Metataxonomic Analysis of Bacterial Diversity in Pigeon Pea after Soaking in Water

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Posted Date: 16 October 2023

doi: 10.20944/preprints202310.0945.v1

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Article

Metataxonomic Analysis of Bacterial Diversity in Pigeon Pea after Soaking in Water

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Abstract: With the increase in demand for non-dairy starter cultures and probiotic bacteria as carriers, the use of legumes (also called pulses) as an alternative has gained momentum. In this study, we investigated the diversity of bacterial communities in samples of pigeon pea (*Cajanus cajan* L. Millsp.) soaked in water for 12 h and 24 h. We soaked 500 g of pigeon pea in sterile distilled water at room temperature (± 25 °C) for 12 h and 24 h; 10 mL of the soaking water was then collected to measure the bacterial diversity using a metataxonomic analysis. The V1–V9 regions on the 16S ribosomal RNA gene were amplified using 27F and 1492R primers under specific polymerase chain reaction conditions for the bacterial identification. Genomic DNA (130 ng) was sequenced on a R9.4 flow cell by Oxford Nanopore Technologies using a GridION sequencer. Library preparations were initiated using a Native Barcoding Kit 24 V14 (SQK-NBD114.24). Primary data were acquired using MinKNOW version 22.05.7. A total of 13 bacterial families and 89 genera were identified in the pigeon pea sample soaked for 12 h; 26 families and 90 genera were identified in the pigeon pea sample soaked for 24 h. Among the bacterial families identified, the five predominant families in both samples were Enterobacteriaceae, Erwiniaceae, Yersiniaceae, Pectobacteriaceae, and Lactobacillaceae. According to the relative abundance of the identified bacterial genera, the following nine genera were predominant in both samples: *Enterobacter*, *Klebsiella*, *Citrobacter*, *Pantoea*, *Kosakonia*, *Pseudoenterobacter*, *Pluralibacter*, *Leclercia*, and *Kluyvera*. At a genus level, a slight increase in the abundance of *Klebsiella*, *Kosakonia*, and *Pluralibacter* and a slight decrease in the abundance of *Citrobacter* were observed after prolonged incubation from 12 h to 24 h. The values of five diversity indices revealed that the sample soaked in water for 24 h had a richer bacterial abundance and diversity than the 12 h sample. Shannon and Simpson values revealed a higher bacterial diversity in the sample collected at 24 h than the sample collected at 12 h. Species observations and abundance-based coverage estimator (ACE) values demonstrated that the sample collected at 24 h harbored a higher bacterial richness than the sample collected at 12 h. These findings indicated that the bacterial diversity in the pigeon pea samples increased with the soaking time. The bacterial communities during the soaking of the pigeon pea samples were dominated by the Enterobacteriaceae family and *Enterobacter* genus. The presence of bacterial genera such as *Lacticaseibacillus*, *Lentilactobacillus*, and *Secundilactobacillus* was notable because of their importance as starter cultures for fermented plant-based milk products, including pigeon pea beverages for lactose-intolerant individuals or individuals with malnutrition.

Keywords: pigeon pea; bacterial diversity; metataxonomic analysis

1. Introduction

Functional foods have positive effects on human health. Their physiological properties are determined by their bioactive components. These include dietary fiber, antioxidants, phytochemicals,

 doi:10.20944/preprints202310.0945.v1

polyunsaturated fatty acids, prebiotics, probiotics, postbiotics, and synbiotics. In Indonesia, the utilization of local food sources for functional foods is essential to meet the protein needs of rural communities. One example of functional foods produced using local food sources is tempeh, a traditional fermented soybean dish prepared using *Rhizopus* that is consumed in various countries around the world. [1] reported the health benefits of tempeh and other fermented soy foods due to their antioxidative, antihypertensive, anti-inflammatory, anticancer, and neuroprotective properties. Certain microbes degrade proteins to amino acids during tempeh fermentation; this determines the flavor and aroma of tempeh. The native microbes in soybeans affect the chemical composition and aroma of the final product [2].

Pigeon pea (*Cajanus cajan* L. Millsp.; local name: *kacang gude*) is another legume that is widely used as a raw ingredient for tempeh fermentation. It belongs to the Fabaceae family [3]. Indonesian pigeon pea has several advantages over other legume crops, including being tolerant of drought and infertile soils [4] due to its deep roots [5]. Pigeon pea crops can be cultivated in dry areas where soybean plants do not grow well. Its deep rooting does not interfere with the nutrition absorption of other plants; thus, pigeon pea can be intercropped [6].

Pigeon pea is cultivated in several regions in Indonesia due to its edible seeds. The fruit of pigeon pea is a pod that is 4–10 cm long, hairy, flat, and green in color. Pigeon pea seeds are round and small; the number of seeds per pod ranges from four to nine [3]. The pods are straight or crescent in shape and the seed-coat color can be grayish white, cream, yellow, purplish brown, or black. The seed coats are smooth and shiny; the seed weight varies between 4 and 26 g per 100 grains [7]. Pigeon pea seeds comprise a seed coat (14%), an embryo (1%), and cotyledons (85%). The nutritional content of pigeon pea seeds per 100 g is 21.7 g protein, 1.5 g fat, 62.8 g carbohydrates, 12.7 g water content, and 336 kcal total energy [8]. Pigeon pea has traditionally been used as a medicinal plant [9]; however, it contains cyanide and antinutritional compounds. Processing is required to reduce these compounds. Pigeon pea fermentation increases the availability of nutrients as well as the health benefits of pigeon pea as a functional food [10].

