Prevalence of spoilage microorganism, *Pseudomonas* spp. on restaurants cutting boards collected in Seri Kembangan, Malaysia.

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Abstract

Due to the tropical climate that promotes the growth of most microorganisms, the food service industry in Malaysia faces a lot of challenges in order to ensure quality foods are served to the customers. One of the bacteria that are associated with food spoilage is *Pseudomonas* spp., and the presence of these bacteria on cutting boards could cross-contaminate food items during food handling. This study performed pyrosequencing analysis to investigate the existence of microbial communities on cutting boards collected from restaurants in Seri Kembangan, Malaysia. Most of the samples were dominated by *Flavobacteriales, Enterobacteriales, Lactobacillales*, and *Pseudomonadales*. This study discovered that, all samples of cutting boards were contaminated with *Pseudomonas* spp. With the mean bacterial count of 1.4×10^6 cfu/cm^2, 5.1×10^5 cfu/cm^2 and 5.6×10^6 cfu/cm^2 on samples collected from clean, moderately clean and less clean food premises, respectively. The study shows that the contamination level of *Pseudomonas* spp. was not significantly different in different grades of food premises. Regardless of the status and grades of the food premises, they have the same likelihood to introduce spoilage bacteria like *Pseudomonas* spp. from cutting board to food if they neglect correct food handling measures. Therefore, all food handlers need to prioritize safe food handling to avoid food contamination and spoilage.

Keywords: Cutting board, *Pseudomonas* spp., Food spoilage, Grade of premises.

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Introduction

Malaysia is a hot and humid country, thus, this promotes the growth of most microorganisms. The abuse of temperature control and poor food handling could encourage the growth of microorganisms which leads to contamination and spoilage of food, especially among high-risk foods such as sprouted seeds, raw vegetables, bakery products, and unpasteurized juices [1]. In contrast to foodborne pathogens, spoilage organisms are not usually associated with human illness. However, they could reduce the overall quality of the food, by causing the formation of bad odors and slime, which makes it undesirable for consumption [2].

In several food service establishments, foods are cooked in advance and served in a buffet style without temperature control. This will promote the growth of microorganisms as the foods are left on the counter for a few hours. Additionally, some uneaten food will be chilled to be used the next day. Although this practice is prohibited by the authority, a number of food service establishments neglect the law in order to reduce wastage and prevent loss of profit.

Pseudomonas is an aerobic, Gram-negative bacterium that is commonly found in soil. It can grow in a wide range of temperature levels, from 2°C to 35°C [2], and can be easily found in chilled food products, as well as food prepared at room temperature. In the food industry, various foods harbor very diverse *Pseudomonas* species. Most of the isolates have the ability to grow at a low temperature and are capable of secreting enzymes that can affect the overall quality of the food products, including cold-stored food [3].

Some of the enzymes produced by *Pseudomonas* are heat

resistant. For instance, proteases can withstand heat treatment or cooking processes, and cause coagulation in certain food products [4]. Four species of *Pseudomonas*, namely, *P. fluorescens, P. fragi, P. lundensis,* and *P. viridiflava*, are the main food spoilage organisms that produce enzymes and form a biofilm, thus, causing spoilage in refrigerated food [5]. For instance, *P. fluorescens* has been associated with spoilage of chicken carcasses. When its population reaches 10^8 cfu/ml, it could cause the production of a strong foul smell [6]. In addition, *P. fragi* is commonly known to spoil milk and meat spoiler [2]. This could also lead to the production of odour and slime in food products. Reusing the ingredients stored at room temperature for few hours pose risk to consumers especially if they are immunocompromised [7]. Apart from being a spoilage microorganism, *Pseudomonas* spp. could cause urinary and blood stream infection. This is due to the fact that they develop resistance to certain antibiotics [8].

Numerous studies had focused on foodborne pathogenic bacteria. However, the data on spoilage microorganisms is lacking. The data in this study provide an overview of the presence of *Pseudomonas* spp. on cutting boards, which can give a general idea on the food safety of a food premise and the risk introduced.

The methods used to investigate microbial diversity are constantly improved, and this study applied the pyrosequencing technique that can provide fast and reliable results. The aim of this study is to determine the prevalence of *Pseudomonas* spp. on cutting board samples as well as the correlation of the contamination level of *Pseudomonas* spp. and the grades of food premises.

