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# Metagenomic analysis of bacterial diversity in pigeon pea after soaking in water

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**ABSTRACT** This study investigated the diversity of bacterial community in the samples of pigeon pea (*Cajanus cajan* L. Millsp.) soaked in water for 12 h and 24 h. The detection of certain bacterial species in the samples that can be isolated and potentially be used as starter cultures in the development of pigeon pea-based functional foods is the importance of this study. For bacterial identification, the V1–V9 regions on the 16S ribosomal RNA gene were amplified using 27F and 1492R primers under specific polymerase chain reaction conditions. Genomic DNA (130 ng) was sequenced on the R9.4 flow cell by Oxford Nanopore Technologies using a GridION sequencer. Library preparations were conducted 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, and 26 families and 90 genera were identified in the pigeon pea soaked for 24 h. The values of five diversity indices showed that the sample soaked in water for 24 h had richer bacterial abundance and diversity than for 12 h. Shannon and Simpson values revealed the higher bacterial diversity in the samples collected at 24 h than in those collected at 12 h. Species observation and abundance-based coverage estimators (ACE) values demonstrated that the samples collected at 24 h harbored higher bacterial richness than those collected at 12 h. Bacterial communities during soaking of the pigeon pea were dominated by the family Enterobacteriaceae and genus *Enterobacter*. The presence of bacterial genera like *Lacticaseibacillus*, *Lentilactobacillus*, and *Secundilactobacillus* is interesting because of their importance as starter cultures for fermented plant-based milk products.

**KEYWORDS** Bacterial diversity; Lactic acid bacteria; Metagenomic analysis; Pigeon pea; Soaking time

## 1. Introduction

Functional foods have positive effects on human health. Its physiological properties are determined by its bioactive components, such as dietary fiber, antioxidants, phytochemicals, 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 the rural community. One example of functional foods produced using local food sources is tempeh, a traditional fermented soybean dish prepared using Rhizopus and that has been consumed in various countries around the world. Qiao et al. (2022) reported the health benefits of tempeh and other fermented soy foods due to their antioxidative, antihypertensive, anti-inflammatory, anticancer, and neuroprotective properties. During tempeh fermentation, certain microorganisms degrade proteins to amino acids that determine the flavor and aroma of tempeh. These native microorganisms in soybeans affect the chemical composition and aroma of final product (Nurdini et al. 2015).

Apart from soybean, pigeon pea (*Cajanus cajan* L. Millsp., local name: *kacang gude*) is another local legume that is widely used as the raw ingredient for tempeh fermentation, which belongs to the family Fabaceae (Valenzuela and Smith 2002). Indonesian pigeon pea has several advantages over other legume crops, such as being drought- and infertile soils-tolerant (Varshney et al. 2012) due to its deep roots. Therefore, pigeon pea can be cultivated in dry areas where soybean plants do not grow well. Its deep rooting does not interfere with nutrition absorption of other plants, thus pigeon pea can be intercropped (Sheahan 2012).

Pigeon pea is cultivated in several region in Indonesia due to its edible seeds. The fruit of pigeon pea is a pod that is 4–10 centimeter long, hairy, flat, and green in

color. Pigeon pea seeds are round and small, with the number of seeds per pod ranging from four to nine (Valenzuela and Smith 2002) and the pods are straight and crescent in shape, and the seed coat color varies from being grayish white, cream, yellow, purplish brown, or black. In addition, the seed coats are smooth and shiny, and the seed weight varies between 4 and 26 g per 100 grains (Maesen 1985). Pigeon pea seeds are composed of the seed coat (14%), embryo (1%), and cotyledons (85%). The nutritional content of pigeon pea seeds per 100 g is 21.7 g of protein, 1.5 g of fat, 62.8 g of carbohydrates, 12.7 g of water content, and 336 kcal total energy provided (Abebe 2022). Pigeon pea has been traditionally used as a medicinal plant (Akande et al. 2010); however, it contains cyanide and antinutritional compounds. Hence, further processing is warranted to reduce such compounds. Pigeon pea fermentation increases the availability of nutrients and health benefits of pigeon pea as a functional food (Nwosu et al. 2013).

Soybean and many other pulses are an excellent food source due to their high amounts of either dietary fibers, proteins, or micronutrients and phytochemicals. However, the consumption of pulse products has been somewhat limited because of intestinal disturbances such as flatulence, which is caused by the presence of oligosaccharides, such as raffinose and stachyose, which are nondigestible and not assimilated in the small intestine by the human GI enzymes, are fermented by the microbiota and thus responsible for flatulence (Liu et al. 2022).