Soybean and many other pulses are excellent food sources due to their high amount of dietary fibers, proteins, or micronutrients and phytochemicals. The consumption of pulse products has been somewhat limited due to intestinal disturbances such as flatulence. This is caused by the presence of oligosaccharides such as raffinose and stachyose, which are non-digestible and not assimilated in the small intestine by human GI enzymes; rather, they are fermented by the microbiota and are thus responsible for flatulence [11].

Fermentation is a food biotransformation process that provides positive nutritional and sensory properties to products depending on the microbes being used as starter cultures [12,13]. Certain microbes can synthesize vitamins such as folic acid, riboflavin, niacin, thiamin [14,15], and B12 [16] from the fermentation of grain legumes as a substrate. Other microbes can metabolize n-hexanal and pentanal, which assign a beany flavor to the products [17]. Fermentation can reduce the level of oligosaccharides that cause postprandial flatulence from legumes [18,19] and can decrease antinutritional components such as tannins, phytic acid [20], and trypsin inhibitors [21]. Different strains of the same species can have completely distinct metabolic patterns; consequently, this affects the taste and texture of the product [22].

The process of producing tempeh from pigeon pea is performed in two stages. The first stage is to soak the pigeon pea for approximately 24 h. Soaking results in pigeon pea acidification, which occurs through bacterial activity. At this stage, the pH drops from 7 to 4, which is important for the growth of *Rhizopus oryzae*. This species is inoculated with pigeon pea in the second stage, which lasts approximately 48 h. Information regarding the types of bacteria that play a role in the acidification of pigeon pea tempeh is limited. Our research intention was to determine the bacteria responsible for pigeon pea acidification and select those that could be used as starter cultures for the preparation of pigeon-pea-based functional food products. In this study, we performed a metataxonomic analysis by sequencing 16S ribosomal RNA (16S rRNA) genes that have been widely used to monitor microbial populations. Our aims were to detect and identify all the bacteria that played a role in the

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acidification of pigeon pea samples soaked for 12 and 24 h and to measure their abundance in water-immersed pigeon pea samples.

2. Materials and Methods

2.1. Pigeon Pea Collection and Sample Preparation

Pigeon pea samples were collected from Nusa Tenggara Timur Province in Indonesia. These were sorted to obtain peas with good or intact pods. In brief, 500 g of pigeon pea was soaked in sterile distilled water at room temperature (± 25 °C) for 12 and 24 h. Afterward, 10 mL of the soaking water was collected to measure the bacterial diversity using a metagenomic analysis.

2.2. Genomic DNA Extraction

In brief, 10 mL of each soaking-water sample was centrifuged at 2500 × *g* for 10 min. The obtained pellets were first washed with saline and then with sterile distilled water. These were then used for genomic DNA extraction with a ZymoBIOMICS DNA Miniprep Kit D4300 (Zymo Research, Cambridge, UK). The DNA concentration was determined using NanoDrop spectrophotometers and a Qubit fluorometer (Thermo Fisher, Waltham, MA, USA). The DNA quality was assessed via agarose gel electrophoresis followed by visualization using a Gel-Doc EZ imager (Bio-Rad, CA, USA).

2.2.1. Amplification of the 16s rRNA V1–V9 Regions

The V1–V9 regions of the 16S rRNA gene for the identification of bacterial species were amplified using 27F and 1492R primers under the following PCR conditions: preliminary denaturation at 95 °C for 3 min followed by 5 cycles at 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s; 30 cycles at 95 °C for 15 s, 62 °C for 15 s, and 72 °C for 30 s; and a final extension at 72 °C for 1 min.

2.2.2. Library Preparation and Sequencing

A library was prepared using a Native Barcoding Kit 24 V14 (SQK-NBD114.24) (Oxford Nanopore Technologies, Oxford, UK). The genomic DNA (130 ng) was sequenced using Oxford Nanopore Technology (ONT, Oxford, UK), which provided the long-read sequencing that covered the full-length sequence of the 16S rRNA gene. Sequencing was conducted on an R9.4 flow cell by ONT using a GridION sequencer (ONT, Oxford, UK). Primary data were acquired using MinKNOW version 22.05.7; this is the software used with nanopore sequencing devices.

2.2.3. Bioinformatics Analysis

Base calling was performed using Guppy version 6.1.5 with a high-accuracy model [23]. The quality of FASTQ files was visualized using NanoPlot [24]. The reads were classified using a centrifuge classifier [25]. An index for bacteria and archaea was built using the NCBI 16S RefSeq database (https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/). The downstream analysis and visualizations were performed using Pavian (https://github.com/fbreitwieser/pavian), Krona Tools (https://github.com/marbl/Krona), and RStudio (R version 4.2.0) (https://www.R-project.org/).

3. Results and Discussion

3.1. Metataxonomic Data

The bacterial diversity in the soaking water of the pigeon pea samples soaked for different lengths of time (12 and 24 h) was examined by sequencing the hypervariable regions (V1–V9) of the 16S rRNA gene. The 16S rRNA gene is well-preserved and uniquely found in all bacteria and archaea, suggesting that it could specifically target and identify the bacteria and archaea present in the samples. As all the informative sites of the 16S rRNA gene were considered, the full-length 16S rRNA sequences provided a high level of taxonomic and phylogenetic resolution for our bacterial identification [26].

