International Journal of Food Microbiology 299 (2019) 33–38

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Persistence of *Bacteroidales* and other fecal indicator bacteria on inanimated materials, melon and tomato at various storage conditions

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ARTICLE INFO

Keywords:
Produce contamination
Escherichia coli
Risk assessment
Fecal indicators
Bacteroidales

ABSTRACT

In order to determine the microbial safety of produce, conventional fecal indicator bacteria (CFIB) such as *Escherichia coli* and *Enterococcus* are quantified as a standard practice. *Bacteroidales* are also fecal indicators mostly used for water samples; however, their use and persistence in foods has been rarely studied. In this study, persistence of both CFIB and genetic markers of host-specific *Bacteroidales* was determined in artificially contaminated materials and vegetables with different textured surfaces under different storage conditions. Sterile feces were contaminated with *E. coli*, *E. faecalis*, *Bacteroides thetaiotaomicron* (human origin), and *Bacteroidales* from porcine and bovine origin. Feces were applied to filters of mixed cellulose esters and tomatoes (smooth surface) and flat cork coupons and melons (rough surface) and stored at 10 °C/95% relative humidity (RH) and 25 °C/65%RH for up to 25 days. *Bacteroidales* markers were analyzed by real-time polymerase chain reaction (qPCR), whereas CFIB were plated onto selective agars. CFIB detection on filters and cork surfaces declined over time. *E. coli* decreased 2.9 log CFU and 1.2 log CFU per filter and cork, respectively, at 10 °C/95%RH and 5.8 log CFU and 1.8 log CFU per filter and cork, respectively, at 25 °C/65%RH. *E. faecalis* decreased 1.9 log CFU on filters and 1.3 log CFU on cork at 10 °C/95%RH and 2.6 log CFU/filter and cork under both storage conditions. Although *E. coli* levels in tomatoes slightly increased during storage, the levels decreased by the end of the assays. However, CFIB levels in melons stored at 10 °C/95%RH increased after 20 days; when stored at 25 °C/65%RH, these levels increased after five days.

Bacteroidales levels (universal and host-specific markers) in inanimated material and produce did not show significant differences ($P \leq 0.01$) over time. Stability and persistence of *Bacteroidales* genetic markers make them superior to CFIB as markers and are alternatives for determining the risk of exposure to feces-contaminated produce.

1. Introduction

With increasing frequency, outbreaks of foodborne illness have been attributed to fresh and fresh-cut fruits and vegetables worldwide. Contamination of produce with human pathogens has public health and economic consequences (Lynch et al., 2009). Produce contamination can occur at different steps during the production chain, including in the field (soil, water), during harvesting, packaging, distribution, and at the market (Castro-Ibáñez et al., 2017; León et al., 2009). Most outbreaks of foodborne diseases, in which produce has been the pathogen's vehicle, were associated with direct or indirect contact with animal or human feces at some point in the production chain (Conrad et al., 2017; Olaimat and Holley, 2012).

Conventional fecal indicator bacteria (CFIB) such as the total and/or fecal coliforms, *E. coli* and *E. faecalis*, are a group of bacteria normally found in human and animal gastrointestinal tracts (Odagiri et al., 2015). Determination of CFIB in foods is a standard practice for assessing the microbial risk of exposure to human or animal feces. This procedure requires fewer resources than testing directly for enteric pathogens (Ahmed et al., 2016).

Bacteroidales are an order of bacteria that are widely used as fecal indicators and for microbial source tracking in water. These bacteria are highly prevalent in feces (constituting 26%–36% of the intestinal microbiome with fecal levels ranging from 10^9 to 10^{11} colony forming units [CFU]/g), are strict anaerobes, and exhibit high host-specificity in addition to limited survival in the environment; these anaerobes are

Abbreviations: SS, means 0.85% (w/v) saline solution; MST, means microbial source tracking; GEC, means genomic equivalent copies; RH, means relative humidity; CFIB, means conventional fecal indicator bacteria

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https://doi.org/10.1016/j.ijfoodmicro.2019.03.015
Received 25 January 2019; Received in revised form 22 March 2019; Accepted 25 March 2019
Available online 27 March 2019
0168-1605/

also intolerant to oxidative stress preventing their growth and multiplication in the environment, with their genetic material to be present at more constant levels than CFIB under different environmental conditions (Ahmed et al., 2016; Merino-Mascorro et al., 2018). Although most studies have reported the utility of *Bacteroidales* as a fecal contamination indicator in water samples, recent reports have suggested their use as an indicator in the agricultural environment (Merino-Mascorro et al., 2018; Ravaliya et al., 2014).

