donated by the Laboratório de Referência Nacional para Enteroinfecções Bacterianas (LRNEB) from Fundação Oswaldo Cruz in Rio de Janeiro, Brazil.

Electrophoresis of PCR products was performed in 1.5% agarose gel (Invitrogen), stained with Sybr Safe™ (Invitrogen) and viewed on a transilluminator.

Table 1 Genes and sequence of primers used in the study

Pathogen	Gene	Product	Sequence (5'-3')	Size of product (bp)	Reference
ETEC	*est1b*	Thermostable toxin	F TGTCTTTTTCACCTTTCGCTC R CGGTACAAGCAGGATTACAACAC	171	Chandra et al, 2013
EPEC	*eae*	Intimin	F TCAATGCAGTTCCGTTATCAGTT R GTAAAGTCCGTTACCCCAACCTG	482	Chandra et al, 2013
EIEC	*ipaH*	Invasion antigen	F CTCGGCACGTTTTAATAGTCTGG R GTGGAGAGCTGAAGTTTCTCTGC	933	Chandra et al, 2013
EAEC	*agg*	Aggregative fimbria	F ACGCAGAGTTGCCTGATAAAG R AATACAGAATCGTCAGCATCAGC	400	Müller et al, 2007
EHEC	*stx1*	Shiga toxin	F GATGTTACGGTTTGTTACTGTGACAGC R AATGCCACGCTTCCCAGAATTG	244	Chandra et al, 2013
Salmonella	*invA*	Invasion protein	F TGAAATTATCGCCACGTTCGGGCAA R TCATCGCACACGTCAAAGGACC3	284	Rahn et al, 1992

Analysis of *Salmonella* sp.

The amount of 25 grams of the sample was homogenized with 225 ml of buffered peptone water (OXOID) and incubated at 35°C for 18 hours. For selective enrichment, 1 ml of broth was transferred to Muller's tetrathionate broth (SIGMA) and incubated at 35°C for 24 hours and 0.1 ml was added to Rappaport Vassilidis broth (OXOID) and then it was incubated at 42°C for 24 hours. In the differential selective plating, the broth was streaked on the following media: Hektoen enteric agar (DIFCO), Bismuth sulfite (BS, DIFCO) agar, and Xylose Lysine Deoxycholate agar (OXOID). The plates were incubated at 35ºC for 24 hours and characteristic colonies were submitted to biochemical tests in TSI, Lysine Iron agar (DIFCO) and urea agar (OXOID) [20]. PCR was used to detect *invA*, a gene that is present in all species of *Salmonella sp.*, according to Rahn et al [21].

Antimicrobial susceptibility testing

The antimicrobial susceptibility profile of *E. coli* and *Salmonella sp.* was analyzed by disk diffusion method, as recommended by the National Committee for Clinical Laboratory Standards or Clinical and Laboratory Standards Institute (CLSI) [22]. The antibacterial agents evaluated were: nalidixic acid (30 µg), ampicillin (10 µg), ceftadizime (30 µg), ciprofloxacin (5 µg), chloramphenicol (30µg), trimethoprim/ sulfamethoxazol (25 µg).

Results and discussion

Frozen fruit pulps analysis

All 100 samples of frozen fruit pulps presented counts of molds and yeast below 10^2 CFU.g^{-1} and all pH values were also in agreement with

the standard of Normative Instruction nº 1/2000 of the Ministry of Agriculture (MAPA) [23]. Low amounts are associated with a pasteurization process that destroys molds and yeasts [24] and the proper storage temperature for frozen fruit pulps is -18ºC, as lagging chemical reactions and inhibits the growth of microorganisms[25].

Counts of thermotolerant coliforms in fruit pulps were lower than 3 MPN.g^{-1}, but a sample of unpasteurized mango pulp was positive for *E. coli* in the presence/absence test. The isolates from this sample were positive for *est1b* that encodes the virulence factor of ETEC thermostable toxin (ST), and negative for the other analyzed genes: *ipaH, eae, agg* and *stx*. The presence of *E. coli* with pathogenic potential in frozen fruit pulp demonstrates its ability to survive freezing temperature and acidic environments, such as mango, which presented pH 4.3 (±0.2).