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Shannon and Simpson diversity indices were applied to measure the bacterial diversity in the samples. Both consider the number of species living in a habitat and their relative abundance [27]. The observed species, Chao1, and abundance-based coverage estimator (ACE) indices reflected the sample richness. The values of these five diversity indices are shown in Figure 1. The sample collected at 24 h had a richer genus abundance and diversity than the sample collected at 12 h. The Shannon and Simpson values revealed that the sample collected at 24 h harbored a higher bacterial diversity than the sample collected at 12 h. The species observation and ACE values revealed that the sample collected at 24 h harbored a higher sample richness than the sample collected at 12 h. These findings indicated that the bacterial diversity in the pigeon pea samples increased with the soaking time.

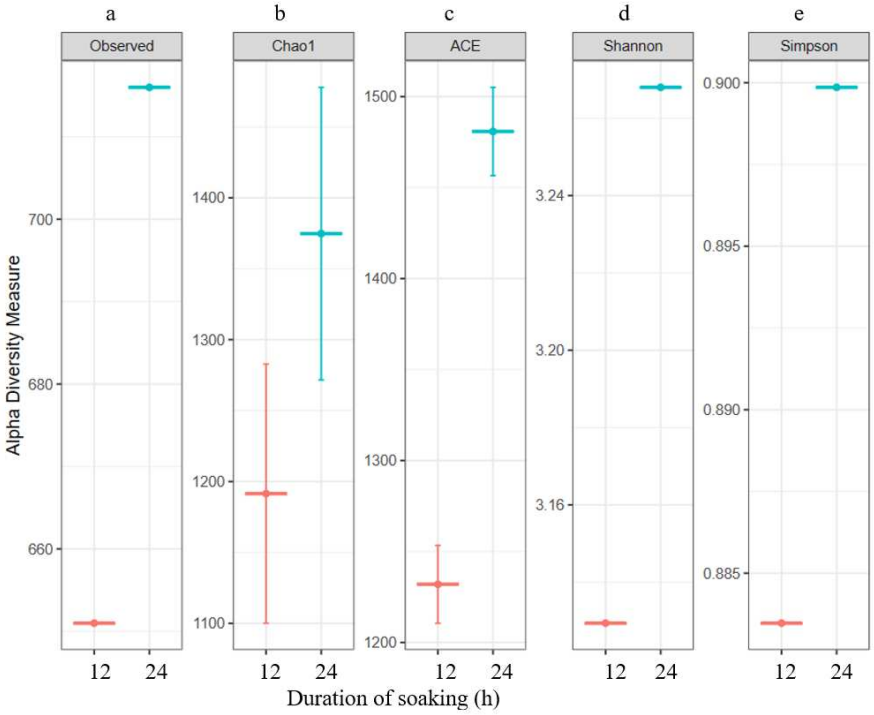

Figure 1. Diversity index box figure of bacteria in pigeon pea samples at different soaking times: (**a**) observed species index; (**b**) Chao1 index; (**c**) ACE index; (**d**) Shannon index; (**e**) Simpson index.

After being soaked in water for 12 and 24 h, the dominant phylum of the pigeon pea samples was Proteobacteria (Figure 2a,b). Within Proteobacteria, Enterobacteriaceae was the predominant family and *Enterobacter* and *Klebsiella* were the most abundant genera in the samples (Figure 2a,b). At the species level, *Enterobacter ludwigii* (15%), *Enterobacter asburiae* (11%), and *Enterobacter cloacae* (8%) were the most abundant in the sample soaked in water for 12 h. These three *Enterobacter* species remained the most dominant species after the pigeon pea sample was soaked in water for 24 h. Other phyla were detected, including *Firmicutes* and *Actinobacter*; however, their relative abundance remained below 1.00%. Nur et al. [28] reported that *Firmicutes* and *Actinobacteria* were consistently observed in tempeh fermentation; *Firmicutes* was the most dominant bacteria.

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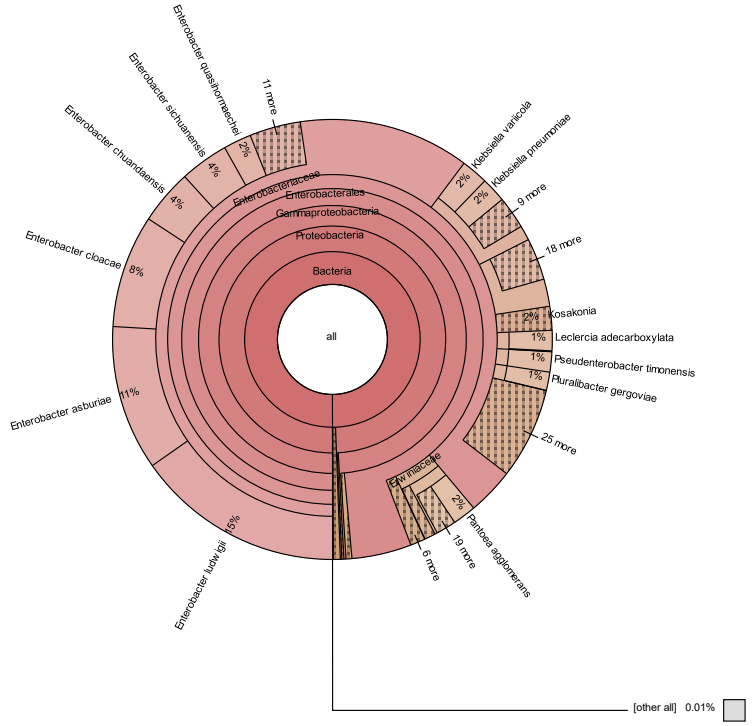
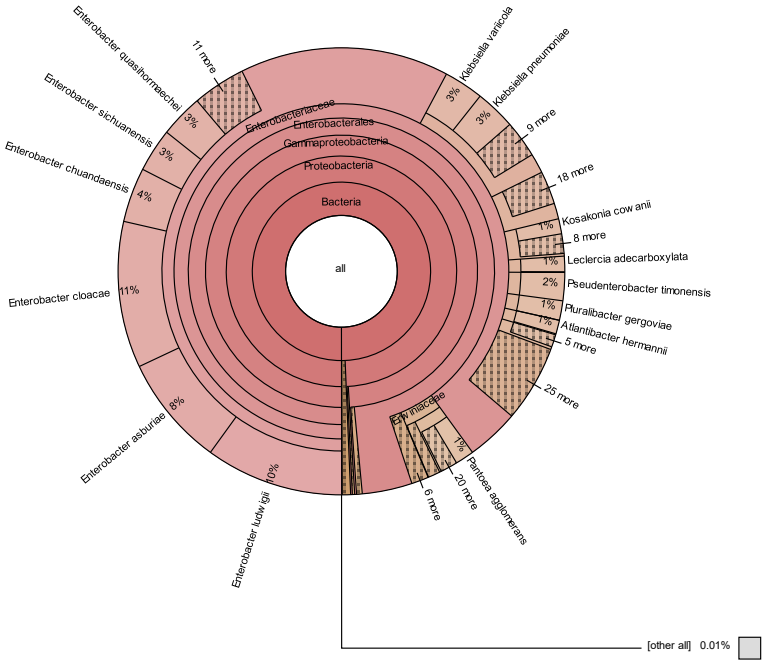