Since *Bacteroidales* are usually inactive in the environment, various sequences of their 16S RNA gene have been designed to detect fecal pollution from specific hosts (humans, pigs, ruminants, dogs, birds, and wild animals), showing high levels of sensitivity and specificity, whereas other sequences are common to all *Bacteroidales* (Allbac markers) (Mieszkin et al., 2010; Ravaliya et al., 2014).

One of the attributes that a good fecal indicator microorganism must have is the capability of persisting at detectable levels over a long period of time (such as many pathogens do). *Bacteroidales* persistence in the environment is controversial, and studies in marine and freshwater samples have reported fast decay rates and clear effects of various environmental factors (Bae and Wuertz, 2015; Green et al., 2011), whereas others indicated long persistence and non-affectation by environmental factors (Mattioli et al., 2017; Wanjugi et al., 2016).

Studies in our lab showed that *Bacteroidales* markers (DNA) can be detected in fresh produce (Ravaliya et al., 2014). *Bacteroidales* markers specific for human, canine, or bovine samples were also detected in strawberries and tomatoes artificially contaminated with ≥0.1 mg of host-specific feces (Merino-Mascorro et al., 2018). In the latter case, however, detection was made immediately after produce contamination. Thus, persistence of these markers over time needs to be determined, including the influence of texture and storage conditions (for example temperature and humidity), which constitute this study's objectives. Furthermore, the behaviors of host-specific *Bacteroidales* from human, bovine, and porcine samples were also determined and compared with CFIB.

2. Materials and methods

2.1. Bacterial strains

The strains used as controls were *Bacteroides thetaiotaomicron* ATCC 29148 and *Escherichia coli* transformed with a 4071-bp plasmid encoding a specific *Bacteroidales* 16S rRNA sequence (AllBac, *Bacteroidales* universal marker). Both were donated by Dr. Lee-Ann Jaykus (North Carolina State University, Raleigh). *E. coli* ATCC 25922 was kindly donated by Dr. Lynne McLandsborough (University of Massachusetts, Amherst), and *Enterococcus faecalis* ATCC 19433 was purchased from ATCC (Manassas, VA, USA). Strains were maintained as stock cultures at −80 °C in brain heart infusion (BHI) broth (Bioxon, Becton-Dickinson, Mexico) with 20% glycerol (Sigma-Aldrich Mexico). Subcultures of *E. coli* and *E. faecalis* were obtained by transferring an aliquot to tubes containing BHI agar, and after 48 h, they were stored at 4 °C. Fresh cultures were obtained by transferring an aliquot to tubes with 5 ml of BHI broth and incubated for 24 h to yield an A_{595nm} of 0.6 and 0.4 (10^9 and 10^6 CFU/ml respectively, as determined by bacterial count in agar) for *E. coli* and *E. faecalis*, respectively.

Fresh cultures of *B. thetaiotaomicron* were obtained by inoculating frozen aliquots into tubes containing 7 ml of liquid thioglycolate medium (Difco, La Point de Craix, France) and incubating at 37 °C for 48 h. The suspension was adjusted with phosphate saline buffer (PBS) to an $A_{595\ nm}$ of 0.9 corresponding to 10^9 CFU/ml as determined by bacterial count in agar (EPA, 2010).

2.2. Isolation of host-specific strains of Bacteroidales

For the inoculation of produce with feces containing host-specific *Bacteroidales*, it was necessary to isolate the host-specific strains. In order to obtain them, human, porcine, and bovine fecal samples were obtained from human volunteers and from pigs and cows at the Facultad de Agronomía of the Universidad Autónoma de Nuevo León.

Pools of feces from five individuals from each species (humans or animals) in apparently healthy condition were prepared. Pools were diluted (1:10) with 0.85% (w/v) saline solution (SS), and a loopful of the sample was plated onto *Brucella* agar with hemin and vitamin K1 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% of blood, 0.0075 g/l vancomycin, and 0.1 g/l kanamycin and incubated at 37 °C for 24 to 48 h anaerobically (GasPack™ EZ Anaerobe Pouch System, BD Diagnostics, Japan. This system produces an anaerobic atmosphere within 2.5 h with < 1.0% oxygen, and greater than or equal to 13% carbon dioxide within 24 h. Bacic and Smith, 2008).