Fermentation is a food biotransformation process that provides positive nutritional and sensory properties to the products depending on the microorganisms being used as starter cultures (Gan et al. 2017; Tiwari et al. 2020). Certain microorganisms can synthesize vitamins, such as folic acid, riboflavin, niacin, thiamin (Yang et al. 2015), and B12 (Gu et al. 2015) from the fermentation of grain legumes as a substrate. Other microorganisms can metabolize n-hexanal and pentanal that give a beany flavor to the products (Desai et al. 2002). Furthermore, fermentation can reduce the level of oligosaccharides, which can cause postprandial flatulence from legumes (Sandberg 2011), and decrease antinutritional components, such as tannins, phytic acid (Fredrikson et al. 2002), and trypsin inhibitors (Fredrikson et al. 2002). Different strains of the same species can have completely distinct metabolic patterns, which consequently affect the taste and texture of the product (Hickisch et al. 2016).

The process of making tempeh from pigeon pea is carried out in two stages. The first stage is soaking the pigeon pea for approximately 24 h. Soaking is carried out for 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. However, information regarding the types of bacteria that play a role in the acidification of pigeon pea tempeh is limited. Our interest is to determine the bacteria responsible for pigeon pea acidification and select those that can be used as starter cultures for the preparation of pigeon pea-based functional food products. In this study, we use metagenomic analysis by sequencing 16S ribosomal RNA (16S rRNA) genes that have widely been used for monitoring microbial population. This study aims to detect and identify all bacteria that play a role in the acidification of pigeon pea soaked for 12 and 24 h and measure their abundance in water-immersed pigeon pea. Soaking pigeon pea for 12 and 24 h has previously been reported to increase hydration of grain legumes and accelerate the growth of lactic acid bacteria, and increase the acidity which provides a suitable pH for the growth of *Rhizopus oryzae* (Prativi et al. 2023).

## 2. Materials and Methods

### 2.1. Pigeon pea collection and sample preparation

Pigeon pea samples were collected from Nusa Tenggara Timur Province in Indonesia (-10.110251059792311, 123.81441076672097) and were sorted to obtain peas with good or intact pods. In brief, 500 g of pigeon pea were 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 metagenomic analysis (Demarinis et al. 2022; Yarlina et al. 2022).

### 2.2. Genomic DNA extraction

In brief, 10 mL of each soaking water sample was centrifuged at 2,500 × g for 10 min. The obtained pellets were washed first with saline NaCl 1.5 M and then with sterile distilled water and used for genomic DNA extraction with ZymoBIOMICS DNA Miniprep Kit D4300 (Zymo Research, Cambridge, UK). DNA concentration was determined using NanoDrop spectrophotometers and Qubit fluorometer (Thermo Fisher, Waltham, Massachusetts, USA), and DNA quality was assessed via 1% agarose gel with electrophoresis 100 V, followed by visualization using Gel-Doc EZ imager (Bio-Rad, California, USA).

### 2.3. Amplification of the 16s rRNA V1–V9 region

The V1–V9 regions of the 16S rRNA gene for bacterial species identification were amplified using 27F and 1492R primers (final concentration 10 µM) with MyTaqTM HS Red Mix 2X (Bioline, Essex, UK). The following PCR conditions were applied: preliminary denaturation at 95 °C for 3 min; followed by 5 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s; 30 cycles of 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.

### 2.4. Library preparation and sequencing

A library was prepared using a Native Barcoding Kit 24 V14 (SQK-NBD114.24) (Oxford Nanopore Technologies, Oxford, UK). The amplicons from the 16S rRNA amplification (130 ng) was sequenced using Oxford Nanopore Technology (ONT, United Kingdom), which provides the

long-read sequencing that covers the full-length sequence of the 16S rRNA gene. Sequencing was conducted on the R9.4 flow cell by ONT using a GridION sequencer (ONT, United Kingdom). Primary data were acquired using MinKNOW, version 22.05.7, the operating software that operates nanopore sequencing devices.