(**a**)



(**b**)

Figure 2. Bacterial composition of pigeon pea samples after being soaked in water: (**a**) 12 h; (**b**) 24 h.

3.2. Bacterial Diversity

The bacterial 16S rRNA gene sequences were classified at the family and genus levels to discover the contents of the bacterial communities observed in pigeon pea samples soaked in water. A total of

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13 families (Table 1) and 89 genera (Table 2) were identified in the pigeon pea sample soaked in water for 12 h; 26 bacterial families (Table 3) and 90 genera were identified in the pigeon pea sample soaked in water for 24 h (Table 4).

Table 1. Classification of the bacterial families of the pigeon pea sample soaked in water for 12 h using the Centrifuge *nt* database.

Family Name	taxID	taxRank	Number of Uniquely Classified Reads
Enterobacteriaceae	543	Family	2607
Erwiniaceae	1903409	Family	22
Pectobacteriaceae	1903410	Family	10
Calotrichaceae	2661849	Family	4
Halomonadaceae	28256	Family	2
Morganellaceae	1903414	Family	2
Vibrionaceae	641	Family	2
Alteromonadaceae	72275	Family	1
Balneolaceae	1813606	Family	1
Flavobacteriaceae	49546	Family	1
Intrasporangiaceae	85021	Family	1
Kofleriaceae	224464	Family	1
Micrococcaceae	1268	Family	1

Table 2. Classification of the bacterial genera of the pigeon pea sample soaked in water for 12 h using the Centrifuge *nt* database.

Genus Name	taxID	taxRank	Number of Uniquely Classified Reads
Enterobacter	547	Genus	9484
Citrobacter	544	Genus	1516
Klebsiella	570	Genus	819
Pantoea	53335	Genus	138
Kluyvera	579	Genus	100
Kosakonia	1330547	Genus	98
Serratia	613	Genus	85
Tatumella	82986	Genus	54
Erwinia	551	Genus	46
Cronobacter	413496	Genus	30
Vibrio	662	Genus	23
Pectobacterium	122277	Genus	21
Bacillus	1386	Genus	18
Dickeya	204037	Genus	14
Rahnella	34037	Genus	14
Buttiauxella	82976	Genus	13
Mangrovibacter	451512	Genus	12
Planctopirus	1649480	Genus	12
Pseudocitrobacter	1504576	Genus	11
Cedecea	158483	Genus	10
Lentilactobacillus	2767893	Genus	10
Mixta	2100764	Genus	9
Acinetobacter	469	Genus	9
Franconibacter	1649295	Genus	8
Chimaeribacter	2716544	Genus	7
Lelliottia	1330545	Genus	7
Edwardsiella	635	Genus	6
Actinobacillus	713	Genus	5

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Escherichia	561	Genus	5
Lacticaseibacillus	2759736	Genus	5
Pseudomonas	286	Genus	4
Yersinia	629	Genus	4
Izhakiella	1780190	Genus	3
Leclercia	83654	Genus	3
Shigella	620	Genus	3
Acetomicrobium	49894	Genus	2
Bradyrhizobium	374	Genus	2
Brenneria	71655	Genus	2
Brevitalea	2048911	Genus	2
Gibbsiella	929812	Genus	2
Haemophilus	724	Genus	2
Lonsdalea	1082702	Genus	2
Paraburkholderia	1822464	Genus	2
Psychromonas	67572	Genus	2
Raoultella	160674	Genus	2
Trabulsiella	158851	Genus	2
Xenorhabdus	626	Genus	2
Actinoplanes	1865	Genus	1
Aeribacillus	1055323	Genus	1
Aeromonas	642	Genus	1
Atlantibacter	1903434	Genus	1
Brucella	234	Genus	1
Burkholderia	32008	Genus	1
Caldimonas	196013	Genus	1
Catenulispora	414878	Genus	1
Crinalium	241421	Genus	1
Croceicoccus	1295327	Genus	1
Flavisolibacter	398041	Genus	1
Gloeobacter	33071	Genus	1
Halomonas	2745	Genus	1
Humidesulfovibrio	356	Genus	1
Hyphomicrobium	81	Genus	1
Iningainema	1932705	Genus	1
Ktedonobacter	363276	Genus	1
Leucothrix	45247	Genus	1
Marichromatium	85076	Genus	1
Massilia	149698	Genus	1
Mathylobacillus	404	Genus	1
Micromonospora	1873	Genus	1
Microvirga	186650	Genus	1
Morganella	581	Genus	1
Motilimonas	1914248	Genus	1
Niastella	354354	Genus	1
Novosphingobium	165696	Genus	1
Oceanisphaera	225143	Genus	1
Paraglaciecola	1621534	Genus	1
Pedomicrobium	47494	Genus	1
Permianibacter	1649479	Genus	1
Phenylobacterium	20	Genus	1
Providencia	586	Genus	1