Presumptive *Bacteroidales* colonies (small to medium size [0.5 to 5 mm], round, and white to beige) were transferred to tubes with 5 ml of cooked meat broth (Oxoid, England) and liquid thioglycolate medium and incubated for 24 h at 37 °C. From the tubes that showed growth, the Allbac marker and host-specific sequences of *Bacteroidales* (Mieszkin et al., 2009 and Kildare et al., 2007) were confirmed by polymerase chain reaction (PCR) as described by Merino-Mascorro et al. (2018).

2.3. Produce samples

Samples of tomato (*Lycopersicon esculentum* var. Saladett) of 1 to 2 ripeness grade (USDA, United States Department of Agriculture, 1991) and melon (*Cucumis melo* var. Cantaloupe) of 1 to 3 ripeness grade (León, 2007) with no visible damage (fungal growth, putrefaction, or discontinuous skin) were obtained from local supermarkets in Metropolitan Monterrey, Nuevo Leon, Mexico, and transported to the laboratory.

Organic material and dirt were removed from the tomatoes and melons by gentle swabbing with plain liquid soap (Salvo, Procter & Gamble Manufactura, Mexico) for 1 min and rinsed with sterile distilled water. Vegetables were disinfected by spraying the surface with 70% ethanol (Sigma Aldrich, Mexico), and placed into Nasco bags containing 500 ml of detergent (Likofen B10, Productos Blitzer, S.A. de C.V., Mexico). After agitation for 1 min, samples were washed with sterile distilled water and dried with a paper towel, followed of gentle swabbing with 95% alcohol for 1 to 5 min and 2.5% (v/v) sodium hypochlorite (Cloralex, Alen del Norte S.A. de C.V., México) for 1 to 5 min, rinsed three times with sterile distilled water, dried for 1 h at room temperature in a flow chamber (Laminar Flow Cabinet, Streamline®, Vertical), and exposed to ultraviolet (UV) light (254 nm) on each side for 20 min. A sub-sample of cleansed produce was analyzed to confirm removal of viable bacteria and for the presence of specific DNA sequences by PCR (Merino-Mascorro et al., 2018).

2.4. Fecal contamination

Human, porcine, and bovine fecal samples were sterilized (121 °C/30 min) and exposed to UV light (254 nm) for 10 min four times (Ahn et al., 2013). A sub-sample of the feces was analyzed to confirm that *Bacteroidales* genetic markers were undetected by PCR and bacteria by plating in culture media (Ravaliya et al., 2014; Kildare et al., 2007; Mieszkin et al., 2009).

Feces were diluted 1:10 with SS, vortexed, and filtered through Whatman no. 5 paper (Buckinghamshire, UK) in order to remove any remaining solids. Human, porcine, and bovine fecal samples were then mixed (1:1:1) and inoculated with *E. coli* (final concentration 10^8–10^9 CFU/ml), *E. faecalis* (final concentration 10^6–10^7 CFU/ml), and *Bacteroidales* from pigs and cows and *B. thetaiotaomicron* (human) at final concentrations of 10^9 to 10^{11} CFU/ml. The selected bacterial concentrations corresponded to fecal concentrations that can be found in human or animals (McQuaig et al., 2012; Mieszkin et al., 2010; Silkie and Nelson, 2009).

2.5. Influence of surface texture and storage conditions in Bacteroidales and CFIB persistence

Bacterial persistence was analyzed in two inanimate materials and two vegetables with different texture surfaces. The inanimate materials were 47 mm diameter cellulose mixed ester sterile filters with 0.45 μm pores (S-Pak®, EMD Millipore Corp, Billerica, MA), which simulated a smooth surface, and sterile coupons of flat cork, 47 mm diameter and 2 mm wide, which simulated a rough surface.

Ten 10-μl aliquots from the contaminated fecal pool were spotted over the previously decontaminated materials. Contaminated material was replicated in amounts sufficient enough for sampling at different times. Filters and cork coupons were placed over SS-wet sterile sponges and stored under refrigerated conditions (10 °C/95% relative humidity [RH]) and under room conditions (25 °C/65%RH) for 25 days in a Bioclimatic Chamber (Forma Environmental Chamber, Thermo Scientific, Marietta OH). Samples of filter and cork coupons were taken at days 0, 13, 19, and 25 and placed into a Whirl-Pak® bag (Nasco, Ft. Atkinson, WI) containing 10 ml of SS. After 60 s of manual homogenization, suspension samples were used for CFIB and host-specific *Bacteroidales* enumeration.

