Research article

The prevalence of foodborne pathogenic bacteria on cutting boards and their ecological correlation with background biota

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Abstract: This study implemented the pyrosequencing technique and real-time quantitative PCR to determine the prevalence of foodborne pathogenic bacteria (FPB) and as well as the ecological correlations of background biota and FPB present on restaurant cutting boards (CBs) collected in Seri Kembangan, Malaysia. The prevalence of FPB in high background biota (HBB) was lower (0.24%) compared to that of low background biota (LBB) (0.54%). In addition, a multiple linear regression analysis indicated that only HBB had a significant ecological correlation with FPB. Furthermore, statistical analysis revealed that the combinations of *Clostridiales, Flavobacteriales,* and *Lactobacillales* orders in HBB had significant negative associations with FPB, suggesting that these bacteria may interact to ensure survivability and impair the growth of pathogenic bacteria.

1. Introduction

Foodborne pathogenic bacteria (FPB) are carried by food and can cause infections in the gastrointestinal system [1,2]. Most FPB and bacteria indicator of fecal contamination belong to the *Enterobacteriaceae* family, under the *Proteobacteria* phylum, and are usually associated with bacteria such as *Listeria monocytogenes, Escherichia coli, Salmonella, Campylobacter, Yersinia enterocolitica, Cronobacter* spp. and *Shigella* [3]. Foodborne diseases have always been a concern in food service establishments such as restaurants, canteen, cafeteria and food stalls. Additionally, to trigger an illness in humans, FPB must exceed the infectious dose level, which differs among species. Examples of infectious doses are 10^5 organisms of *Salmonella* and *E. coli* [4], 700 organisms of *E. coli* O157:H7 [5], 10^4 to 10^5 CFU/ml of *Bacillus cereus* [6], 10 CFU/g of *Cronobacter sakazakii* [7] and 10^5 to 10^6 cells of *Yersinia enterocolitica* [8]. This indicates that the bacterial number, and not the percentage of FPB in an environment, can determine the potential risk of developing foodborne illnesses.

Moreover, dirty conditions or low-grades food premises, do not necessarily harm consumers, because of the protective effect of the human immune system and microflora in the gut [9,10]. Various studies have documented the ability of indigenous microflora to affect the growth of pathogens [11], which also have a greater antagonistic effect in a mixed culture of bacteria [12]. Previous studies have also reported that certain bacteria can impair the growth of pathogenic bacteria, such as the ability of lactic acid bacteria (LAB) and *Pseudomonas* to produce antimicrobial compounds that eliminate competing bacteria [13–17]

Pyrosequencing analysis has been widely used to identify bacteria from food and food-related samples [18–21]. In the present report, an extended and enhanced analysis from a previous study by Abdul-Mutalib et al. [22] was implemented. Our initial observations indicated that the level of cleanliness of food premises was not significantly associated with the number of FPB on CBs. The previous study also found that some high-grade food premises harboured a high number of total bacteria. Therefore, the present study determined whether the total bacteria and background biota could influence FPB populations. The total bacteria were divided into two groups: high background biota (HBB) and low background biota (LBB). The aim of this study was to determine the prevalence of FPB in high and low background biota and the ecological relationship between FPB and other bacteria present in the CB samples, through pyrosequencing analysis.

2. Methods

2.1. DNA isolation

Methods in this study were identical to a previous study by Abdul-Mutalib et al. [22]. In brief, 26 CBs were obtained from various food premises around Seri Kembangan, Malaysia. The CBs were requested from the food handlers who work in the premises. All CBs made of wood or plastic were

accepted as were and brought to the laboratory for analysis. For the bacterial DNA extraction, an approximately 10 cm^2 centre of the CB was swabbed using wet sterile sponges 3.8 $\times$ 7.6 cm (3M^{TM}, USA) using 40-vertical S-strokes, followed by immersion in 90 mL of sterile buffered peptone water (Difco, USA) prior to homogenization for 10 minutes. DNA extraction was performed using UltraClean Microbial DNA Isolation Kit (MO Bio Laboratories, USA) from 1.8 mL of samples according to the manufacturer's instructions. Concentrations and quality of DNA were determined using Nanodrop 2000 (Thermo Scientific, USA) [23].

