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Impact of consumer handling on bacteriological quality of orange and red grape juices: A preliminary laboratory-based study

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Bacillus spp; consumer handling; food consumption; food safety

KEY CONTRIBUTION
Potentially pathogenic bacteria belonging to *Bacillus* spp. recovered from fruit juice after consumer handling. Fruit juice consumed directly from a juice pack and stored at ambient temperature may pose a food safety risk to consumers.

ABSTRACT
This study examined if the direct consumption of fruit juices from the juice packs, at intervals and irrespective of storage conditions between the consumption, could predispose fruit juices to bacterial contamination and consumers to health risks. The test group consumed either orange or red grape juices directly from the juice pack at 3-h intervals for a 6-h drinking regime, while storing the juices at ambient conditions or 4 °C. Control juice had no direct contact with the consumer's mouth. Total bacterial counts (TBC) in the juices consumed directly from the packs and stored at ambient temperature were higher than TBC observed in the juice samples of one consumer. The 16S rRNA sequencing analysis of selected isolates revealed potentially pathogenic bacteria sharing similarities with *Bacillus amyloliquefaciens*, *B. atrophaeus*, *B. cereus, B. subtilis,* and *B. tequilensis*. Some of the identified strains exhibited amylase and hemolytic potentials *in vitro*, while *eae* gene, coding for adherence factors mostly in *Escherichia coli*, was detected in one strain of *B. amyloliquefaciens* in this study. Consumers are advised to pour juices into pre-washed cups before the consumption, and to store leftovers at 4 °C or even lower.

Introduction

The consumption of industrially processed fruit juices has become commonplace in a large proportion of households within the suburban and urban regions of many low- and middle-income countries such as Nigeria. Fruit juices are mostly consumed by children (Heyman and Abrams 2017), but are also taken by some adults (Agarwal *et al*. 2019; Benton and Young 2019). Typically, fruit juices can be processed from raw fruits or concentrates of fruits such as oranges, red grape, apples and pineapples (Clemens *et al*. 2015). Thus, fruit juices can contain an array of nutrients, including carbohydrates, minerals and vitamins (Heyman and Abrams 2017).

One critical stage during fruit juice processing is the microbial inactivation step, which involves the application of techniques such as pasteurization, pulsed-electric field and radiation to kill potentially pathogenic and spoilage microorganisms (Zhang *et al*. 2010; Tremarin *et al*. 2017; Zhu *et al*. 2018). Consequently, industrially processed fruit juices are considered safe for consumers at the retail point of the supply chain. Nevertheless, bacteria such as *Escherichia coli*, and acid-tolerant species within the *Bacillus* genera have been reported in industrially processed fruit juices (Mahgoub and El-Shourbagy 2015; Soltani and Mahdavi 2018). These reports suggest that some fruit juice processors adopt poor manufacturing practices. When conditions are favourable, these bacteria proliferate in the juice, leading to rapid deterioration of nutrients and spoilage. On the other hand, depending on the type and number of potentially pathogenic bacteria in contaminated juice, the quantity of juice consumed, and the age of the consumer, the consumption of such contaminated fruit juice could lead to food poisoning, with adverse health effects such as abdominal pain, diarrhea, and nausea (Ceuppens *et al*. 2011).

Despite the available data on bacterial contamination of industrially processed fruit juice in Nigeria (Braide *et al*. 2012; Ndife *et al*. 2013; Ogodo *et al*. 2016), there is a paucity of data on the bacteriological quality of industrially processed fruit juices mediated by consumer handling practices. Specifically, there is limited data on whether direct and intermittent drinking of fruit juices from the juice packs, while storing the juice under various conditions in between consumption could predispose fruit juices to contamination by pathogenic and food spoilage bacteria. In Nigeria, it is common practice for young adults, especially students of tertiary institutions who are on busy schedules, to consume purchased fruit juices “on the go”. When this happens, the consumption happens directly from the juice packs since the access to the cups may not be feasible. It is also common practice to retain fruit juice consumed during the late morning hours until the early evening hours (approximately six hours duration) when lectures are over, with intermittent sips during this period. On the contrary, in some households, opened fruit juices are refrigerated at temperatures of approximately 4 °C until the consumption. Consequently, it is important to examine the potential health risks associated with these fruit juice consumption practices on consumer health. This study, therefore, investigated the impact of consumer handling on the bacteriological quality of orange and red grape juices.