Batches of produce composed of three tomatoes or one melon that had been previously decontaminated were inoculated by applying 10-μl aliquots of contaminated feces (10 aliquots per each tomato and 30 aliquots per melon). Produce samples were placed into a bioclimatic chamber at 10 °C/95%RH or 25 °C/65%RH. Immediately after (0 days), 10, and 20 days at 10 °C/95%RH or 0, 5, and 10 days under room conditions, batches were placed into sterile 16 oz. Whirl-Pak® bag (Nasco) containing 300 ml of SS and manually shaken for 60 s. Rinsates were used to quantify traditional CFIB and *Bacteroidales*.

2.6. CFIB enumeration

The method proposed by Heredia et al. (2015) was followed. Rinsates from filters, cork coupons, and produce items were diluted 10-fold, and aliquots were inoculated onto Petri dishes containing *Rapid'E.coli* 2™ agar (Biorad, USA) for *E. coli* growth and KF agar (Neogen, Michigan, USA) for *Enterococcus* growth. Plates were incubated at 37 °C for 24 to 48 h before enumeration of typical colonies (pink to purple for *E. coli* and red-centered colonies for *E. faecalis*).

2.7. Bacteroidales detection

Different volumes of the rinsates (5 ml from filters and cork coupons or 150 ml of produce rinses) were passed through sterile membrane filters (0.45 μm pores, 47 mm diameter, mixed cellulose esters, S-Pak®, EMD Millipore Corp, Billerica, MA) using a sterile vacuum-manifold filtration system (Pall Corp, Port Washington, NY). DNA for *Bacteroidales* analysis was extracted from the filters following the method described by Ravaliya et al. (2014) with some modifications using the commercial kit ISOLATE Fecal DNA (Bioline, London, England) according to the manufacturer's instructions.

The *Bacteroidales* marker (AllBac) found in all *Bacteroidales* (Ravaliya et al., 2014), and host-specific *Bacteroidales* sequences were evaluated by real-time (q)PCR from the resulting DNA (Table 1) using a Pikoreal 96 (Thermo Fisher Scientific Oy, Vantaa, Finland) and a SensiFAST Probe master mix (Bioline, London, UK). Thermocycler conditions consisted of several parameters: (1) 1 cycle 2 min 95 °C; (2) 40 cycles 15 s 95 °C; (3) 30 s 53 °C (Allbac and human), 54 °C (cow), or 60 °C (porcine); and (4) 30 s 72 °C.

Absolute quantification curves for AllBac and host-specific (human, cow, and pig) *Bacteroidales* were developed. Transformed *E. coli* containing plasmids with AllBac and host-specific *Bacteroidales* sequences were used (Merino-Mascorro et al., 2018). The plasmid DNA concentrations in the dilutions were determined by measuring absorbance A_{260} using a Nanodrop system (mod. 2000, Thermo Scientific, Massachusetts, USA). Serial DNA dilutions were made, and the genomic equivalent copies (GEC) were calculated using the formula (Staroscik, 2004):

$$GEC = (A_{260} \times 6.022 \times 10^{23})/(\text{length} \times 1 \times 10^{9} \times 650)$$

Host-specific *Bacteroidales* sequences for quantitation calibration curves based on qPCR assays were obtained from *E. coli* DH5α transformed in our laboratory with host-specific *Bacteroidales* sequences (human, bovine, and porcine, Table 1) using the TOPO® TA Cloning® Kit following the manufacturer protocol. Ten colonies (from each plate) of transformed *E. coli* DH5α were subjected to PCR to confirm the presence of the specific *Bacteroidales* sequences. The amplicon sizes were 166 bp for human, 145 bp for bovine, and 116 bp for pig. Once the DH5a *E. coli* cells were transformed, the isolates were grown in LB/ampicillin agar plates and incubated at 37 °C/24 h. The resulted colonies were inoculated in LB/ampicillin medium for 16 h, and the plasmid was extracted by alkaline lysis, following the standard mini prep methodology (Sambrook and Russell, 2001).

2.8. Statistical analysis

All experiments were performed at least twice, each experiment in duplicate. Differences in the reduction of CFIB and *Bacteroidales* over time under the two types of storage conditions were determined by analysis of variance ([ANOVA]; $\alpha = 0.05$), and the comparison between the host-specific *Bacteroidales* was done by Tukey test. For all analyses, the SPSS Version 21 software (IBM SPSS Statistics) was used.

3. Results

3.1. Bacteroidales marker detection

Thirty-five presumptive *Bacteroidales* isolates were obtained from different hosts' fecal samples (12 from human samples, 14 from cow samples, and 9 from pig samples). The isolates were subjected to analysis of their respective host-specific sequences. One isolate of each host which did not cross reacted with other host-specific sequences was selected, and used in the assays.