2.2. Pyrosequencing and real-time PCR

Table 1. Tag sequences inserted for pyrosequencing.

Samples name	Tag sequences	DNA concentration (ng/ μl)
cb1	CAGTACGTACT	26.8
cb2	CGATACTACGT	33.4
cb3	CTACTCGTAGT	22.9
cb5	ACGATGAGTGT	14.1
cb6	ACGTCTAGCAT	8.1
cb7	ACTCACACTGT	50.6
cb8	ACTCACTAGCT	36.6
cb9	ACTGATCTCGT	23.4
cb10	ACTGCTGTACT	12.3
cb11	AGACACTCACT	74.4
cb12	AGACGTGATCT	33.0
cb13	AGATACGCTGT	53.0
cb14	AGTATGCACGT	42.5
cb15	AGTCTGTCTGT	15.0
cb16	ATCGTCAGTCT	19.6
cb17	ATCTGAGACGT	14.5
cb18	ATGCTACGTCT	82.0
cb19	CACTACGATGT	30.3
cb20	CAGTCTCTAGT	43.9
cb21	CGAGACACTAT	46.5
cb22	CGTATAGTGCT	35.5
cb23	CTAGACAGACT	36.5
cb24	CTATCGACACT	30.1
cb25	CTCACGTACAT	56.4
cb26	AGTACGAGAGT	27.2
cb27	AGTAGACGTCT	46.3

Samples were amplified using a forward primer, F357 (5'-CCTACGGGAGGCAGCAG-3') and a reverse primer, R926 (5'-CCGTCAATTCCTTTRAGTTT-3'). Short tag sequences (2.5 µL) specific for each sample, were also inserted for analysis (Table 1). PCR amplifications were performed in a total volume of 50 µL containing 25 µL of Premix Ex Taq (Takara Bio, Japan), 2.5 µL of forward and reverse primer (10 µM), 2.0 µL of the template and 18 µL of dH$_2$O. The following conditions of the thermal cycler (Takara, Japan) were set: 94 ℃ for 5 minutes; 30 cycles of 94 ℃ for 40 seconds; 50 ℃ for 40 seconds; and 72 ℃ for 1 minute; followed by 72 ℃ for 5 minutes, and finally, holding temperature at 4 ℃. Pyrosequencing analysis was performed by 454 GS FLX Titanium XL+ Platform (Roche, Switzerland).

Real-time quantitative PCR (qPCR) was used to determine total bacterial population. The 16S rDNA was amplified using TaqMan probe (5'-CGTATTACCGCGGCTGCTGGCAC-3'), forward primer, 340f (5'-TCCTACGGGAGGCAGCAGT-3') and reverse primer, 781r (5'-GGACTACCAGGGTATCTAATCCTGTT-3'). A total volume of 20 µL of the reaction mixture consisted of 10 µL of TaqMan Fast Advanced Master Mix (Applied Biosystems, USA), 0.72 µL of each forward and reverse primer (25 µM), 0.34 µL of TaqMan probe, 6.22 µL of nuclease-free water and 2 µL of DNA template (1 pg to 100 ng) was used for amplification. The qPCR was performed in a Real-Time PCR System (Applied Biosystems, USA) using the following thermal profiles: 50 ℃ for the first 2 minutes, 95 ℃ for the next 20 minutes, followed by 40 cycles of 95 ℃ for 1 second, and at 60 ℃ for 20 seconds.

2.3. Analysis of the pyrosequencing data

Raw sequence data were processed through demultiplexing, chimera detection, as well as deletion of low quality and barcoded sequences. Operational taxonomy unit (OTU) picking and diversity analysis was completed using Quantitative Insights into Microbial Ecology (QIIME) software [24]. Finally, using the Ribosomal Database Project (RDP) Classifier (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) all bacterial DNA sequences were confirmed. Using statistical analysis, multiple regressions were used to evaluate the ecological correlation of FPB and different bacterial phyla, classes, orders, and families. Additionally, correlation analysis was performed to determine the relationship between FPB and bacterial orders as well as bacterial genera.