Low counts (10^1MPN.g^{-1}) of thermotolerant coliforms were found in pulps of passion fruit, cashew and açaí by Santos et al [26] and Neto et al. [27], also in agreement with the standards required by the Brazilian legislation, RDC nº 12 of 2001 [28], as recommends a maximum value of 10^2 MPN.g^{-1}.

Salmonella sp. was not found in all 100 samples of fruit pulps analyzed. The absence of this pathogen in the pulps can be explained by the pasteurization process performed by most industries and freezing, as injures the microbial cells and inhibits their development, low pH value in most samples (3.5 to 5.3) and storage temperature as a limiting factor for the development of microorganisms [29].

Minimally processed fruits analysis

In the minimally processed fruit samples, 36.25% (29/80) of the samples had high mold and yeast counts, above 4.2x10^3 CFU/g (Table 2). Counts between 10^3 and 2.1x10^3 CFU.g^{-1} were observed in 26.25% (21/80), while 37.50% (30/80) presented counts lower than 1.2x10^2 CFU.g^{-1}.

The highest counts (>5x10^3 CFU.g^{-1}) were observed in samples of fruit salad, sliced honeydew melon and crenshaw melon, totaling 91.1% (11/12), 77.7% (7/9), and 77, 7% (7/9), respectively, which can be explained by the intense handling (Figure 1). And the lowest counts (<10^2 CFU.g^{-1}) were observed in coconut, tangerine and papaya.

High counts of molds and yeasts were also observed in other studies. Pinheiro et al [30] found counts ranging from 10^2 to 10^7 CFU.g^{-1} in 75% (75/100) of fruit samples and Bruno et al [31] reported 4.8x10^3 to 1.8x10^5 CFU.g^{-1}.

Table 2 Higher counts of molds and yeasts on minimally processed fruits

Fruits	Number of samples	Count (CFU.g^{-1})
Crenshaw melon	7	4.2x10^3 to 5.1x10^6
Honeydew melon	7	3.8x10^4 to 6.7x10^4
Piel del sapo melon	1	3.9x10^5
Watermelon	1	3.1x10^4
Pineapple	2	5.9x10^3 to 2.0x10^4
Fruit salad	11	4.2x10^5 to 7.2x10^6
Total	**29**	

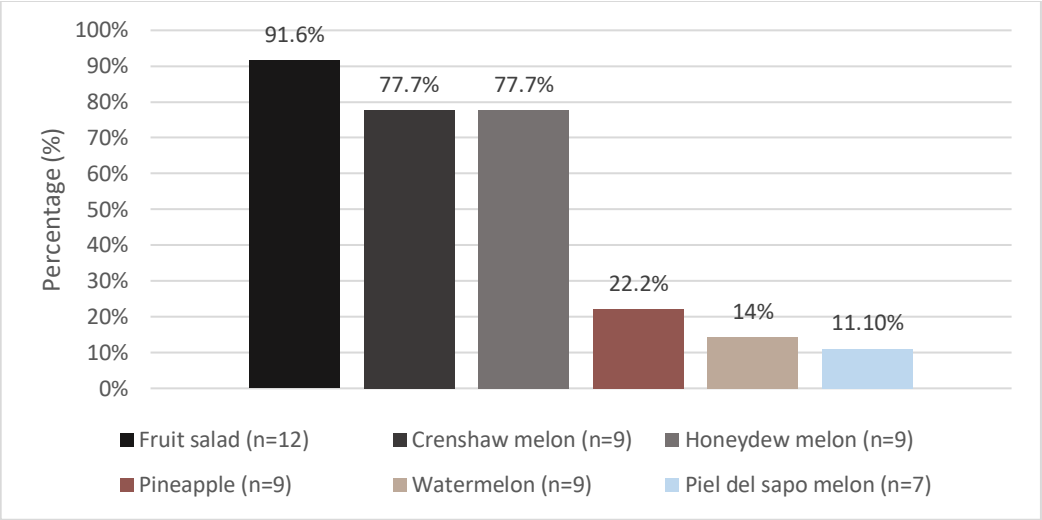

Figure 1 Percentage of minimally processed fruit samples with yeast and mold counts above $5x10^3$ CFU.g^{-1}