Considering that *Bacteroidales* cells have 4–7 copies of their 16S rRNA gene (Layton et al., 2006), the limit of detection for the Allbac marker in this study was 172 GEC/μl ($R^2 = 0.98229$), which is equivalent to 26 CFU per10 ml (in the case of membrane or cork), or per 150 ml of produce batch (1 melon or 3 tomatoes). Similar limits of detection were obtained for the cow and pig -specific *Bacteroidales* ($R^2 = 0.96832$ and 0.96499, respectively), whereas for humans, the limit of detection was 58 GEC/μl ($R^2 = 0.96904$), which is equivalent to 9 CFU per 10 ml (in the case of membrane or cork), or per 150 ml of produce batch (1 melon or 3 tomatoes).

3.2. Influence of texture and storage condition in persistence of CFIB and Bacteroidales

When filter and cork coupons were contaminated with the inoculated feces, *Bacteroidales* levels (Allbac and host-specific markers) did not show significant differences ($P \leq 0.01$) over time (Table 2). However, the levels of CFIB in filter and cork coupons decreased significantly ($P \leq 0.01$) over time (Table 2). In the case of *E. coli*, a decrease of 2.9 log CFU/filter (from 7.1 ± 1.1 to 4.2 ± 1.1 log CFU/ml) and 1.2 log CFU/cork (from 6.5 ± 1.1 to 5.3 ± 1.2 log CFU/cork) were detected at storage day 25 at 10 °C/95%RH. However, when the storage condition was 25 °C/65%RH, the observed reduction was 5.8 log CFU/filter (from 7.2 ± 1.2 to 1.4 ± 1.4 log CFU/filter) to 1.8 log CFU/cork (from 6.6 ± 1.2 to 4.8 ± 1.1 log CFU/cork). More discreet, but significant ($P \leq 0.01$), reductions were observed for *E. faecalis*. When the filter was stored for 25 days at 10 °C/95%RH, the microorganism decreased 1.9 log CFU/filter (from 6.8 ± 1.2 to 4.9 ± 1.2

Table 1
Primer sequences for the amplification of host-specific *Bacteroidales*.

Feces origin	Marker name	Gene amplified	Primer sequence 5′-3′	Size (bp)	Reference
All feces	AllBac	AllBac296f	GAGAGGAAGGTCCCCCAC	106	Kildare et al., 2007
		AllBac412r	CGCTACTTGGCTGGTTCAG		
		AllBac375Bhqr	CCATTGACCAATATTCCTCACTGCTGCCT		
Human	Bvulg	BVulgF1	CATCATGAGTCCACATGTTCA	166	
		BFDRev	CGTAGGAGTTTGGACCGTGT		
		BFDFAM	CTGAGAGGAAGGTCCCCCACATTGGA		
Cow	BoBac	BoBac367f	GAAG(G/A)CTGAACCAGCCAAGTA	109	
		BoBac467r	GCTTATTCATACGGTACATACAAG		
		BoBac402Bhqr	TGAAGGATGAAGGTTCTATGGATTGTAAACTT		
Pig	Pig-2-Bac41F	BacCan-545f1	GCATGAATTTAGCTTGCTAAATTTGAT	116	Mieszkin et al., 2009
	Pig-2-Bac163Rm	BacUni-690r1	ACCTCATACGGTATTAATCCGC		

log CFU/filter). When it was in cork coupons, it decreased 1.2 log CFU/cork (from 6.9 ± 0.2 to 5.6 ± 1.1 log CFU/cork), and when it was stored at 25 °C/65%RH, *E. faecalis* decreased 2.6 CFU/ml in both filter and cork coupons.

When contaminated feces were inoculated onto tomato or melon, *Bacteroidales* markers also remained at stable levels over time under both types of storage conditions (Table 3). However, contrasting behaviors of CFIB were found in which *E. coli* decreased ($P \leq 0.01$) 0.3 log CFU/item (from 4.1 ± 0.3 to 3.8 ± 0.4 log CFU/item) when tomatoes were stored at 10 °C/95%RH and decreased 2.2 log CFU/item (4.2 ± 0.3 to 2.0 ± 0.4 log CFU/item) when these were stored at 25 °C/65%RH for 10 days. However, in melons, an increase of 0.7 log CFU/ml (from 4.8 ± 0.3 to 5.5 ± 0.2 log CFU/item) was observed when stored at 10 °C/95%RH for 20 days. When melons were stored at 25 °C/65%RH for 10 days, an increase of 1.8 log CFU/item (from 4.5 ± 0.4 to 6.3 ± 0.3 log CFU/item) was observed at day 10. *E. faecalis* levels in tomatoes under both types of storage conditions were relatively stable over time (Table 3); however, in melon, levels increased 0.6 log CFU/item (from 4.9 ± 0.3 to 5.5 ± 0.2 log CFU/item) when stored at 10 °C/95%RH and 1.4 log CFU/item (from 5.1 ± 0.2 to 6.5 ± 0.3 log CFU/item) when stored at 25 °C/65%RH at the end of the two storage periods.