The number of bacteria was divided into two groups of high and low background biota. This is to study the effect of their number towards the prevalence of FPB and *E. coli*. The separation value for the statistical analysis of HBB and LBB was 5.0×10^6 bacteria cell/cm^2. The boundary was determined through a trial and error method by entering values of 1.0×10^5 to 1.0×10^7. Values that were lower or higher than this resulted in an unevenness of the variable numbers in each category. For example, the sample number for HBB would be very low, and the sample number for LBB would be very high for a separation value of 1.0×10^7 bacteria cell/cm^2, and could not be analysed statistically. This value of 5.0×10^6 bacteria cell/cm^2 was also chosen because it allowed for high R^2 value in the correlation analysis.

3. Results and Discussion

3.1. Classification of bacteria into high and low background biota and their impact on FPB growth

Pyrosequencing analysis has been widely used to study microbial communities in various samples such as abomasal ulcers [25], blood cultures [26], drinking water distribution systems [27] and refrigerators [18]. The present work is one of the studies that applied pyrosequencing analysis to characterize microbial communities on CB samples collected from food service establishments. The bacterial DNA obtained from the extraction was enough and suitable for the pyrosequencing analysis. DNA concentration for all samples are shown in Table 1. The pyrosequencing result can be referred to the previous study by Abdul-Mutalib et al. [22] that include the taxonomy summary of bacterial phyla and genera as well as alpha and beta diversity of all CB samples.

In this study, 13 samples were classified as HBB, and 13 samples were classified as LBB. As mentioned earlier, for LBB, a total bacteria number of less than 5.0×10^6 bacterial cells/cm^2 was selected, and for HBB, total background biota of 5.0×10^6 bacterial cells/cm^2 or more was chosen. Foodborne pathogenic bacteria and bacteria indicator of fecal contamination identified in this study were *Bacillus cereus, Cronobacter sakazakii, Cronobacter turicensis, Escherichia coli* and *E. coli* O157:H7, *Salmonella* spp., and *Yersinia enterocolitica*. Foodborne pathogenic bacteria were dominated by *Cronobacter sakazakii* and *Salmonella enterica*, with few samples contained a high number of *Escherichia coli*. Low and high bacteria group were dominated by *Enterobacteriales, Flavobacteriales* and *Pseudomonadales* with an addition of *Lactobacillales*, in high bacteria group (Figure 1).
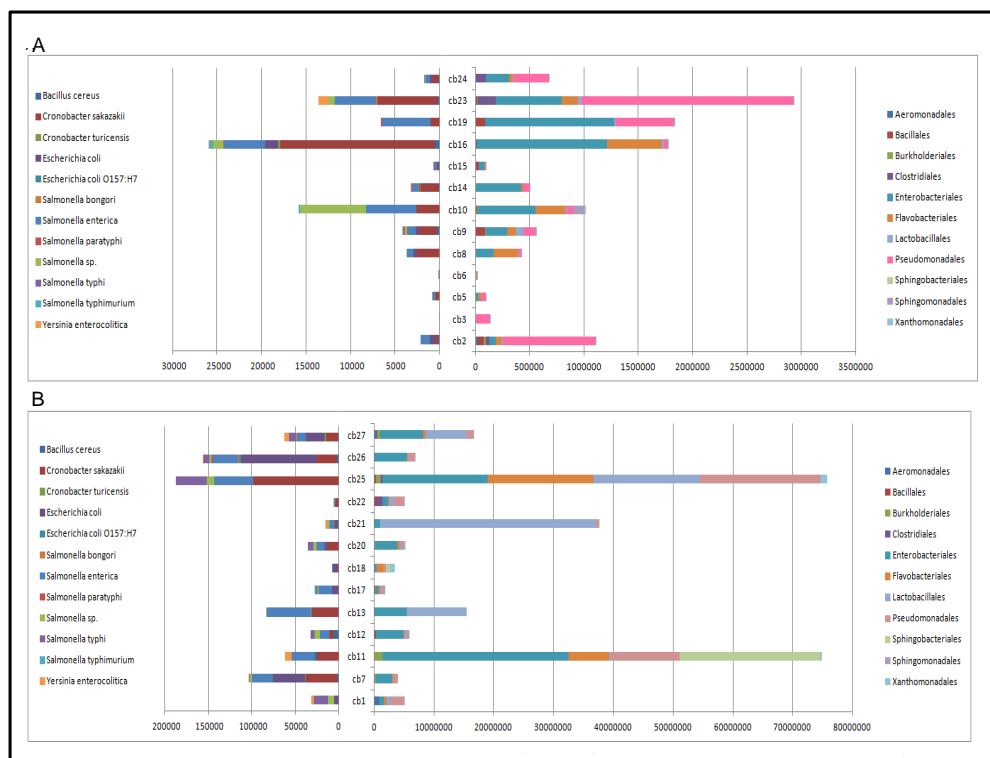