### *2.5. Bioinformatics analysis*

Base calling was performed using Guppy version 6.1.5 with high-accuracy model (Wick et al. 2019). The quality of FASTQ files was visualized using NanoPlot 1.40.0 (De Coster et al. 2018). Reads were classified using Centrifuge classifier (Kim et al. 2016). An index for bacteria and archaea was built using NCBI 16S RefSeq database (https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/). Downstream analysis and visualizations were performed with Pavian (https://github.com/fbreitwieser/pavian), Krona Tools (https://github.com/marbl/Krona), and RStudio using R version 4.2.0 (https://www.R-project.org/) to obtain diversity index (observed species index, Chao1 index, ACE index, Shannon index, and Simpson index), and bacterial composition.

## 3. Results and Discussion

### *3.1. Metagenomic data*

The bacterial diversity in the soaking water of the pigeon pea samples soaked for different times (12 and 24 h) was examined by sequencing the hypervariable regions (V1–V9) of the 16S rRNA gene. This 16S rRNA gene is well preserved and uniquely found in all bacteria and archaea, suggesting that it specifically targets and identifies bacteria and archaea present in the samples. Given that all the informative sites of 16S rRNA genes are considered, the full-length 16S rRNA sequences can provide a high level of taxonomic and phylogenetic resolution for bacterial identification (Bahram et al. 2019). Using this technology, we identified a total of 13 bacterial families, 89 genera, and 495 species in the pigeon pea sample soaked for 12h; 26 families, 90 genera, and 533 species in the pigeon pea sample soaked for 24 h.

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 (Fang et al. 2015). Meanwhile, the observed species, Chao1, and abundance-based coverage estimators (ACE) indices reflect sample richness. The values of these five diversity indices are shown in Figure 1. The sample collected at 24 h had richer genera abundance and diversity than that collected at 12 h. The Shannon and Simpson values showed that the samples collected at 24 h harbored higher bacterial diversity than those collected at 12 h. Species observation and ACE values revealed that the samples collected at 24 h harbored higher sample richness than those collected at 12 h. All these findings indicated that the bacterial diversity in pigeon pea increases with the soaking time.

After being soaked in water for 12 and 24 h, pigeon pea's dominant phylum was Proteobacteria (Figure 2a and b). Among Proteobacteria, Enterobacteriaceae was the predominant family, and *Enterobacter* and *Klebsiella* were the most abundant genera in the samples (Figure 2a and b). At the species level, *Enterobacter ludwigii* (15%), *Enterobacter asburiae* (11%), and *Enterobacter cloacae* (8%) were the most abundant species in the samples soaked in water for 12 h. These three *Enterobacter* species were still the most dominant species after soaking pigeon pea in water for 24 h. Other phyla were detected, such as Firmicutes and Actinobacter; however, their relative abundance remained below 1.00%. Nur et al. (2020) reported that Firmicutes and Actinobacteria were consistently found in tempeh fermentation, with Firmicutes be-

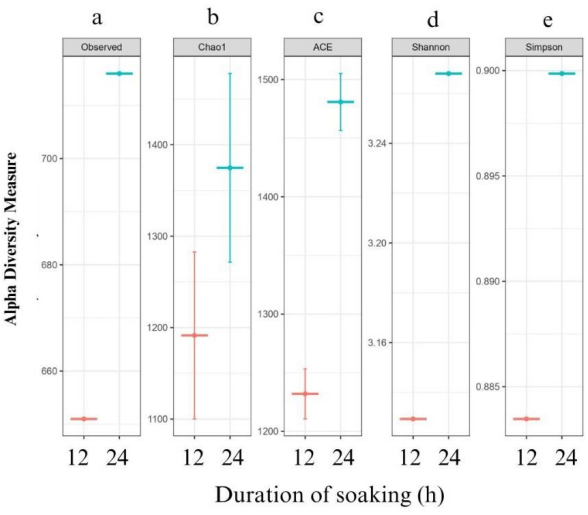
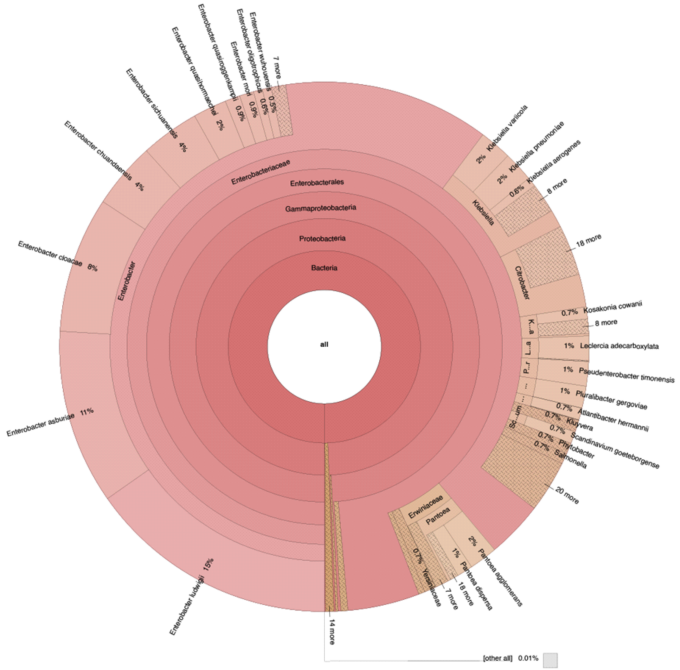