4. Discussion

Controlling pathogens in fruits and vegetables is complicated since contamination can occur from various sources and during all steps in the farm-to-consumer continuum, including in the field, during harvesting, packaging, distribution, at retail, and even handling at home (Garcia and Heredia, 2017). Investigations of outbreaks on farms and in packing facilities suggest that sources of produce contamination may include animal droppings, farmworkers' hands, soil, agricultural water, tools, equipment, and other contact surfaces (Bartz et al., 2017).

Because of limitations for direct pathogen monitoring, it is a standard practice to monitor CFIB such as *E. coli* and fecal *Enterococci* in order to predict the presence of fecal pollution (Field and Samadpour, 2007). Produce contaminated with human feces are regarded as a greater risk to human health as these are more likely to contain human-specific enteric pathogens; however, animals can also serve as reservoirs for a variety of enteric pathogens (Blaustein et al., 2015).

Because of their abundance in the intestinal tract of humans and animals, *Bacteroidales* have been proposed as indicators of fecal contamination in water (Bäckhed et al., 2005; Odagiri et al., 2015). The *B. thetaiotaomicron* used in this study has been reported to possess good human-specific correlation, and the presence of human pathogens such as *Campylobacter* spp. and *Salmonella* spp. was highly correlated with the presence of this microorganism in the analyzed samples (Converse et al., 2009; Jokinen et al., 2010; Haugland et al., 2010; Marti et al., 2017).

Several factors such as produce type, cultivar, physiological state of the plant, microbial pathogen (Critzer and Doyle, 2010), capability of forming a bacterial biofilm in addition to plant surface hydrophobicity among others can influence the presence of pathogens on vegetable surfaces (Olaimat and Holley, 2012). Also, high levels of humidity and organic matter on the produce's surface support microbial life, and the static charge is determinant in their capability to survive in this environment, allowing microbial adherence, colonization, and persistence (Torres-Aguilar et al., 2015). In this study, the influence of different storage conditions on the persistence of CFIB and *Bacteroidales* markers on rough and smooth surfaces of inanimate surfaces and vegetables was analyzed. Our data showed that texture of surfaces (filter, cork, tomatoes, and melon) did not significantly influence the persistence of *Bacteroidales* over time. An interesting finding was that bacterial recovery in many cases was higher on inanimate materials (membranes

Table 2
Quantitation of CFIB and *Bacteroidales*-microbial source tracking (MST) from contaminated filter and cork coupons (smooth and rough surfaces) under different storage conditions. Each value represents the mean ± standard deviations of three independent repetitions ($n = 3$).