Among the samples of minimally processed fruit samples, 27.5% (22/80) presented counts of thermotolerant coliforms $>10^2$ MPN.g^{-1} (Table 3) and among these, 11 samples (1 of honeydew melon and 10 of fruit salad) were in disagreement with the Brazilian legislation, RDC nº 12 [28], which allows utmost $5x10^2$ MPN.g^{-1}.

Among the most contaminated samples, the fruit salad is the most contaminated one, totaling 83% (10/12) of the samples with counts above $5x10^2$ MPN.g^{-1} (Table 3). Santos et al [26] also found samples of fruit salads marketed by street sellers in Juazeiro do Norte, in Bahia, contaminated with thermotolerant coliforms. The fruit salads are submitted to more intense handling than sliced fruits or haft of fruit; then they are mixed with each other and remain at room temperature for a long period, which favors contamination and multiplication of microorganisms [26].

The source of fruit contamination may be the low quality of irrigation water or the soil where they were cultivated. Farther, chopped fruits have high moisture content and nutrients, favoring microbial growth [6].

One sample of grated coconut with a count of thermotolerant coliforms of 240 MPN.g^{-1} presented *E. coli*; however, neither of the isolates had the virulence genes analyzed. The presence of *E. coli* in natural foods indicates fecal contamination and possible presence of pathogens such *Salmonella* sp. Lately, dry coconut and frozen grated coconut were considered the reasons of outbreaks of salmonellosis [32]. *E. coli* O157:H7 was not found in any sample of the minimally processed fruits.

Table 3 Counts of thermotolerant coliforms in minimally processed fruits.

Fruit	Number of samples	Count (MPN.g^{-1})
Fruit salad	10	≥1100
Honeydew melon	6	150 to 1100
Crenshaw melon	4	120 to 240
Papaya	1	290
Coconut	1	240
Total	**22**	

In actual research, *Salmonella* sp. was isolated from a sample of sliced honeydew melon (1.25%, 1/80). Higher contamination of minimally processed fruits (papaya, pineapple and fruit salad) was observed by Bruno, et al [31], which 26.6% (4/15) of the samples were contaminated with *Salmonella*, making them inadequate for consumption. In addition, studies have shown that *Salmonella* sp. can growth in fruit products at refrigerated temperature. *Salmonella* Enteritidis grew in melon pulp (pH 5.87) at 10°C [36]. In fresh sliced cantaloupe and honeydew melons, *Salmonella* sp. outlived at 5°C and grew at 10°C [37].

Melons have already been involved in several outbreaks of salmonellosis in the United States [33, 34] and Australia [35] Recently, *Salmonella* Newport was considered the cause of an outbreak implying the consumption of pre-sliced fruits (watermelon and cantaloupe melon) where 18 people had sickened in the United States [34]. Therefore, minimally processed foods request proceedings that prioritize microbiological safety for consumer health, since these foods are directly consumed without any treatment. The temperature needs be tightly controlled including, during preparation and storage to avoid the development of pathogenic microorganisms.

The minimally processed fruits analyzed in this study were purchased in supermarkets, where the employees handled and cut the fruits. Sanitation of the employees' hands and tools used in fruit cutting and the handling conditions were probably not adequate, outcoming in fruit contamination. The source of contamination may be the fruit itself which has not undergone proper washing and sanitizing process, considering fruit peel is a potential source of contamination by microorganisms, which can contaminate the edible parts of the fruit while it is cut. In addition, contamination may result from poor hygienic and sanitary conditions of the environment, utensils and employees.