Methodology

Sample collection

The methods in this study were similar to a previous study by Abdul-Mutalib, et al. [9]. Cutting board samples (N=26) were collected from various food service eslishments around Seri Kembangan, Malaysia, for analysis. The grades of food premise were also recorded with A, B, and C, which represents clean (86% to 100%), moderately clean (71% to 85%), and not clean (51% to 70%), respectively. The grade of the food premises was given by the City Council or the Ministry of Health, and the grades are shown at the counter. These grades were based on the cleanliness of the food preparation area, serving area, food handlers, facilities, water supply, and maintenance program [10].

The restaurants that were chosen for this study served classic Malaysian food like rice, vegetables, chicken, beef, and seafood using self-service selling method and ran a small to medium scale operation. The customers took their preferred dishes at the serving area and made payment to the cashier. The dishes were cooked in advance in the morning and were served until late afternoon. Cutting boards were collected randomly from 26 food premises. Mainly the cutting boards were used to handle a combination of food items and were usually washed using tap water and dishwashing liquid. Permission was asked from the food handlers and the cutting boards were either swabbed at the restaurant or taken to the laboratory for further analysis. All of the restaurants allowed for only one cutting board to be sampled.

Pyrosequencing analysis

DNA of bacteria was extracted from the samples according to the procedure of Gómez, et al. [11], with slight modifications. The procedure started with swabbing the center of the cutting board with wet sterile sponges (3MTM, USA) using 40-vertical S-strokes. Then, the sponges were immersed in 90 mL sterile buffered peptone water (Difco, USA), prior to a 10 mins homogenization process in a sterile stomacher bag (Interscience, France). Bacterial DNA was directly extracted without culturing using UltraClean Microbial DNA Isolation Kit (MO Bio Laboratories, USA) according to the manufacturer's instructions. A total of 1.8 mL of the sample was used during the extraction process. Samples were preserved at -20°C until further analysis. The concentration of bacterial DNA was determined using NanoDrop 2000 (Thermo Scientific, USA).

For pyrosequencing analysis, the 16S rRNA gene fragments were amplified by PCR using the forward primer, F357 (5′-CCTACGGGAGGCAGCAG-3′) and reverse primer, R926 (5′-CCGTCA ATTCCTTTRAGTTT-3′). Short tag sequences specific to each sample were also inserted during the analysis (Table 1). All of the primers used were commercially purchased.

A total of 50 µL PCR reaction mixtures were used for the analysis. The mixtures consisted of 25 µL of Premix Ex Taq (Takara Bio, Japan); 2.5 µL of forward and reverse primer (10 µM), respectively; 2.0 µL of template; 18 µL of dH2O; and 2.5 µL of short tag sequences. The thermal cycler for the

Table 1. *Samples, tag sequences, bacterial number and percentage of foodborne pathogens in all samples.*

Samples and premise grades*	Tag sequences	Total number of bacteria per cm^2	*Pseudomonas* number per cm^2 (%)
cb1A	CAGTACGTACT	2.4×10^7	2.07×10^6 (8.8)
cb2A	CGATACTACGT	2.2×10^6	9.37×10^5 (42.2)
cb3B	CTACTCGTAGT	1.5×10^5	1.35×10^5 (92.8)
cb5B	ACGATGAGTGT	1.2×10^5	4.33×10^4 (35.9)
cb6B	ACGTCTAGCAT	2.5×10^4	2.96×10^3 (12.0)
cb7A	ACTCACACTGT	5.4×10^6	6.95×10^5 (12.9)
cb8C	ACTCACTAGCT	5.0×10^5	4.64×10^3 (0.9)
cb9B	ACTGATCTCGT	6.8×10^5	7.87×10^4 (11.6)
cb10C	ACTGCTGTACT	1.3×10^6	6.78×10^4 (5.1)
cb11C	AGACACTCACT	7.6×10^7	7.33×10^5 (1.0)
cb12B	AGACGTGATCT	7.7×10^6	5.67×10^5 (7.4)
cb13B	AGATACGCTGT	1.6×10^7	3.35×10^4 (0.2)
cb14B	AGTATGCACGT	5.3×10^5	5.72×10^4 (10.9)
cb15B	AGTCTGTCTGT	1.2×10^5	6.47×10^3 (5.4)
cb16B	ATCGTCAGTCT	2.6×10^6	2.45×10^4 (0.9)
cb17B	ATCTGAGACGT	6.2×10^6	6.86×10^5 (11.1)
cb18B	ATGCTACGTCT	5.7×10^6	6.77×10^5 (11.9)
cb19B	CACTACGATGT	1.9×10^6	5.83×10^5 (31.1)
cb20B	CAGTCTCTAGT	6.0×10^6	6.34×10^5 (10.5)
cb21B	CGAGACACTAT	3.9×10^7	1.62×10^6 (4.1)
cb22B	CGTATAGTGCT	5.6×10^6	1.83×10^6 (32.9)
cb23A	CTAGACAGACT	3.1×10^6	2.09×10^6 (67.7)
cb24B	CTATCGACACT	9.6×10^5	3.49×10^5 (36.4)
cb25C	CTCACGTACAT	1.3×10^8	2.15×10^7 (17.3)
cb26A	AGTACGAGAGT	7.3×10^6	1.16×10^6 (15.9)
cb27B	AGTAGACGTCT	2.2×10^7	1.42×10^6 (6.6)
*A: Clean, B: Moderately Clean, C: Not Clean.			