Storage conditions	Day	Membrane (smooth surface)						Cork coupon (rough surface)					
		Bacteroidales (log CFU/filter)				CFIB (log CFU/filter)		*Bacteroidales* (log CFU/cork)				CFIB (log CFU/cork)	
		Allbac	Human	Cow	Pig	*E. coli*	*E. faecalis*	Allbac	Human	Cow	Pig	*E. coli*	*E. faecalis*
10 °C 95% RH	0	6.2 ± 1.2	4.9 ± 2.2	2.0 ± 1.5	3.4 ± 1.2	7.1 ± 1.1	6.8 ± 1.2	5.7 ± 1.2	5.7 ± 1.8	2.3 ± 1.2	3.5 ± 1.5	6.5 ± 1.1	6.9 ± 0.2
	13	6.7 ± 1.4	5.1 ± 2.1	2.4 ± 1.5	3.4 ± 1.3	6.6 ± 1.2	6.3 ± 1.2*	5.7 ± 1.2	5.5 ± 1.3	2.2 ± 1.3	3.6 ± 1.1	7.1 ± 1.1	6.2 ± 1.1
	19	5.9 ± 1.3	5.3 ± 1.7	3.0 ± 1.8	3.8 ± 1.3	6.9 ± 1.1	6.2 ± 1.1*	5.6 ± 1.2	5.3 ± 1.7	2.1 ± 1.6	2.7 ± 0.1	7.4 ± 1.2	6.1 ± 1.2
	25	5.7 ± 1.4	5.9 ± 1.2	3.0 ± 1.8	3.3 ± 1.3	4.2 ± 1.1*	4.9 ± 1.2*	5.9 ± 1.3	5.5 ± 1.4	2.2 ± 1.2	3.4 ± 1.1	5.3 ± 1.2*	5.6 ± 1.1*
25 °C 65% RH	0	5.8 ± 1.2	5.9 ± 1.6	2.2 ± 0.3	3.0 ± 1.2	7.2 ± 1.2	6.7 ± 1.3	6.1 ± 1.1	4.8 ± 1.0	1.9 ± 1.7	3.3 ± 1.3	6.6 ± 1.2	6.1 ± 1.2
	13	5.6 ± 1.2	4.6 ± 1.3	3.7 ± 1.2	3.4 ± 1.1	2.5 ± 1.4*	4.8 ± 1.2*	5.9 ± 1.1	5.1 ± 1.6	2.3 ± 1.2	3.5 ± 1.0	4.8 ± 1.2*	4.7 ± 1.2*
	19	5.5 ± 1.2	4.9 ± 1.7	2.7 ± 2.1	3.3 ± 1.0	1.8 ± 1.4*	4.1 ± 1.2*	6.0 ± 1.3	4.4 ± 1.2	1.2 ± 1.4	3.4 ± 0.1	4.6 ± 1.2*	4.7 ± 1.2*
	25	5.8 ± 1.2	4.9 ± 1.7	3.0 ± 1.2	4.0 ± 1.1	1.4 ± 1.4*	4.1 ± 1.2*	4.6 ± 1.3	5.2 ± 1.5	3.0 ± 1.8	3.4 ± 1.3	4.8 ± 1.1*	3.5 ± 0.1*

* Treatment presented statistical differences ($P \leq 0.01$) when compared with Day 0.

Table 3
Quantitation of CFIB and *Bacteroidales*-MST from contaminated tomatoes and melons under different storage conditions. Each value represents the mean ± standard deviations of three independent repetitions ($n = 3$).

Storage conditions	Day	Tomato						Melon					
		Bacteroidales (log CFU/item)				CFIB (log CFU/item)		*Bacteroidales* (log CFU/item)				CFIB (log CFU/item)	
		Allbac	Human	Cow	Pig	*E. coli*	*E. faecalis*	Allbac	Human	Cow	Pig	*E. coli*	*E. faecalis*
10 °C 95% RH	0	3.1. ± 0.4	3.5 ± 1.7	2.8 ± 1.1	3.2 ± 0.9	4.1 ± 0.3	4.9 ± 0.2	3.5 ± 1.3	3.2 ± 1.3	2.5 ± 1.2	3.5 ± 0.2	4.8 ± 0.3	4.9 ± 0.3
	10	3.0. ± 0.4	3.2 ± 1.0	2.6 ± 0.2	3.2 ± 0.4	5.0 ± 0.4*	4.0 ± 0.4*	3.7 ± 0.0	4.1 ± 1.7	2.6 ± 1.0	3.7 ± 0.1	4.8 ± 0.1	4.4 ± 0.2
	20	3.0. ± 0.4	3.1 ± 2.0	2.7 ± 1.2	3.1 ± 0.0	3.8 ± 0.4*	5.1 ± 0.4	3.0 ± 0.4	4.3 ± 1.5	3.4 ± 1.1	3.5 ± 0.1	5.5 ± 0.2*	5.5 ± 0.2*
25 °C 65% RH	0	3.7 ± 0.2	4.2 ± 1.6	3.0 ± 1.1	3.0 ± 0.7	4.2 ± 0.3	4.9 ± 0.2	3.3 ± 0.5	4.1 ± 0.9	2.6 ± 0.8	3.5 ± 0.3	4.5 ± 0.4	5.1 ± 0.2
	5	3.0 ± 0.5	4.0 ± 2.6	2.5 ± 1.3	3.2 ± 0.1	4.8 ± 0.3	5.1 ± 0.4	2.7 ± 0.3	3.9 ± 1.5	2.5 ± 0.6	3.3 ± 0.2	5.9 ± 0.5*	6.0 ± 0.3*
	10	3.0 ± 1.1	4.9 ± 2.4	2.1 ± 1.2	2.8 ± 0.7	2.0 ± 0.4*	5.0 ± 0.4	4.1 ± 0.3	3.7 ± 1.8	2.6 ± 1.2	3.5 ± 0.3	6.3 ± 0.3*	6.5 ± 0.3*