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Pseudoxanthomonas	83618	Genus	1
Rhabdothermincola	2820403	Genus	1
Rhodoplanes	29407	Genus	1
Salmonella	590	Genus	1
Sphingomonas	13687	Genus	1
Thermithiobacillus	119979	Genus	1
Thermosynthropha	54293	Genus	1
Virgisporangium	65504	Genus	1

Table 3. Classification of the bacterial families of the pigeon pea sample soaked in water for 24 h using the Centrifuge *nt* database.

Family Name	taxID	taxRank	Number of Uniquely Classified Reads
Enterobacteriaceae	543	Family	2836
Yersiniaceae	1903411	Family	34
Erwiniaceae	1903409	Family	23
Vibrionaceae	641	Family	6
Calotrichaceae	2661849	Family	5
Pectobacteriaceae	1903410	Family	5
Bacillaceae	186817	Family	4
Burkholderiaceae	119060	Family	2
Methylococcaceae	403	Family	2
Acidobacteriaceae	204434	Family	1
Aeromonadaceae	84642	Family	1
Anaerolineaceae	292628	Family	1
Bruguierivoracaceae	2812006	Family	1
Flavobacteriaceae	49546	Family	1
Gomontiellaceae	1892255	Family	1
Halanaerobiaceae	972	Family	1
Halomonadaceae	28256	Family	1
Heliobacteriaceae	31984	Family	1
Hyphomicrobiaceae	45401	Family	1
Intrasporangiaceae	85021	Family	1
Microbacteriaceae	85023	Family	1
Morganellaceae	1903414	Family	1
Pasteurellaceae	712	Family	1
Pseudomonadaceae	135621	Family	1
Rhodospirillaceae	41295	Family	1
Succinivibrionaceae	83763	Family	1

Table 4. Classification of the bacterial genera of the pigeon pea sample soaked in water for 24 h using the Centrifuge *nt* database.

Genus Name	taxID	taxRank	Number of Uniquely Classified Reads
Enterobacter	547	Genus	11233
Klebsiella	570	Genus	1061
Citrobacter	544	Genus	842
Kluyvera	579	Genus	150
Kosakonia	1330547	Genus	144
Pantoea	53335	Genus	98
Serratia	613	Genus	68
Tatumella	82986	Genus	58
Erwinia	551	Genus	45

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Buttiauxella	82976	Genus	33
Cronobacter	413496	Genus	31
Dickeya	204037	Genus	23
Mangrovibacter	451512	Genus	21
Pectobacterium	122277	Genus	18
Vibrio	662	Genus	17
Planctopirus	1649480	Genus	16
Edwardsiella	635	Genus	13
Rahnella	34037	Genus	12
Shigella	620	Genus	12
Pseudocitrobacter	1504576	Genus	11
Lacticaseibacillus	2759736	Genus	10
Lentilactobacillus	2767893	Genus	9
Acetomicrobium	49894	Genus	8
Lelliottia	1330545	Genus	8
Escherichia	561	Genus	7
Trabulsiella	158851	Genus	6
Chimaeribacter	2716544	Genus	5
Brevitalea	2048911	Genus	4
Rosenbergiella	1356488	Genus	4
Yersinia	629	Genus	4
Gibbsiella	929812	Genus	3
Raoultella	160674	Genus	3
Salmonella	590	Genus	3
Xenorhabdus	626	Genus	3
Bradyrhizobium	374	Genus	2
Burkholderia	32008	Genus	2
Crinalium	241421	Genus	2
Franconibacter	1649295	Genus	2
Gloeobacter	33071	Genus	2
Legionella	445	Genus	2
Microbacterium	33882	Genus	2
Miltoncostaea	2843200	Genus	2
Providencia	586	Genus	2
Pseudaeromonas	1929090	Genus	2
Reyranella	445219	Genus	2
Salinicola	404432	Genus	2
Shewanella	22	Genus	2
Thalassotalea	1518149	Genus	2
Achromobacter	222	Genus	1
Aeromonas	642	Genus	1
Alkalimonas	265980	Genus	1
Ammonifex	42837	Genus	1
Bosea	85413	Genus	1
Caballeronia	1827195	Genus	1
Cephalothrix	1844514	Genus	1
Chloroflexus	1107	Genus	1
Clostridium	1485	Genus	1
Conexibacter	191494	Genus	1
Defluviitalea	1185408	Genus	1
Dongia	1146845	Genus	1
Euryhalinema	2661529	Genus	1