Figure 1. Comparison of the number of FPB and orders of bacteria in LBB (A) and HBB (B). Note that the values of the x-axis are not the same.

The bacterial populations from each sample differ from one sample to another (Table 2): the average number of FPB in HBB was higher than in LBB, which had more than 2.4×10^7 differences. Although this was the case, the prevalence of FPB in HBB was lower (0.24%) compared to the LBB group (0.54%), with more than 5.6×10^4 differences. This indicates that the total number of background biota could influence pathogenic bacteria numbers, as well as influence the interactions between background biota and pathogenic bacteria. Additionally, the finding was in agreement with Jay [28] who observed that the high population of background biota could result in insignificant numbers of pathogenic bacteria. This study also identified the possible interaction of background biota that suppressed pathogenic bacteria growth. Because bacteria grow in complex communities comprising other species of bacteria, they might produce antimicrobial component to suppress the growth of FPB and *E. coli*. This is based on the fact that bacteria interact with each other in the human body, especially in the intestines, to ensure health and reduce the number of harmful microorganisms [29,30].

Table 2. Prevalence of FPB in high and low background biota.

	Mean total bacteria number per cm^2 (SD)	Mean FPB number per cm^2 (SD) [%]
Low background biota	$1.1 \times 10^6 (1.0 \times 10^6)$	$6.0 \times 10^3 (7.8 \times 10^3) [0.54]$
High background biota	$2.6 \times 10^7 (3.6 \times 10^7)$	$6.2 \times 10^4 (5.7 \times 10^4) [0.24]$

3.2 Multiple regression and correlation analysis

A multiple regression analysis was performed to determine the ecological correlation of FPB and different bacterial phyla, classes, orders, and families. This analysis is used to make prediction based on the relationship between the variables. In this analysis, only orders in HBB showed the significant result ($p = 0.020$; Table 3), whereas bacterial phyla, classes and families gave insignificant result. The table shows that 11 bacterial orders can be used the predict the population of FPB and *E. coli*. Therefore, based on this result a subsequent analysis specifically correlation test was performed on the bacterial orders in the HBB group only. The equation for the regression line was

y = 3.511 − 0.970 (*Aeromonadales*) − 0.462 (*Bacillales*) + 0.617 (*Burkholderiales*) + 0.057 (*Clostridiales*) − 0.016 (*Enterobacteriales*) − 0.230 (*Flavobacteriales*) − 0.037 (*Lactobacillales*) + 0.019 (*Pseudomonadales*) − 0.064 (*Spingobacteriales*) − 0.451 (*Spingomonadales*) + 0.091 (*Xanthomonadales*)

Further correlation analyses were performed to determine the negative associations between bacterial orders and FPB and *E. coli*. Statistical test revealed that the population of *Clostridiales, Flavobacteriales* and *Lactobacillales* were negatively correlated with FPB (Figure 2). This means that as the population of *Clostridiales, Flavobacteriales* and *Lactobacillales* increases, the population of FPB and *E. coli* decreases.

Table 3. Multiple linear regression of factors (bacterial orders) related to the percentage of FPB.