PCR reaction was set at 94°C for the first 5 min, followed by 30 cycles of denaturation (94°C, 40 s), annealing (50°C, 40 s) and extension (72°C, 1 min). Next, the temperature was set at 72°C for an additional 5 min, and finally at 4°C as the holding temperature. Finally, pyrosequencing analysis was continued using the amplified fragments with the help of 454 GS FLX Titanium XL+Platform (Roche, Switzerland).

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was implemented to determine the total bacterial population in each sample. The standard curve was produced using *E. coli* BW25113, with the equation y=−3.711x+54.73 (R2=0.9941; efficiency value=86%). DNA samples previously prepared for pyrosequencing were reused for the qPCR. Forward primer, 340f (5′-TCCTACGGGAGGCAGCAGT-3′),andreverseprimer,781r (5′-GGACTACCAGGGTATCTAATCCTGTT-3′), were used for the amplification while 5′-CGTATTACCGCGGCTGCTGG CAC-3′ was applied as TaqMan probe.

A total volume of 20 µL reaction mixture was used for the analysis, which consisted of 10 µL of TaqMan Fast Advanced Master Mix (Applied Biosystems, USA); 0.72 µL of 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). The profile of Real-Time PCR System (Applied Biosystems, USA) was set as follows: 2 min at 50°C for the uracil-N-glycosylase incubation, 20 min at 95°C for polymerase activation, followed by 40 cycles of denaturation (1 s at 95°C) and simultaneous annealing and extension (20 s at 60°C).

Data analysis

After pyrosequencing was completed, raw sequence files went through demultiplexing, deletion of low quality (<25, Phred score ≥20) and barcoded sequence, as well as chimera detection [12]. The operational taxonomy units (OTUs) selection and diversity analysis were performed using The Quantitative Insights into Microbial Ecology (QIIME) software 13. In order to match the sequences obtained with microbial genus or species, Ribosomal Database Project (RDP) Classifier (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro. jsp) was adopted. One-way ANOVA and correlation test was performed using SPSS Version 22 to determine the mean difference of bacterial counts and the association between *Pseudomonas* contamination level and the grades of the food premise [13].

Results

Heat map of bacterial orders (Figure 1) obtained from the study illustrates that most of the samples contained less than 20% of a single bacterial order. Nevertheless, one bacterial order, *Enterobacteriales*, dominated most of the samples and made up to over 40% in almost half of the samples. Sample cb14 can be seen to be dominated by more than 70% of this bacterial order. *Enterobacteriales* are Gram-negative bacteria mostly found in the gastrointestinal tract of animals and humans. Examples of members in this order are *Enterobacter* and *Escherichia*. The presence of *Lactobacillales* was high in sample cb13 (50% to 70%) and cb21 (70%). The percentage of *Flavobacteriales* was also high in samples cb6, cb10 (20% to 30% respectively) and cb8 (40% to 50%). The heat map also shows domination of *Pseudomonadales* in some cutting board samples.