Materials and methods

Source of fruit juices

Sixteen samples of available industrially processed fruit juices, consisting of eight orange and eight red grape juice varieties, were randomly purchased from a food mall in Ilishan-Remo, Ogun State, Nigeria. The juice samples were 100% fruit juice and all of the same brand, based on food labels given by the manufacturer. All the juice samples were in pre-packed containers of 315 mL and within the shelf-life period stipulated as printed on the expiry date labels on the juice packs.

Experimental design for fruit juice consumption

In this preliminary experiment, two adults (aged 21 and 23) were recruited to voluntarily participate in the study as consumers of the fruit juices. Prior to recruitment, the purpose of the study was explained to each consumer and their consent was obtained. For the experimental set-up, care was taken as much as possible to simulate the "drink as you go" approach practiced among young people. One of the consumers was required to drink one fruit juice variety directly from the juice pack at drinking intervals of 3 h for a 6-h duration (i.e., three drinking times of 0, 3 and 6 h) while the other consumer consumed the other juice variety at the same intervals. The control experiment consisted of juices consumed without direct contact with the consumer mouth. Each experimental group consumed duplicate juice samples. From each group of control and test juices, two sub-groups, based on storage temperatures during the drinking intervals, were set up. One set of the juices was stored at ambient temperature while the other set was refrigerated (at 4 °C). At each time interval, each juice (test and control) was thoroughly homogenized by hand-shaking for 10 seconds, and 10 mL aliquots were aseptically taken for pH analysis and bacterial enumeration. All experiments were conducted in a controlled laboratory environment. This study received approval from the Babcock University Health Research Ethics Committee under the reference number: BUHREC 288/17.

Determination of pH and enumeration of bacteria from fruit juices

The pH of each fruit juice at the sampling intervals was measured using a pH meter (Surgifield Medical, England) as described in AOAC (2000). For the enumeration and isolation of bacteria in the fruit juices, 1 mL aliquots of the fruit juices were serially diluted ten-folds in sterile water and pour-plated on freshly prepared molten nutrient agar (NA; Oxoid Ltd, UK) plates. For control fruit juices, 1 mL aliquots were pour-plated directly on freshly prepared NA plates in duplicate. All the inoculated plates were incubated aerobically at 37 °C for 24 h. Thereafter, total bacterial counts (TBC) of the juice samples were enumerated. Distinct colonies were subcultured repeatedly on freshly prepared NA plates in order to obtain pure isolates.

16S rRNA gene analysis of isolates

Genomic DNA extraction and Polymerase chain reaction (PCR)

Genomic DNA isolation was performed from overnight cultures of bacterial isolates (in NA) by colony PCR. Briefly, a single bacterial colony was picked with a sterile pipette tip and resuspended in 20 µL of Milli-Q® water. The bacterial suspension was homogenized by vortexing and microwaved at 1000 W for 2 min to lyse the cells. Thereafter, cell lysate was centrifuged at 9000 rpm for 90 s and placed immediately on ice. One microliter of the supernatant was used as a DNA template for PCR amplification of the 16S rRNA gene. For the PCR amplification, oligonucleotide primers 27F (5'-AGAGTTTGATCCTGGCTCAG- 3') and 1492R (5'-GGTTACCTTGTTACGACTT- 3') were used. PCR components comprised 12.5 µL of 2X PCR master mix (0.4 mM of each dNTPs, 0.05 U/µL Taq DNA Polymerase reaction buffer and 4 mM $MgCl_2$) (Thermo Fisher Scientific, Waltham, MA, USA), 1 µL of each primer, 1 µL of cell lysate and 9.5 µL nuclease-free water (Thermo Fisher Scientific) in a 25 µl reaction. The optimized thermocycling (BioRad® ThermoCycler; Bio-Rad, USA) conditions were 94 °C for 3 min, 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and final extension at 72 ° C for 10 min. Amplicon sizes (bp) were verified by electrophoresis on 1% (w/v) agarose gel. PCR amplicons were purified using AMpure XP beads (Illumina, CA, USA).