Variables	High background biota				Low background biota			
	Unstandardized coefficient		Standardized coefficient		Unstandardized coefficient		Standardized coefficient	
	B	SE	β	*p* value	*B*	SE	β	*p* value
Constant	3.511	0.112		0.020	0.988	1.493		0.628
Aeromonadales	-0.970	0.022	-1.492	0.015*	0.175	0.321	0.409	0.683
Bacillales	-0.462	0.012	-1.385	0.016*	-0.009	0.033	-0.203	0.827
Burkholderiales	0.617	0.012	0.861	0.013*	-0.719	0.931	-1.032	0.581
Clostridiales	0.057	0.007	0.440	0.077	-0.031	0.071	-0.260	0.738
Enterobacteriales	-0.016	0.001	-0.595	0.033*	-0.003	0.016	-0.173	0.899
Flavobacteriales	-0.230	0.005	-1.921	0.013*	0.004	0.022	0.146	0.886
Lactobacillales	-0.037	0.001	-1.542	0.021*	0.018	0.091	0.197	0.878
Pseudomonadales	0.019	0.004	0.212	0.141	-0.008	0.014	-0.600	0.673
Spingobacteriales	-0.064	0.001	-0.822	0.011*	-0.058	2.681	-0.019	0.986
Spingomonadales	-0.451	0.022	-0.441	0.031*	0.056	0.151	0.324	0.774
Xanthomonadales	0.091	0.004	0.531	0.029*	0.164	0.878	0.157	0.883

F-test for high background biota give significant result (*p* value= 0.020)

Multiple regression analysis of bacterial phyla, classes and families showed insignificant relationship.

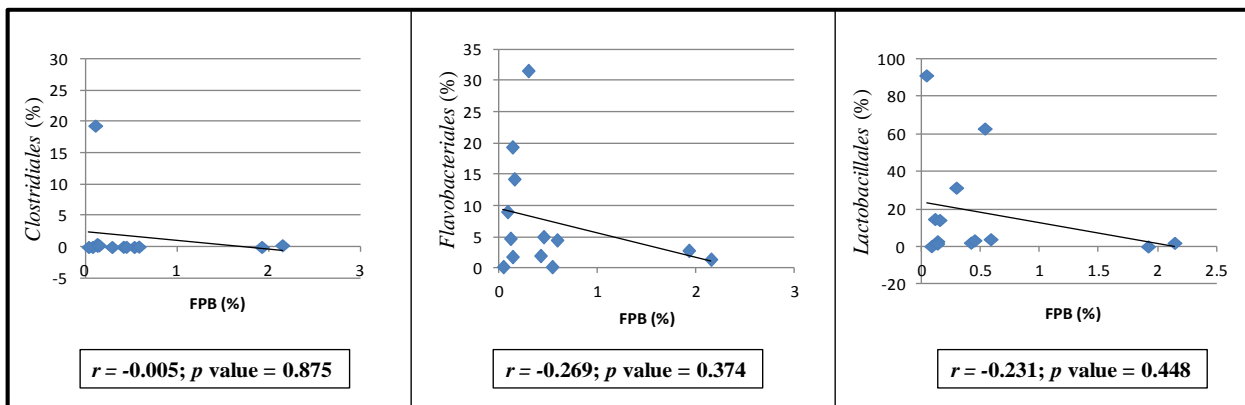Multiple regression of bacterial genera could not be analyzed due to high number of variables.

Figure 2. Correlation analysis of FPB and *Clostridiales*, *Flavobacteriales* and *Lactobacillales* in HBB.

Clostridiales are composed of bacteria in the *Firmicutes* phylum, which is also a dominant order in the healthy gut [31]. The *Clostridiales* and *Lactobacillales* orders present in healthy individuals and are considered autochthonous or good bacteria [32,33]. Many members of this order such as *Clostridium* and *Bacillus* can produce spores [34] and some members of *Clostridiales* order like *Caprococcus* were found to be beneficial to the host, especially when they were supported by other bacteria from the *Lactobacillales* order [35]. A study by Baumgart *et al.* [36] discovered that a reduction in *Clostridiales* could enhance the growth of *E. coli*. Members in this order also impair the colonization of pathogenic bacteria such as *Clostridium difficile, E. coli* and *Salmonella* [37–39], suggesting the importance of *Clostridiales* order to reduce the growth of pathogenic bacteria.