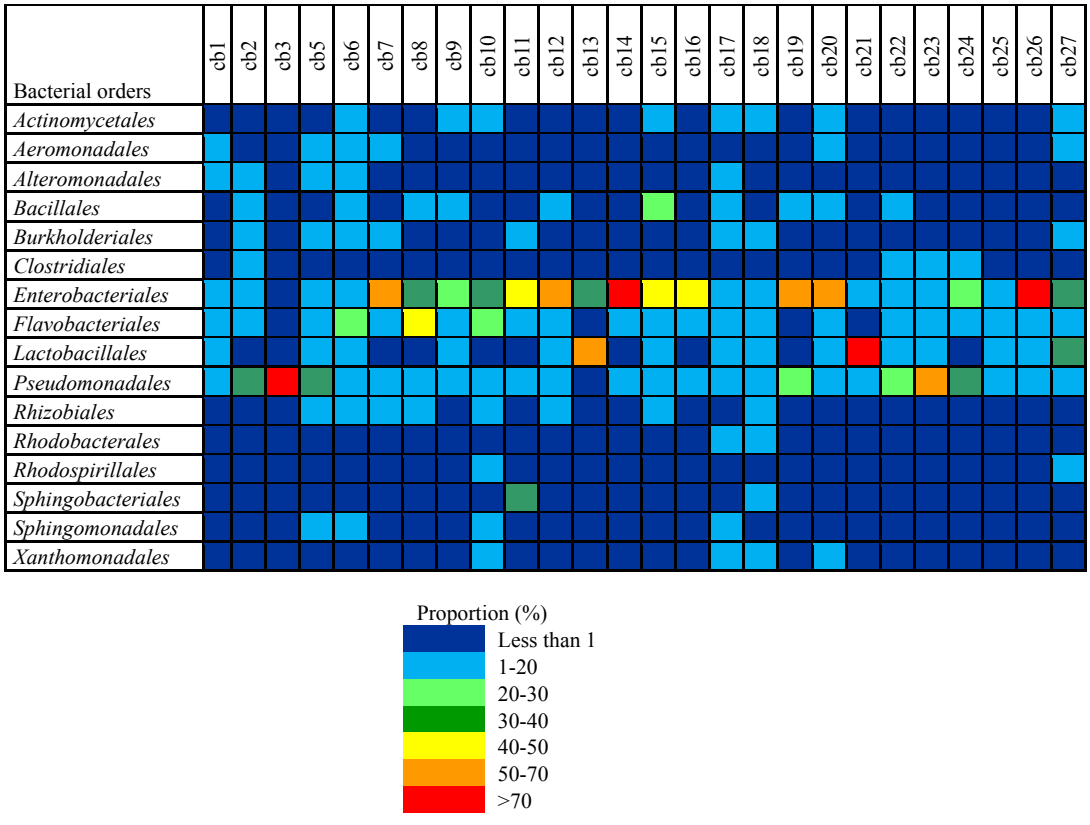

Figure 1. *Composition of the microbiome in the cutting board samples based on bacterial orders.*

The highest percentage of *Pseudomonas* spp. was discovered in sample cb3 (grade B), followed by sample cb23 (grade B) and sample cb2 (grade A) (Table 1). This result indicates that even a clean premise can harbor a high percentage of spoilage bacteria. Comparing the number of *Pseudomonas* spp. among different food premises, the cutting boards collected from grade A restaurants documented a high bacterial count (Table 1). However, based on the statistical test (Table 2), the numbers of *Pseudomonas* spp. isolated from three different grades of food premises was not significantly different. In addition, for further analysis, the *Pseudomonas* spp. was grouped into high (>10^6), medium (10^4 to 10^6) and low (<10^4) contamination level. Consequently, the result showed that the contamination level of *Pseudomonas* spp. was not significantly associated with premise grades (Table 3). This suggests that all grades of premises had the same potential to introduce these spoilage microorganisms to food items especially if the food handlers ignore correct food handling. Hence, food meant to be refrigerated can be easily contaminated, spoiled, and unsuitable for consumption.

Discussion

Pyrosequencing analysis is one of the techniques used to study microbial communities in numerous samples such as abomasal ulcers [14], blood cultures [15], drinking water distribution systems [16], and refrigerators [17]. The present work is a continuation from Abdul-Mutalib et al. [9,18] who also applied the same technique to study microbial communities on cutting boards collected from several food premises in Seri Kembangan, Malaysia. The results showed a high diversity of microorganisms present on the cutting board samples, including few species of foodborne pathogens such as *Bacillus cereus, Salmonella* spp., *Chronobacter* and *E. coli*.

The high occurrence of these bacteria was likely due to the samples being taken from cutting boards that might have been used to handle animal carcasses. This is in parallel with a study conducted by Säde, et al. [19], which discovered a high percentage (60%) of enterobacteria in meat and poultry samples. Some of the samples were contaminated with more than 10^6 cfu/g of bacteria, which can easily contribute to food spoilage. The high percentage of *Lactobacillales* in sample cb13 (50% to 70%) and cb21 (70%) was likely because the cutting boards were used to handle fermented foods such as shrimp paste, tempeh, soy sauce, and pickled vegetables. Fermented foods contain a high amount of bacteria such as *Lactobacillus plantarum, L. pentosus, L. brevis, L. acidophilus, L. fermentum, Leuconostoc fallax,*and *L. mesenteroides* [20]. This is in agreement with the studies by Li, et al. [21] and Liu and Tong [22], which discovered the presence of a high population of *Firmicutes*, specifically lactic acid bacteria, in fermented food.