Sequencing

Sequencing of purified amplicons was conducted at the Unit for Environmental Sciences and Management, North-West University, South Africa. Briefly, sequencing PCR reactions was performed using the BigDye Terminator chemistry (Big Dye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems) and universal primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3'). The sequence products were analysed on a SeqStudio™ Genetic Analyzer (2019) (Applied Biosystems, Life Technologies, Singapore). Sequence electropherograms were manually inspected and edited for ambiguous nucleotide positions and initial poor-quality sequences at the 5´ and 3´ ends using Finch TV (version 1.4) software (Geospiza Inc.). The closest phylogenetic relatives of each isolate were identified by comparison of the 16S rRNA gene sequence to the EzBioCloud database (https://www.ezbiocloud.net/; Yoon et al. 2017). Nucleotide sequences obtained in this study are available in the NCBI GenBank under the accession numbers MT355873–MT355882.

Amylase test and hemolysis assay

To determine the *in vitro* pathogenic and spoilage potential of the isolates, screening of each isolate for hemolysin and amylase production was performed. The amylase test was done on freshly prepared starch agar (28 g/L NA supplemented with 5% v/v antibiotic-free human blood), while the hemolysis assay was performed on freshly prepared blood agar (5% v/v antibiotic-free human blood, 28 g/L Nutrient agar). All plates were incubated at 37 °C for 24 h and observed visually for zones of clearance.

Detection of virulence genes

The presence of the *hlyA* and *eae* virulence genes in the isolates was investigated using primers presented in Table 1. For the detection of each gene, the PCR reaction mixture contained 12.5 µL Dream Taq PCR master mix (Thermo Fisher Scientific), 1 µL of each primer (reverse and forward), 1 µL of 20 ng/µL DNA template and 9.5 µL nuclease-free water. The PCR conditions were an initial denaturation at 95 °C for 10 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min, and extension at 72 °C for 1 min and a final extension at 72 °C for 10 min (Paton and Paton 1998).

Table 1. Primers used for virulence gene detection in this study.

Target gene	Name	Sequence (5´- 3´)	Size(bp)	Reference
Eae	*eae*- F	GACCCGGCACAAGCATAAGC	384	(Paton & Paton, 1998)
	eae- R	CCACCTGCAGCAACAAGAGG		
hly A	*hly A*-F	GCATCATCAAGCGTACGTTCC	534	(Paton & Paton, 1998)
	hly A-R	AATGAGCCAAGCTGGTTAAGC		

Statistical analysis

Data on TBC of the juice samples were transformed using the equation y = Log_{10} (1+CFU/mL) to create a normal distribution for pictorial representation. Data was further analyzed using IBM® Statistical Package SPSS® version 25 for Windows. Two sample t-test was applied to test for significance (p=0.05) of means for data on TBC between test and control juice samples at both storage conditions.

Results and discussion

pH of the fruit juices

Both juice types were acidic (Fig. 1), and pH values were similar to values previously reported for orange and grape juices (Lima Tribst *et al*. 2009; Mahgoub and El-Shourbagy 2015). Irrespective of storage

conditions, the pH values of the control and test orange juices ranged between 3.17 and 3.38 (mean ± SD: 3.26 ± 0.07), while control and test red grape juices had pH values between 2.46 and 2.7 (mean ± SD: 2.54 ± 0.08) (Fig. 1). The higher pH value of the test juice compared to the control for both fruit juice varieties could be attributed to a higher bacterial load in the test juices compared to the control juices (Fig 2). It is likely that bacteria in the test juices utilized the energy sources (e.g., citric acid) in the juices to produce compounds (e.g., butanediol) capable of raising pH (Celinska and Grajek 2009).