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Halomonas	2745	Genus	1
Iningainema	1932705	Genus	1
Laceyella	292635	Genus	1
Leclercia	83654	Genus	1
Lonsdalea	1082702	Genus	1
Lysobacter	68	Genus	1
Marinicella	863253	Genus	1
Massilia	149698	Genus	1
Motilibacter	1434021	Genus	1
Motilimonas	1914248	Genus	1
Neobacillus	2675232	Genus	1
Nitrospira	1234	Genus	1
Oceanisphaera	225143	Genus	1
Paraburkholderia	1822464	Genus	1
Pelagicoccus	455433	Genus	1
Phenylobacterium	20	Genus	1
Photobacterium	657	Genus	1
Pseudoalteromonas	53246	Genus	1
Pseudomonas	286	Genus	1
Psychromonas	67572	Genus	1
Rhizobium	379	Genus	1
Secundilactobacillus	2767892	Genus	1
Shimwellia	1335483	Genus	1
Solirubrobacter	207599	Genus	1
Tepidimonas	114248	Genus	1
Thermoanaerobacter	1754	Genus	1
Thermodesulfovibrio	28261	Genus	1
Trinickia	2571160	Genus	1
Tumebacillus	432330	Genus	1

The relative abundances of the different bacterial communities in all the samples at the family and genus levels are shown in Figure 3. Among the bacterial families observed and identified from the pigeon pea samples soaked for 12 and 24 h, five were predominant. These were Enterobacteriaceae, Erwiniaceae, Yersiniaceae, Pectobacteriaceae, and Lactobacillaceae (Figure 3a). According to the relative abundance of the observed and identified bacterial genera, nine were predominant (Figure 3b). These were *Enterobacter*, *Klebsiella*, *Citrobacter*, *Pantoea*, *Kosakonia*, *Pseudoenterobacter*, *Pluralibacter*, *Leclercia*, and *Kluyvera* (Figure 3b). At the genus level, a slight increase in the abundance of *Klebsiella*, *Kosakonia*, and *Pluralibacter* and a slight decrease in the abundance of *Citrobacter* were observed after prolonged incubation from 12 h to 24 h (Figure 3b).

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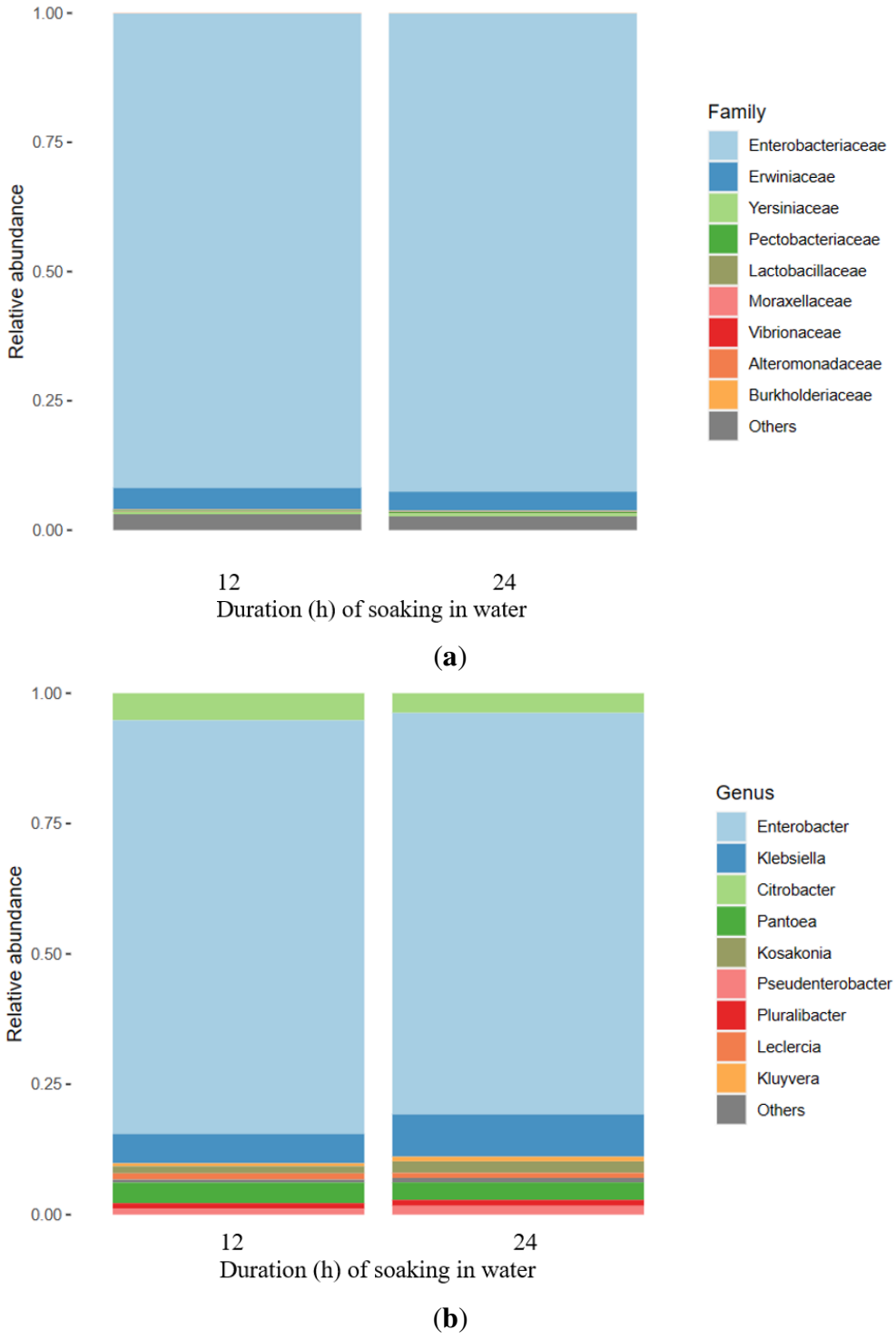

Figure 3. Relative abundance of bacterial families and genera in pigeon pea samples soaked: (**a**) 12 h; (**b**) 24 h.