* Treatment presented statistical differences ($P \leq 0.01$) when compared with Day 0.

and corks) when compared with produce (tomato and melon). These differences could be due to surface characteristics; it is known that substratum surface properties such as morphology, surface chemistry, roughness, and porosity can all exert a strong influence on bacterial attachment to different surfaces (Mitik-Dineva et al., 2009). The types of surfaces and storage conditions also influenced CFIB persistence. A decrease in CFIB levels was observed on the inanimate surfaces in addition to the *E. coli* on the tomato surface; however, viability of CFIB increased on melon surfaces under both types of storage conditions.

Various crops are washed and disinfected postharvest, these processes could disrupt the natural microbiota and the organic material coating and alter bacterial attachment and survival under natural conditions. In this study the crops were washed and disinfected to decontaminate them and conduct the experiments.

E. faecalis is capable of tolerating, surviving, and even replicating in extra-enteric environments, persisting longer that other CFIB (Boehm and Sassoubre, 2014). As expected, persistence of *E. faecalis* was less affected by different storage conditions.

The vegetable type also influenced the persistence of bacteria over time, allowing not only the survival of *E. faecalis* (on tomatoes), but also its growth (on melons) during storage at specific times. Melons have ridges and fissures that could provide a niche and protection from environmental stress, thus favoring microbial growth and entrapment, often forming biofilms on the surfaces that are difficult to remove even by washing treatments (Dobhal et al., 2015). An interesting finding was that of *E. coli* levels on tomatoes slightly increased when stored for 10 days at 10 °C/95%RH and when stored for five days at 25 °C/65% RH; however, the levels had decreased by the end of the assays. This decrease in viability could be due to the stress-related conditions under prolonged storage conditions, especially temperature and humidity reduction (Tiefenthaler et al., 2009). Low humidity will discourage bacteria from growing on fruit and vegetable surfaces and can also lead to dehydration and product shrinkage (Brackett, 1987).

Temperature is a critical factor that affects bacterial survival. Cooler temperatures generally permit longer survival times resulting from growth rate reduction of most microorganisms; however, only a few can survive on vegetables at temperatures used to maintain product quality over long periods (Olaimat and Holley, 2012; Venglovsky et al., 2018). In our study, CFIB stored at 10 °C/95%RH in melons increased in numbers after 20 days, and when stored at 25 °C/65%RH, CFIB increased after five days. Various reports indicate the capability of *E. coli* to obtain diverse nutrients, aiding in its survival in open environments and extreme conditions by means of filamentous structures that help the bacteria attach to surfaces such as melon peel (van Elsas et al., 2011). Owing to this capability, *E. coli* may colonize the grooves of melon peels, thus finding a propitious environment and nutritious conditions that facilitate survival or even growth (van Elsas et al., 2011). *E. faecalis* also can grow on the melon surface when it is stored at these conditions. Tolerance to extreme environments such as dehydration or even radiation have been reported for some strains of *Enterococcus* spp., which allows persistence in unfavorable conditions such as slurry after 115 days at 20 °C (Venglovsky et al., 2018).

In this study, *Bacteroidales* marker levels remained stable over time on all of the analyzed surfaces under both types of storage conditions, indicating that although bacteria were unable to growth outside host (Ravaliya et al., 2014), its DNA remained at detectable levels. The similar prevalence of various host-specific *Bacteroidales* markers (humans, cows, and pigs) suggests that other host markers could have similar behaviors; however, more studies concerning their prevalence should be conducted. Stability is an advantage of using *Bacteroidales* to detect fecal contamination that was the purpose of this study. If a bacterial indicator grows in the environment, then its presence could be the result of its ability to grow in the environment and not as the result of fecal contamination.

In conclusion, this study supports the idea that CFIB detection could be influenced by produce types and storage conditions; furthermore, these can replicate in extra-enteric environments; thus, that they are not effective parameters for the determination of fecal contamination. On the other hand, the persistence and stability of *Bacteroidales* genetic markers provide a better indication of previous fecal contamination than CFIB did under the conditions used in this study.

Acknowledgments

This study was supported by a grant from the Consejo Nacional de Ciencia y Tecnología de México (CONACYT) grant # 213333, and Universidad Autónoma de Nuevo León. G.O.C. acknowledges a postgraduate fellowship from CONACYT.

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