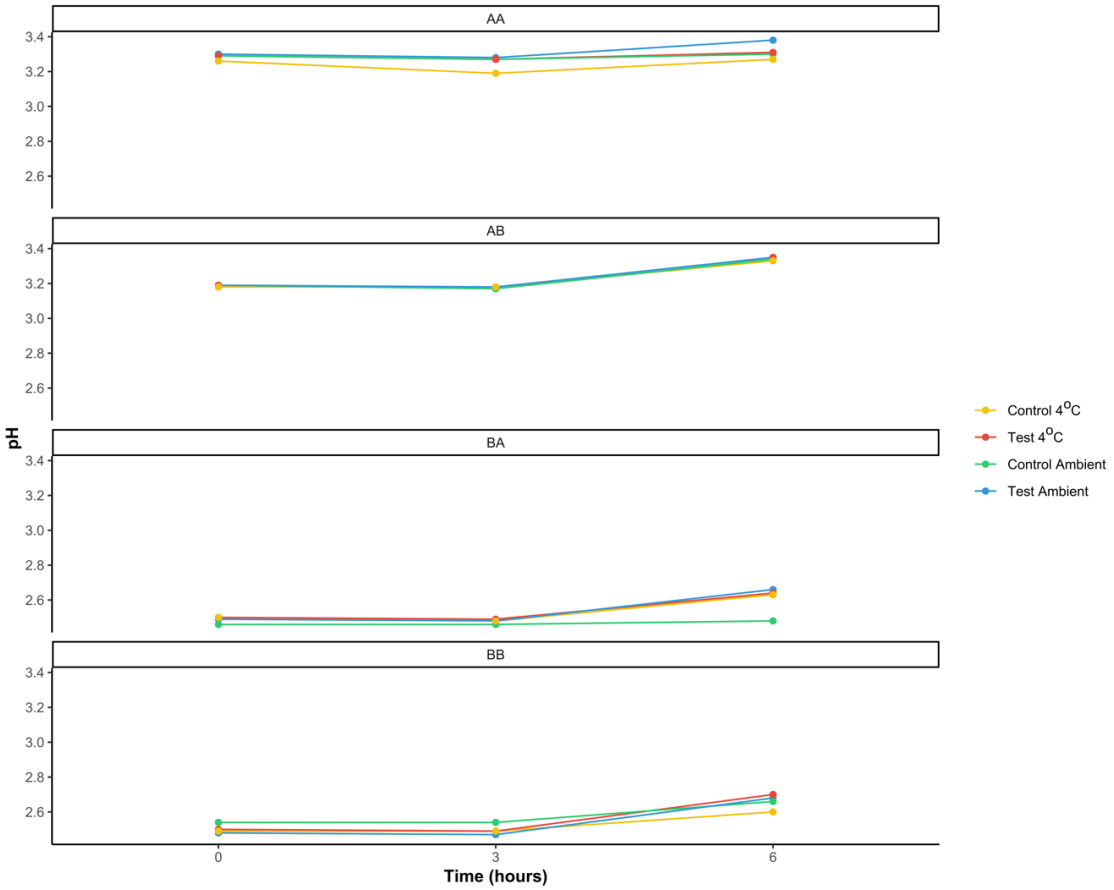

Figure 1. Changes in pH in the fruit juice (**AA:** orange flavoured juice consumed by first consumer; **AB:** orange flavoured juice consumed by second consumer; **BA:** red grape flavoured juice consumed by first consumer; **BB:** red grape flavoured juice consumed by second consumer).