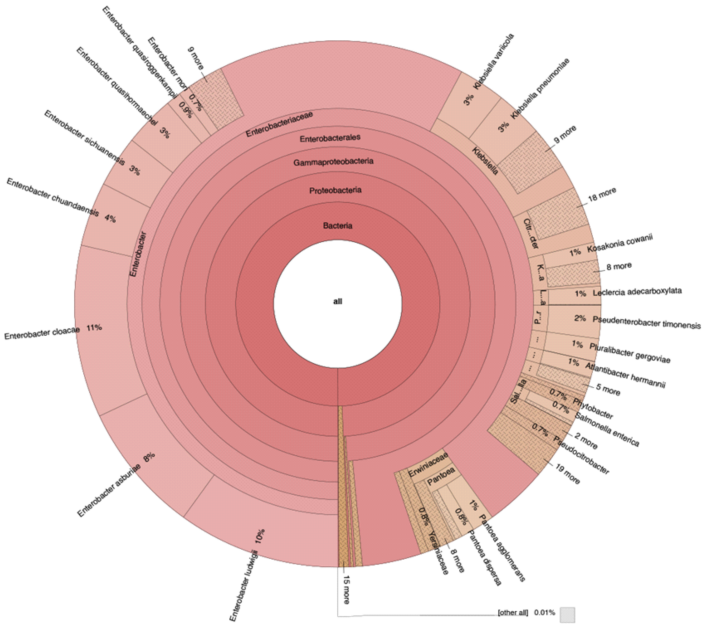

**FIGURE 1** Diversity index box figure of bacteria in pigeon pea at different soaking times. a: Observed species index; b: Chao1 index; c: ACE index; d: Shannon index; e: Simpson index.

**TABLE 1** Classification of the bacterial family of the pigeon pea sample after being soaked in water for 12 h using Centrifuge's 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 |




(a)




(b)

**FIGURE 2** Bacterial composition of pigeon pea after being soaked in water. a. 12 h, b. 24 h.

ing the most dominant bacteria.

Metagenomic analysis was previously used to reveal the bacterial diversity during fermentation process of farmhouse sauce from Northeast China (Hao and Sun 2020). By sequencing the 16S rRNA, they identified 16 phyla, 37 classes, 56 orders, 96 families, 139 genera, and 81 species. *Tetragenococcus*, *Weissella*, *Lactobacillus*, and *Leuconostoc* were all detected during the fermentation process, but *Lactobacillus* was the most important microflora. Metagenomic analysis has also been applied to reveal microbial community in Toddy, a popular fermented palm beverage of India (Das and Tamang 2023). Using shotgun-based metagenomic, they identified 54 phyla, 363 families, 1087 genera and 1885 species. The most abundant bacterial phylum was *Bacillota* (49.3%) and several bacterial species were identified, including *Leuconostoc mesenteroides*, *Leuconostoc citreum*, *Lactobacillus helveticus*, *Lactiplantibacillus plantarum*, *Lac-*

**TABLE 2** Classification of the bacterial genus of the pigeon pea sample after being soaked in water for 12 h using Centrifuge's 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 |
| *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 |
| *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 |

*tococcus lactis*, *Acetobacter malorum*, *Gluconobacter japonicus*, *Gluconacetobacter liquefaciens*, *Fructobacillus durionis*, *Zymomonas mobilis*. Meanwhile, Erhardt et al. (2023) identified 135 lactic acid bacteria species of 7 genera from artisanal cheeses produced in Brazil by amplifying the V3/V4 region of the 16S rRNA with *Lactococcus lactis* as the most abundance species in all samples.