Analysis of antimicrobial susceptibility

Only 20% (1/5) of *Salmonella* sp. isolates from sliced melon presented resistance to sulfamethoxazol/trimethoprim. For others antibiotics analyzed, the isolates were susceptible or presented intermediate resistance. (Figure 2.A)

The bacterial resistance phenotype shows that 100% (14/14) of *E. coli* isolates from mango and grated coconut pulps were resistant to only one antibiotic (ampicillin), 64.3% (9/14) were resistant to two antibiotics, and 14.3% (2/14) were resistant to 3 or more antimicrobial agents tested. The antibiotic with the highest sensitivity was sulfamethoxazol/trimethoprim, presenting 85% (12/14) of isolates (Figure 2.AB). *E. coli* isolated from grated coconut showed more resistance than isolates from mango pulp. It was observed 100% resistance to ampicillin and chloramphenicol, and multi-resistance.

Moura et al [39] evaluated the resistance of the same antibiotics tested in this research. About 99 isolates were collected from children under 5 years old hospitalized with diarrhea: 9 (6.4%) enteropathogenic *E. coli* (EPEC), 4 (2.9%) enteroinvasive *E. coli* (EIEC), 80 (57.1%) of other *E. coli* types, 3 (2.1%) of *Shigella* spp., and 3 (2.1%) of *Salmonella* spp; 82.7% of other *E. coli* types were susceptible to nalidixic acid and nearly 60% to ampicillin and sulfamethoxazol/trimethoprim. For EPEC and EIEC, the susceptibility was 90% or higher for ciprofloxacin, aminoglycosides and third generation cephalosporins.

A research carried out in Malaysia, strains of *E. coli* from food handlers were resistant to several antibiotics, whilst 85.71% of isolates were resistant to penicillin and chloramphenicol, 57.14% to sulfamethoxazole, ampicillin and trimethoprim, 28,57 % to kanamycin and tetracycline, and 14.29% to ciprofloxacin [38].

According to Mota et al [40], the increment of resistance by some pathogenic bacteria occurs faster than the industry's ability to produce new effective drugs. The concern about preventing

resistance causes health professionals to use large spectrum drugs, often resulting in higher treatment costs and occurrence of antimicrobial resistance due to speed of microorganisms acquire multidrug resistance.