Total bacteria count in the fruit juices influenced by consumer and storage practices, and implications for consumer health

The main objective of this study was to determine whether consumer practice of drinking fruit juices directly from the juice pack, at intervals and irrespective of storage conditions between consumption periods, would predispose fruit juices to bacterial contamination and consumers to health risks. At ambient conditions, TBC in the control juice samples ranged between 1.00 and 1.85 (mean ± SD: 1.43 ± 0.67) Log_{10} 1+CFU/mL, while the count in the test juice samples after direct consumption ranged between 1.00 and 5.08 (mean ± SD: 2.8 ± 2.09) Log_{10} 1+CFU/mL (Fig. 2). At 4 °C, TBC in the control juice samples ranged from 1.00 to 1.94 (mean ± SD: 0.61 ± 0.68) Log_{10} 1+CFU/mL, while TBC in the test juice after consumer handling ranged from 4.00 to 5.07 (mean ± SD: 2.2 ± 2.32) Log_{10} 1+CFU/mL (Fig. 2). A higher TBC in juice samples ($p > 0.05$) stored at the ambient temperature compared to 4 °C storage was observed for one of the two consumers, suggesting that storage conditions may influence bacterial load

with cold storage limiting bacterial growth (Soni *et al*. 2018). This results suggests that the mode of drinking juice may influence the bacterial load in the test juice. There is a possibility that drinking juice dispensed into the cup lowered bacterial contamination of fruit juices being consumed intermittently throughout the day.