Flavobacteriales are of the *Bacteroidetes* phylum. In humans, *Flavobacteriales* reside on dry areas of the skin along with β-*Proteobacteria* [40]. They are also ubiquitous in soil and are usually associated with plant rizhosphere and pyllosphere [41]. In general *Flavobacteriales* are one of the beneficial microbial communities to ensure plant health and soil fertility, and can be easily isolated from soil and water [42,43]. *Flavobacteria* possess antimicrobial components and demonstrate antagonistic properties toward other bacteria [44]. Flavocin, an agent with fungisitic and bacteriostatic activities produced by *Flavobacterium* sp. L-30, is widely used to treat various farm crops [45]. *Flavobacteria* were also have an antimicrobial effect against *S. aureus* and *E. coli,* and the interaction was enhanced when combined with α-*Proteobacteria* [46]. *Flavobacteria* are also active against *Bacillus subtilis*, *Candida glabrata*, *Escherichia coli* and *Staphylococcus lentus* [47].

Lactobacillales are comprised of lactic acid bacteria, which are usually associated with fermentation and human nutrition [48]. The members of these orders contain many antimicrobial compounds [15]. Generally, *Lactobacillales,* or LAB, are natural inhabitants of the human gastrointestinal tract [49], and some species are qualified as probiotics that have health-promoting activites [14]. Studies have documented the ability of lactic acid bacteria to produce antimicrobial compounds or bacteriocins such as viridicin [50], pediocins [51] lactococcin and nisin [52].

Table 4. Comparison between different bacterial numbers of HBB.

HBB	Bacterial orders	r	*p*-value
$> 10^7$	*Clostridiales*	-0.247	0.600
	Flavobacteriales	-0.339	0.512
	Lactobacillales	0.223	0.670
	Clostridiales + Flavobacteriales + Lactobacillales	0.186	0.724
$> 10^6$	*Clostridiales*	-0.005	0.875
	Flavobacteriales	-0.269	0.374
	Lactobacillales	-0.231	0.448
	Clostridiales + Flavobacteriales + Lactobacillales	-0.583	0.036*
$> 10^5$	*Clostridiales*	0.012	0.995
	Flavobacteriales	-0.062	0.767
	Lactobacillales	-0.158	0.449
	Clostridiales + Flavobacteriales + Lactobacillales	-0.178	0.394
All 26 samples	*Clostridiales*	0.081	0.693
	Flavobacteriales	-0.011	0.958
	Lactobacillales	-0.032	0.879
	Clostridiales + Flavobacteriales + Lactobacillales	-0.153	0.454

* Correlation is significant at the 0.05 level (2-tailed).

As mentioned above, 5.0×10^6 bacterial cells/cm^2 was chosen as a threshold for low and high background biota. Based on these initial findings, the lowest bacterial number was recorded as 10^4, whereas the highest bacterial number was 10^8. Due to the high differences and inconsistency in the cell number, this study separated the samples into two groups of high and low background biota in order to obtain more reliable data. The purpose of this procedure was to find out whether the total number of bacteria based on bacterial phyla, classes, or orders, influences the population of FPB and *E.coli*. After several trial-and-error methods, the separation value of 10^6 for the minimum number of

HBB was found to be more reliable, which resulted in a strong correlation coefficient and lower p-value (Table 4). This is the main reason why 10^6 was chosen as the separation value.

Additional tests were performed to determine the correlation between FPB and several bacterial orders. The results indicated that the correlation between FPB and the combination of *Clostridiales, Flavobacteriales* and *Lactobacillales* was significant with correlation coefficient of -0.583 (Figure 3). The combination of these three bacterial orders gave a higher correlation coefficient compared to only one order as well as a more significant result. This outcome was in accordance with the authors' assumption that two or more bacterial orders may correlate due to a common environment or to ensure survivability. These bacteria might work synergistically through quorum sensing (QS) to impair FPB growth. This shows that it requires a lot of background biota from different orders with different characteristics to reduce the growth of pathogenic bacteria.