The bacterial communities in the soaking water of the pigeon pea samples were dominated by the bacterial genus *Enterobacter*. The *Enterobacter* species reported in this study included *E. ludwigii*, *E. cloaceae*, *E. asburiae*, *E. chuandaensis*, *E. sichuanensis*, *E. quasiroggenkampii*, *E. oligotrophicus*, *E. wuhouensis*, *E. bugandensis*, *E. cancerogenus*, *E. mori*, and *E. kobei*. Other genera of the Enterobacteriaceae family were also identified in the pigeon pea samples, including *Salmonella* and *Shigella*; these are considered to be pathogenic microbes that cause foodborne diseases (Tables 2 and 4). The Enterobacteriaceae family is the major causative agent of foodborne diseases and it is widely dispersed in nature. The high abundance of this family in the pigeon pea samples was not surprising

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because they are present and have been detected in natural ecosystems, including in the gastrointestinal tract of vertebrates as well as in vegetation and aquatic habitats [29].

Small differences in the bacterial communities were observed in the pigeon pea samples with different soaking times. Figure 4 reveals that 231 and 296 bacteria were identified in the pigeon pea samples soaked in water for 12 and 24 h, respectively. From the total number of observed and identified bacteria, those detected in the pigeon pea sample soaked in water for 12 h accounted for 55% and those detected in the sample soaked for 24 h accounted for 70.4%. This difference increased with the soaking time and was consistent with the results of the diversity indices.

Figure 4. Venn diagram of pigeon pea samples soaked in water for different times.

The presence of a few members of the Lactobacillaceae family in the soaking water of the pigeon pea samples was notable because certain bacterial species of this family are industrially important as starter cultures for dairy fermentation (Figure 5). The bacterial species belonging to the Lactobacillaceae family observed and identified in this study were *Lacticaseibacillus paracasei* (32%), *Lentilactobacillus parabuchneri* (14%), *Lentilactobacillus hilgardii* (7%), *Liquorilactobacillus vini* (9%), *Liquorilactobacillus nagelii* (7%), and *Liquorilactobacillus sicerae* (2%) (Figure 5). The occurrence of a few lactic acid bacteria species during soybean fermentation has also been reported [30,31]. Lactic acid bacteria belong to the Firmicutes phylum. This group is the dominant bacterial group in tempeh and has a role in the acidification of pigeon pea during soaking [2]. The lactic acid bacteria genera *Lacticaseibacillus*, *Lentilactobacillus*, and *Liquoriactobacillus* observed in this study might have important roles in pigeon pea acidification. Radita et al. [32] reported that during fermentation, the low pH of pigeon pea inhibited the growth of spoilage microorganisms.

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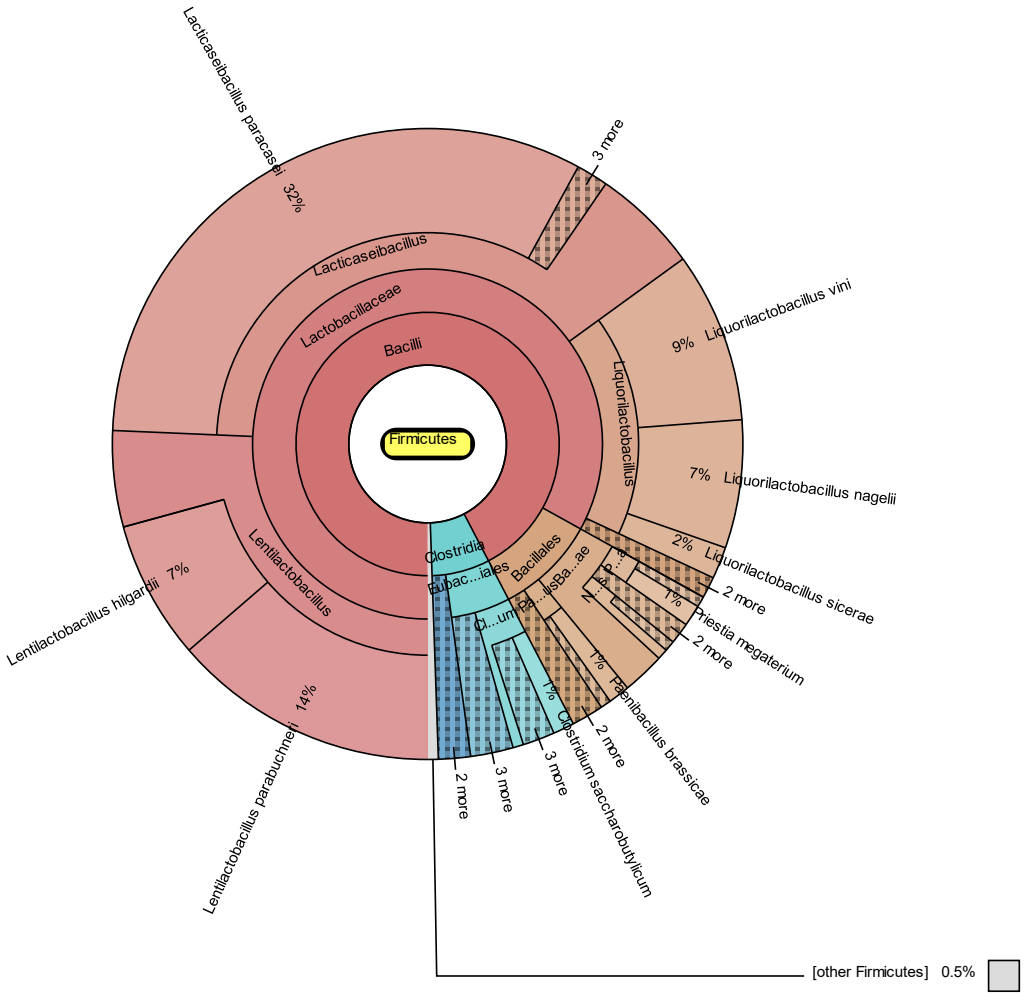

Figure 5. Bacterial composition of Lactobacillaceae family in pigeon pea sample soaked for 24 h.