The identified lactic acid bacteria were *Bavariicococcus*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Marinillactibacillus*, and *Pediococcus*. The bacterial diversity reported by Das and Tamang (2023) was higher than that reported by Hao and Sun (2020), and Erhardt et al. (2023), and by our current study. The findings certainly prove that shotgun metagenome sequencing captures more microbial diversity than 16S rRNA gene sequencing.

### *3.2. Bacterial diversity*

Bacterial 16S rRNA gene sequences were classified at the family and genus levels to find out what makes up the bacterial community in pigeon pea soaked in water. A total of 13 families (Table 1) and 89 genera (Table 2) were identified in the pigeon pea sample soaked in water for 12 h. Meanwhile, 26 bacterial families (Table 3) and 90 genera were identified in the pigeon pea soaked in water for 24 h (Table 4).

The relative abundances of the different bacterial communities in all the samples at the family and genus lev-

**TABLE 3** Classification of the bacterial family of the pigeon pea sample after being soaked in water for 24 h using Centrifuge's 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 genus of the pigeon pea sample after being soaked in water for 24 h using Centrifuge's nt database.

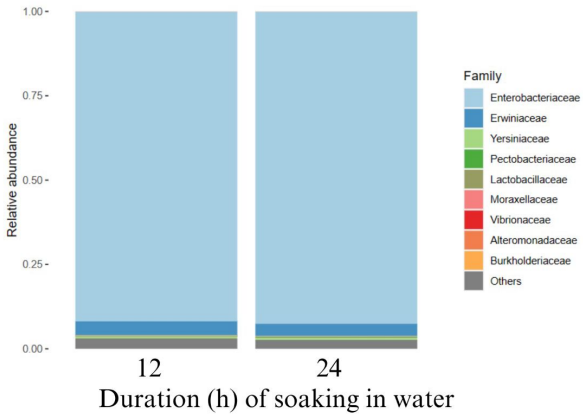
| Genus name | taxID | taxRank | Number of uniquely classified reads |
|---|---|---|---|
| *Enterobacter* | 547 | genus | 11233 |
| *Klebsiella* | 570 | genus | 1061 |
| *Citrobacter* | 544 | genus | 842 |
| *Kluyvera* | 579 | genus | 150 |
| *Kosakonia* | 1330547 | genus | 144 |
| *Pantoea* | 53335 | genus | 98 |
| *Serratia* | 613 | genus | 68 |
| Tatumella | 82986 | genus | 58 |
| *Erwinia* | 551 | genus | 45 |
| *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 |

**TABLE 4** (continued).

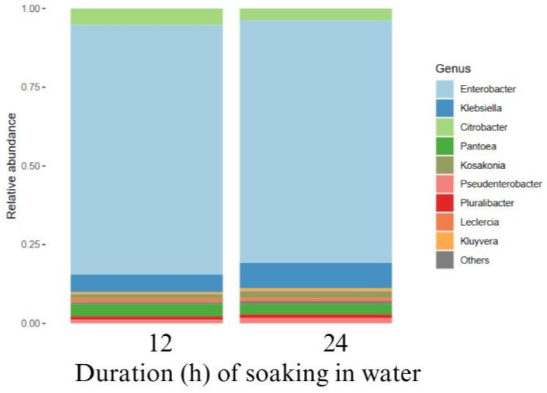
| Genus name | taxID | taxRank | Number of uniquely classified reads |
|---|---|---|---|
| *Thalassotalea* | 1518149 | genus | 2 |
| *Achromobacter* | 222 | genus | 1 |
| *Aeromonas* | 642 | genus | 1 |
| *Alkalimonas* | 265980 | genus | 1 |
| *Ammonifex* | 42837 | genus | 1 |
| *Bosea* | 85413 | genus | 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 |
| *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 |

els are shown in Figure 3. Among the bacterial families observed and identified from the pigeon pea sample soaked for at 12 and 24 h, five were predominant, namely, Enterobacteriaceae, Erwiniaceae, Yersiniaceae, Peptobacteriaceae, and Lactobacillaceae (Figure 3a). Meanwhile, according to the relative abundance of the observed and identified bacterial genera, nine of them were predominant (Figure 3b), namely, *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).