The order *Clostridiales* produces *comQXPA,* a QS system important in encoding four proteins: isoprenyl transferase, pre-peptide signal, histidine kinase, and a response regulator. Upon reaching a specific concentration of these molecules, a large number of cellular responses are activated for competence development, surfactin production, biofilms formation and extracellular DNA release [53]. This allows for background biota to compete with pathogenic bacteria and impair their growth. Few bacterial species in the *Clostridiales* order could also produce the *luxS* gene to synthesise autoinducer-2 (AI-2), an important signalling molecule for the production of biofilms, which ensures bacterial survivability [54].
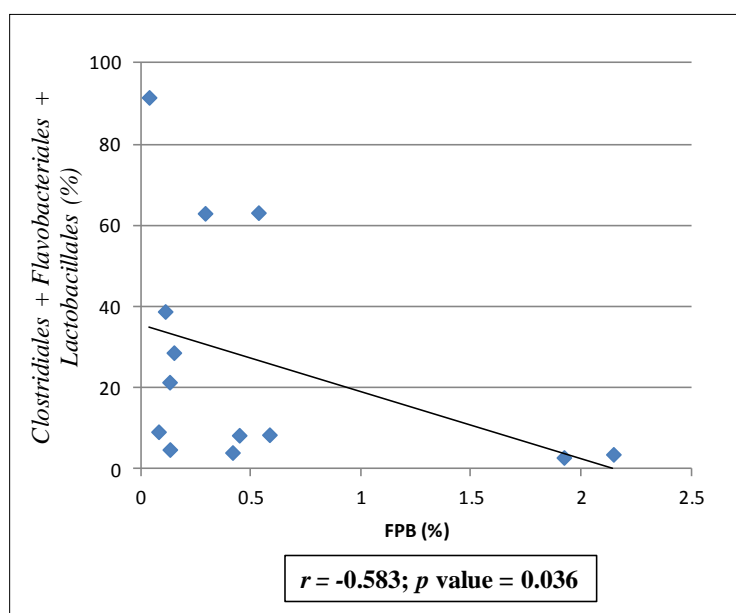

Figure 3. Correlation analysis of FPB and the combination of *Clostridiales*, *Flavobacteriales* and *Lactobacillales* in HBB.

Flavobacteriales (specifically *Chryseobacterium*) produces the *aidC* gene, which displays *N*-Acyl homoserine lactones (AHLs) degrading activity. AHLs are QS signal molecules used by many Gram-negative bacteria. Another study concluded that *Chryseobacterium* (from the *Flavobacteriales* order) produce Aidc, which functions as a AHL lactonase and catalyses the AHL ring opening by hydrolyzing lactones [55]. Some studies have identified the significance of QS inhibition or quorum

quenching by identifying and developing chemical compounds and enzymes that target signalling molecules, signal biogenesis, or signal detection. These therapies are effective against some pathogens but still require further studies [56].

Cell-to-cell communication or QS between LAB occurs when a concentration of a specific molecule is reached and acts as signals for the induction of specific genes, which allows for the production of metabolites such as bacteriocin [57], lactic acid and acetic acid [58]. These metabolites play an important role in controlling food-spoilage and food-borne pathogens [59,60]. Additionally, plantaricin and weisellicin, two types of bacteriocins produced by *Lactobacillus plantarum* and *Weisella hellenica* respectively, were found to be active against foodborne pathogen such as *S. aureus, L. monocytogenes, B. cereus, E. coli, Clostridium perfringens, Salmonella typimurium,* few Gram-positive bacteria as well as some yeasts and molds [42,61,62].

The findings from the present study further support the notion that different bacteria interact with each other either to, increase survivability and to reduce competition. However, information regarding bacterial interactions especially among different species is very limited and requires further investigations.

4.   Conclusion

This study reveals a high diversity of microorganisms including FPB, in CB samples. HBB contain a low percentage of FPB compared to LBB; however, in both cases, the FPB number did not reach the infectious dose level. Through statistical analysis, multiple linear regression analysis showed that only bacterial orders in HBB had a significant ecological correlation with FPB. Additionally, a significant negative association was discovered between FPB and *E.coli,* and the combination of *Clostridiales, Flavobacteriales,* and *Lactobacillales* in HBB. Based on this analysis we propose that, some members of HBB work synergistically with each other to reduce the number of FPB, ensure survivability and eliminate competitors. This study also indicated that the microbial interactions on CB samples were very diverse, and some genera might have the ability to reduce the growth of FPB. Further investigations on these bacteria can be conducted to study their relationship with FPB and possible interactions. Furthermore, future studies on metabolites produced from background biota will be conducted to understand how these metabolites could impact the number of pathogenic bacteria.

Conflict of Interest

The authors declare no conflict of interests in this paper.

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