The Actinobacteria phylum is another subdominant group in tempeh, but it is present in lesser amounts compared with Firmicutes and Proteobacteria. All three bacterial phyla comprise the lipolytic bacteria in tempeh that play an important role in flavor production [28]. Several Proteobacteria species, including *Klebsiella pneumoniae* and *Citrobacter freundii*, play a major role in the production of vitamin B12 in tempeh. Different food products exhibit certain differences in their bacterial communities. Li et al. [33] reported that Firmicutes and Proteobacteria were the predominant phyla in a Chinese traditional fermented broad bean (*Vicia faba* L.) paste. Peng et al. [34] reported a unique microbial diversity of fermented vegetables obtained from different regions of Hainan, China; *Lactobacillus* was the most dominant genus. Within this genus, *Lactobacillus plantarum* was the most abundant species, followed by *L. fermentum* and *L. pentosaceus*. Similarly, an analysis of the microbial composition of kimchi revealed that the main bacteria were lactic acid bacteria, including *Lactobacillus*, *Leuconostoc*, and *Weissella* [35].

Previous studies on the microflora of pigeon pea grains have frequently detected *Lentilactobacillus* and *Lactococcus* [36,37]. Demarinis et al. [36] reported the isolation and identification of lactic acid bacteria and acetic acid bacteria from pigeon pea grains. Among the identified lactic acid bacteria species, *Lentilactobacillus casei*, *Leuconostoc mesenteroides*, and *Gluconobacter hansenii* were the most abundant. Balogun et al. [37] reported the presence of *Lactococcus lactis* and *Enterococcus faecalis* in fermented pigeon pea grains. The *Lentilactobacillus* genus accounted for approximately 30% of the microbes observed in fermented pigeon pea samples examined in North America. One

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Lentilactobacillus species, *Lentilactobacillus hilgardii*, is known to produce exopolysaccharide; this affects the physicochemical quality of products during fermentation. Marsh et al. [38] reported that *Leuconostoc* species are rarely observed in pigeon pea grains. Other bacterial genera observed at low levels in pigeon pea grains included *Acetobacter* and *Gluconoacetobacter* [39]. *Gluconoacetobacter* was initially present in the grain but disappeared after fermentation. *Acetobacteria* was detected at low levels in pigeon pea grains but its role remained unclear [39].

Our metataxonomic analysis results revealed the presence of diverse bacterial families and genera in pigeon pea samples soaked in water. During fermentation, certain microbes with a metabolic activity are able to synthesize vitamins, increase the nutritional content, and produce metabolites that affect the taste and texture of the products. Several lactic acid bacteria were observed in our study, suggesting their importance in pigeon pea fermentation. These species should be isolated to assess their capability as starter cultures to develop pigeon-pea-based functional foods or drinks.

4. Conclusions

The metataxonomic analysis of pigeon pea samples soaked in water for 12 and 24 h revealed a diversity of bacterial families and genera, mainly from the Proteobacteria phylum. The main bacterial families observed and detected were Enterobacteriaceae, Erwiniaceae, Yersiniaceae, Pectobacteriaceae, and Lactobacillaceae. The pigeon pea sample collected after 24 h of soaking revealed a higher bacterial diversity and richness than the sample collected after 12 h. Overall, the bacterial communities in the soaking water of the pigeon pea samples were dominated by the Enterobacteriaceae family and the *Enterobacter* genus. The presence of *Lacticaseibacillus*, *Lentilactobacillus*, and *Secundilactobacillus* was notable because of their importance as starter cultures for the development of fermented pigeon pea beverages for lactose-intolerant individuals. This may prevent malnutrition (e.g., low lysine in pulses) and reduce antinutritional and flatulence factors.

Author Contributions: Conceptualization, W.W., D.W., Y.A.P., and Y.S.; methodology, Y.S., W.W., D.W., Y.A.P., and B.H.L.; validation, Y.S., W.W., D.W., Y.A.P., and B.H.L.; formal analysis, Y.S., W.W., D.W., and Y.A.P.; investigation, Y.S., W.W., D.W., and Y.A.P.; resources, Y.S., and W.W.; data curation, Y.S., W.W., Y.A.P., D.W., and B.H.L.; writing—original draft preparation, Y.S., D.W., Y.A.P., D.W., H.R.P., and B.H.L.; supervision, W.W., D.W., and Y.A.P.; funding acquisition, Y.S., and W.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: All data generated or analyzed during this study have been included in the paper. The raw data are available from the corresponding author on reasonable request.

Acknowledgments: Yuni Sine is a recipient of the LPDP scholarship provided by the Indonesian Government that enables her to pursue a doctoral degree at the Graduate School of Biotechnology, Universitas Gadjah Mada, in Yogyakarta, Indonesia.

Conflicts of Interest: The authors declare no competing interests or personal relationships that could have appeared to influence the work reported in this paper.

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