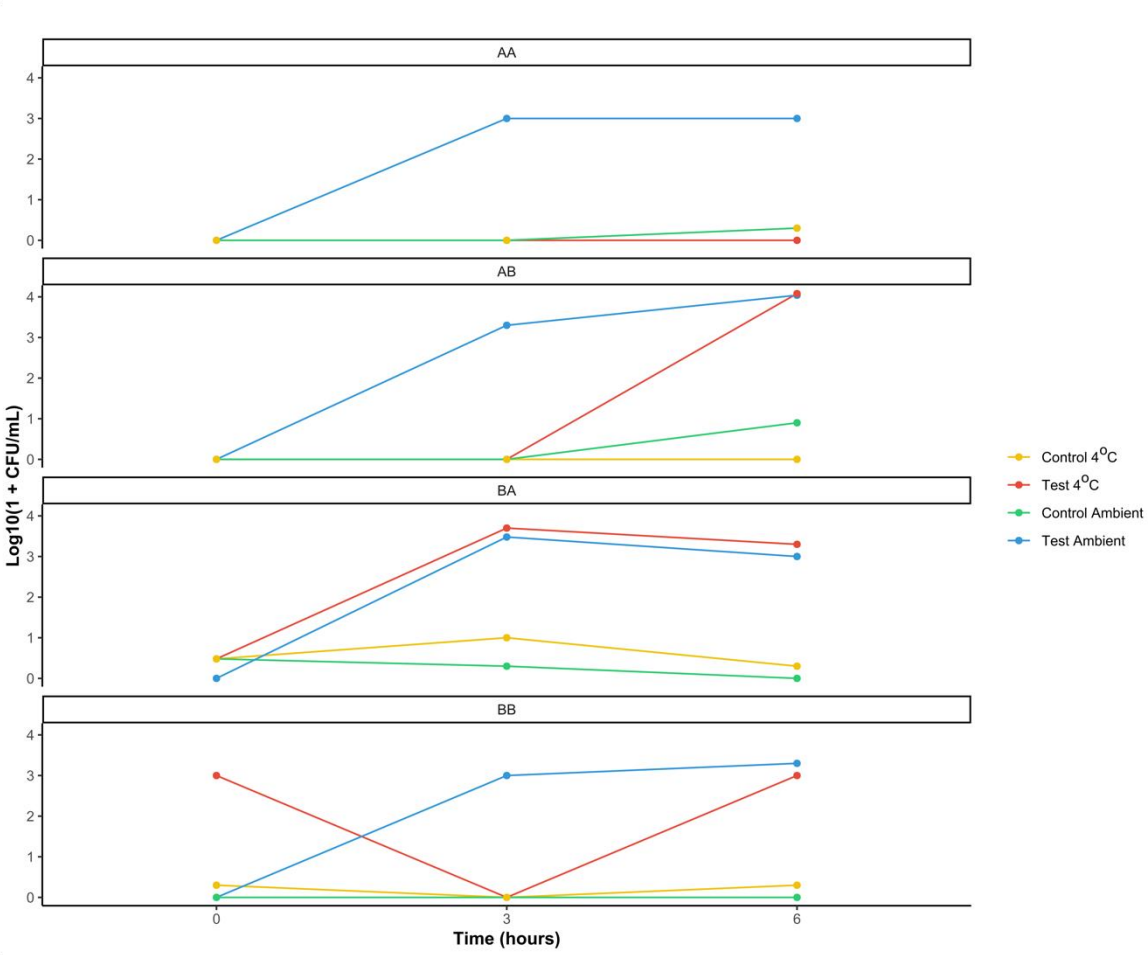

Figure 2. Total bacterial count in the fruit juice (**AA:** orange flavoured juice consumed by first consumer; **AB:** orange flavoured juice consumed by second consumer; **BA:** red grape flavoured juice consumed by first consumer; **BB:** red grape flavoured juice consumed by second consumer).

None of the orange juice variety samples were contaminated with bacteria at 0 h (Fig. 2), suggesting good manufacturing practices during their production. Conversely, bacterial contamination was recorded for the red grape juice variety at 0 h. This suggests poor manufacturing or pre-experimental handling practices during red grape fruit juice processing. Bacterial contamination of industrially-processed fruit juice is not uncommon and has been previously reported in Nigeria (Ndife *et al*. 2013), Egypt (Mahgoub and El-Shourbagy 2015) and Iran (Soltani and Mahdavi 2018). Consequently, fruit juice processors are encouraged to adhere strictly to good manufacturing practices during processing.

Throughout the duration of the experiment, drinking regimes were carefully monitored, but the consumers were allowed to carry on with their daily activities after juice consumption to mimic a real-life scenario. Some of these activities included walking to classrooms to attend lectures and eating. Foodborne pathogens, such as species within the *Bacillus* genus, are ubiquitous, inhabiting several environments (Heyndrickx 2011). As such, it is likely that consumer's hands got contaminated with

bacteria from the environment that were subsequently introduced into the juices during handling/opening of juice packs. In addition, microbiota from the consumer's mouth could have been introduced into the test juices during consumption. This led to the recovery of diverse bacteria from the fruit juices distinct, irrespective of storage conditions. Overall, bacterial load in the two fruit juice varieties examined in the present study were lower compared to bacterial load in other beverages (e.g., 6.74 $\log_{10}$ CFU/mL in tiger nut drink (Ire *et al*. 2020) and 10 x 10^5 CFU/mL in *zobo* drink made from calyx of *Hibiscus sabdariffa* (Oyedele *et al*. 2019). This disparity may be due to the fact that most foodborne pathogens and/or spoilage bacteria grow poorly in acidic environments (Kim *et al*. 2018).

Taxonomy of bacteria recovered from juice samples

Isolates recovered from the test juice samples were initially subjected to preliminary phenotypic identification (data not shown). Based on the results obtained, isolates that shared common phenotypic characteristics were clustered into groups, out of which 10 representatives were selected for 16S rRNA gene sequencing (Table 2). Taxonomically, all the selected isolates were close relatives of *Bacillus* (Table 2). The recovered isolates shared > 99% similarities with *B*. *amyloliquefaciens*, *B*. *atrophaeus*, *B*. *cereus*, *B*. *subtilis* and *B*. *tequilensis*. The recovery of *Bacillus* spp. from the juice samples could be attributed to the ability of species within this genus to form endospores that enable them to withstand harsh environmental conditions such as low temperature and pH (Hornstra *et al*. 2009). Previously, *Bacillus* species have been reported to be dominant in fruit juice from Nigeria (Ogodo *et al*. 2016) and India (Aneja *et al*. 2014). It is important to mention that the colony selection adopted in this study may have resulted in bias regarding species richness. Consequently, other bacterial genera could have been missed out, because phylogenetically distinct species may share similar phenotypic characteristics (Ademola *et al*. 2018).