Table 2. *The number of Pseudomonas spp. and premise grades.*

Premise grades	Mean of *Pseudomonas* number per cm^2 (SD)	*p* value
A	1.4×10^6 (6.5×10^5)	0.084*
B	5.1×10^5 (5.9×10^5)	
C	5.6×10^6 (1.1×10^7)	
*The number of *Pseudomonas* spp., in different grades of food premises was not significantly different.		

Table 3. *Correlation between Pseudomonas spp. contamination level and premise grades.*

	Premise grades	
	r	p-value
Level of *Pseudomonas* contamination	-0.312	0.345
There was no significant association between *Pseudomonas* spp. contamination level and premise grades.		

In this study, most of the samples harboured less than 20% of *Pseudomonadales*. However, few samples recorded a higher percentage of bacteria in this order such as samples cb19 and cb22 (20 to 30%), cb2, cb5 and cb24 (30% to 40%), cb23 (50% to 70%) and cb3 (>70%). The presence of *Pseudomonadales* was high because most of the members of this bacterial order are ubiquitous in environments such as soil and water. A study conducted by Meireles et al. [23] also found that the prevalence of *Pseudomonadales* in a vegetable plant was 49%, which dominated almost half of the community population. Tian et al. [24] also discovered that *Pseudomonas* spp. dominated tomato roots wile acting as a promoter of plant growth. This shows that most of the members in this bacterial order are associated with plants which are widely handled on cutting boards.

The heat map also shows a high number of microorganisms which belongs to the *Enterobacteriales* order. Most foodborne pathogens and bacteria indicator of fecal contamination belong to the *Enterobacteriaceae* family, under the *Enterobacteriales* order. Examples of the microorganisms that can cause illness are *Listeria monocytogenes, Escherichia coli, Salmonella, Campylobacter, Yersinia enterocolitica, Cronobacter* spp. and *Shigella* [25]. To cause illness in human, these microorganisms must reach certain infectious doses such as 10^5 organisms of *Salmonella* and *E. coli* [26], 700 organisms of *E. coli* O157:H7 [27], 10^4 to 10^5 CFU/ml of *Bacillus cereus* [28], 10 CFU/g of *Cronobacter sakazakii* [29] and 10^5 to 10^6 cells of *Yersinia enterocolitica* [30].

This study identified 55 *Pseudomonas* species from the cutting boards. As explained by a few studies, food spoilage is usually associated with *P. fluorescens, P. fragi, P. lundensis* and *P. aeruginosa* [3,31]. In addition to this, the ability of these spoilage bacteria to survive under refrigeration temperatures [32-34] may cause difficulty during the storage of foods.

Pseudomonas spp. can adapt to various environmental conditions, hence, can be easily found in soil, water, animals, and plants [35]. These bacteria are frequently associated with spoilage of food derived from animals and plants. Examples of frequently found *Pseudomonas* that cause food spoilage are *P. fluorescens* and *P. putida*, which were isolated from fish with a prevalence rate of more than 60% [36]. Nevertheless, the contamination of *Pseudomonas* in other meat products should not be ignored. The ability of these microorganisms to grow under refrigeration temperatures is a challenge that should be counteracted [37-39].

Pseudomonas is also able to produce heat-resistant enzymes such as proteases that can have a negative impact on food, even after the cooking process. Apart from being a food spoilage microorganism, *Pseudomonas* could also be linked to several illnesses such as pneumonia, septicaemia, and gastrointestinal diseases [40]. Therefore, the presence of these opportunistic bacteria should be prevented during earlier stages of food preparation. Additionally, during serving, temperature abuse

will lead to spoilage of food such by giving bad odour and taste, which in turn will drive customers away, that can affect sales and reputation of the food service establishments.

Conclusions

In conclusion, the composition of the microbiome showed that all cutting board samples harboured *Pseudomonas* spp. with seven samples were dominated with more than 50% of these bacteria. In addition, the numbers of *Pseudomonas* spp. in all grades of food premises were not significantly different. The correlation test showed that there was no significant association between food premise grades and the contamination level of *Pseudomonas* spp. Hence, all food premises have the same chance to cross-contaminate food with *Pseudomonas* spp., thus, increasing the possibility of food spoilage and reducing the quality of the food served at the food service establishment. Therefore, the cutting board should be properly cleaned and sanitised to reduce cross-contamination and to increase the safety of food served at the establishments.

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