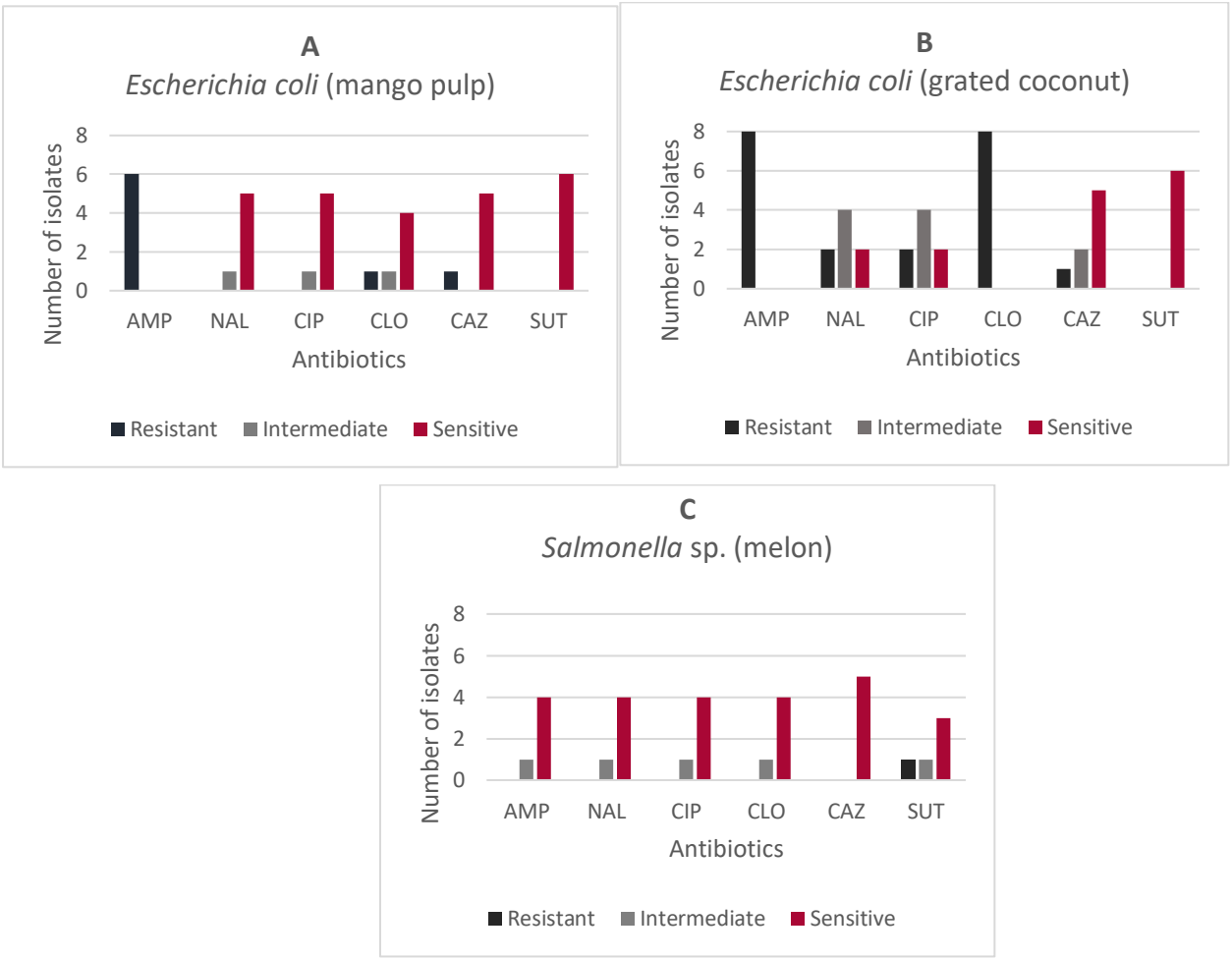

Figure 2: Antimicrobial susceptibility patterns identified in *Escherichia coli* isolated from mango pulp (A); *Escherichia coli* isolated from grated coconut (B); *Salmonella* sp. isolated from sliced melon (C). AMP – ampicillin; NAL – nalidixic acid; CIP – ciprofloxacin; CLO – chloramphenicol; CAZ – ceftadizime; SUT – sulfamethoxazol/trimethoprim.

Conclusions

Most samples of frozen fruit pulps were suitable for consumption, with low counts of thermotolerant coliforms, molds and yeasts and absence of *Salmonella sp.*. However, in one sample of unpasteurized mango pulp, *Escherichia coli* was isolated and it presented *est1b*, the gene encoding ETEC thermolabile toxin. Including the phase of pasteurization in fruit pulp processing is suggested to ensure safe products to consumers.

In minimally processed fruits, 27.5% of the samples were counts of thermotolerant coliforms above 10^2 NMP.g^{-1}, and one sample presented *Salmonella* sp. Higher yeast and mold counts were observed in 36.25% of the samples. Good hygienic and sanitary practices during handling of fruits and adequate storage temperature are recommended to minimize the contamination.

Antimicrobial susceptibility testing showed that *E. coli* isolates are resistant to antibiotics used in the treatment of infections, such as ampicillin, which 100% of *E. coli* isolates showed

resistance. Multidrug resistance to 4 or more antibiotics was observed in 14.3% of *E. coli* isolates. *Salmonella* sp. isolates showed low resistance to the antibiotics tested in this study.

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Conflicts of interest

We have no conflict of interest to declare

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