Table 2. Phylogenetic similarities of 16S rRNA gene of isolates from fruit juice after consumer handling.

Isolate code	EzBioCloud database		Accession number of sequences
	Closest match	Similarity (%)	
FJ1	*Bacillus amyloliquefaciens*	100	MT355873
FJ2	*Bacillus tequilensis*	100	MT355874
FJ3	*B. amyloliquefaciens*	99.67	MT355875
FJ4	*Bacillus subtilis*	99.67	MT355876
FJ5	*B. amyloliquefaciens*	100	MT355877
FJ6	*Bacillus cereus*	100	MT355878
FJ7	*Bacillus atrophaeus*	99.67	MT355879
FJ8	*B. atrophaeus*	99.67	MT355880
FJ9	*B. cereus*	100	MT355881
FJ10	*B. cereus*	99.33	MT355882

Amylase and hemolytic potential of Bacillus from fruit juices

Overall, seven out of the 10 *Bacillus* isolates could produce amylase on starch agar, suggesting an ability to cause rapid deterioration of carbohydrates present in the juice. Such activity potentially leads to spoilage and poor product palatability. Our findings on amylase production by *Bacillus* spp. are consistent with several reports from the literature (Annamalai *et al*. 2011; Abd-Elaziz *et al*. 2020).

In vitro analysis of the 10 *Bacillus* species revealed that one, three and six isolates exhibited beta, alpha and gamma hemolysis, respectively. Due to the observed *in vitro* hemolysis, isolates were further screened for the presence of a hemolytic gene, *hlyA*, which was not detected in any of the *Bacillus* strains. This is in contrast to the report from South Korea, where *hlyA* and other hemolysin genes such as *hlyII* and *hlyIII* were detected in *B*. *cereus* and *B*. *thuringiensis*, which showed hemolysis *in vitro* (Kim *et al*. 2015). Nonetheless, the presence of other hemolysin genes (other than *hlyA*) not investigated in this study in the *Bacillus* isolates cannot be overruled and, as such, merits further investigation. Another highlight of this study was the detection of *eae* gene in one of the *Bacillus* species (*B*. *amyloliquefaciens*). The *eae* gene codes for adherence factors mostly in *Escherichia coli* (Gannon *et al*. 1993; Krause *et al*. 2005; Yang *et al*. 2020). Its role in *Bacillus* is not yet known. Consequently, to the best of our knowledge, we report for the first time on the presence of *eae* in *B*. *amyloliquefaciens* from fruit juice and recommend further studies to unravel its function(s) in *Bacillus* spp. Due to the fact that mothers and/or caregivers sometimes feed leftover fruit juices to infants and young children (IYC), and given the vulnerability of IYC largely typified by a poorly developed immune system (Simon *et al*. 2015), continuous exposure to hemolytic *Bacillus* through the consumption of self-contaminated fruit juices could constitute a health risk to IYC as well as adolescents, young adults and the elderly.

Limitation of the study

This study was limited by low number of consumers due to resource constraints, time and willingness of consumers.

Conclusions

Although this study was limited by a low number of consumers, it provides a novel snapshot and useful data indicating that the direct consumption of fruit juices from juice packs would increase bacterial load in the juices and may lead to deterioration over time. Additionally, individuals who consume leftover fruit juices previously consumed directly from juice packs by them or another consumer (such as family members) and stored at ambient temperature could be at risk of exposure to potential pathogenic bacteria. The results presented herein are highly relevant for households, especially those with vulnerable IYC consumers of fruit juices. We therefore recommend that individuals pour juices into pre-washed cups before the consumption. In addition, in the case that leftover juice will be consumed after 3 h, juices should be kept at 4 °C of lower after the packs are opened.

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***Conflicts of Interest*:** The authors declare no conflict of interest.

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