The bacterial communities in the pigeon pea's soaking water were dominated by bacterial genus *Enterobacter*. *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*. Apart from the genus *Enterobacter*, some other genera of the family Enterobacteriaceae were identified from the pigeon pea's samples, including *Salmonella* and *Shigella* that are considered as pathogenic microorganisms causing foodborne diseases (Tables 2 and 4). The family Enterobacteriaceae is the major causative agent of foodborne diseases and is widely dispersed in nature. The




(a)




(b)

**FIGURE 3** Relative abundance of bacterial (a) family and (b) genera in pigeon pea after being soaked for 12 and 24 h.


**FIGURE 4** Venn diagram of pigeon pea soaked in water for 12 hours (left) and 24 hours (right)

high abundance of this family in the pigeon pea samples is not surprising because they are present and have been detected in natural ecosystems, including in the gastrointestinal tract of vertebrates, vegetation, and aquatic habitats (Janda and Abbott 2021).

Small differences in bacterial communities were observed among the pigeon pea samples under different soaking times. Figure 4 shows 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 samples soaked in water for 12 h accounted for 55%, and those detected in the samples soaked for 24 h accounted for 70.4%. This difference increased with the soaking time and was consistent with the abovementioned results for diversity indices.

The presence of some members of the family Lactobacillaceae in the pigeon pea's soaking water is interesting; some bacterial species of this family are industrially important as starter cultures for dairy fermentation (Figure 5). The bacterial species belonging to family Lactobacilaceae 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 some lactic acid bacteria species during soybean fermentation has been reported (Ma et al. 2022; Sirilun et al. 2017). Lactic acid bacteria belong to the phylum Firmicutes. This group is the dominant bacteria in tempeh because of their role in the acidification of pigeon pea during soaking (Nurdini et al. 2015). The lactic acid bacteria genera *Lacticaseibacillus*, *Lentilactobacillus*, and *Liquoriactobacillus* observed in this study might have important roles for pigeon pea acidification. Radita et al. (2018) reported that during fermentation, the low pH of pigeon pea inhibited the growth of spoilage microorganisms.

The lactic acid bacteria species isolated from the soaking water of pigeon pea samples can be of interest as potential starter fermentation to improve nutritional value, flavor characteristic, and health-promoting effects of soy beverages. The use of *Lacticaseibacillus rhamnosus* GG and *L. rhamnosus* INIA P344 as starters for soy beverage fermentation increased antioxidant capacity in the fermented products (Ruiz de la Bastida et al. 2023). Luo et al. (2023) reported flavor improvement of fermented soybean foods due to co-culture of *Bacillus velezensis* and *Lactiplantibacillus plantarum* which produced acetoin and pyrazines. Promoting health benefit of the fermented pigeon pea products by lactic acid bacteria species as starters was reported by Yogeswara et al. (2023). They concluded the increased γ-aminobutyric acid in pigeon pea fermented by *Lactiplantibacillus plantarum* Dad-13 as starters.

Phylum Actinobacteria is another subdominant group in tempeh but is present in lesser amounts compared with Firmicutes and Proteobacteria. All three bacterial phyla make up the lipolytic bacteria in tempeh that play an important role in flavor production (Nur et al. 2020). Some Proteobacteria species such as *Klebsiella pneumoniae* and *Citrobacter freundii* play a major role in the production of vitamin B12 in tempeh. Different food products exhibit certain differences in bacterial communities. Li et al. (2017) reported that Firmicutes and Proteobacteria were the predominant phyla in a Chinese traditional fermented broad bean (*Vicia faba* L.) paste. Peng et al. (2018) reported the unique microbial diversity of fermented vegetables obtained from different regions of Hainan, China and found that *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 showed that main bacteria were lactic acid bacteria, including *Lactobacillus*, *Leuconostoc*, and *Weissella* (Kim and Chun 2005).

Previous studies on pigeon pea grain's microflora frequently detected *Lentilactobacillus* and *Lactococcus* (Balogun et al. 2021; Demarinis et al. 2022). Demarinis et al. (2022) 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. (2021) reported the presence of *Lactococcus lactis* and *Enterococcus faecalis* in fermented pigeon pea grains. *Lentilactobacillus* genus accounted for approximately 30% of the microorganisms found in the fermented pigeon pea samples examined in North America. One *Lentilactobacillus* species, *Lentilactobacillus hilgardii*, is known to produce exopolysaccharide, which affects the physicochemical quality of products, during fermentation. Marsh et al. (2013) reported that *Leuconostoc* species are rarely found in pigeon pea grains. Other bacterial genera found at low levels in pigeon pea grains included *Acetobacter* and *Gluconoacetobacter* (Gulitz et al. 2011). *Gluconoacetobacter* was initially present in the grain but disappeared after fermentation. Meanwhile, Acetobacteria was detected at low levels in pigeon pea grains, but its role

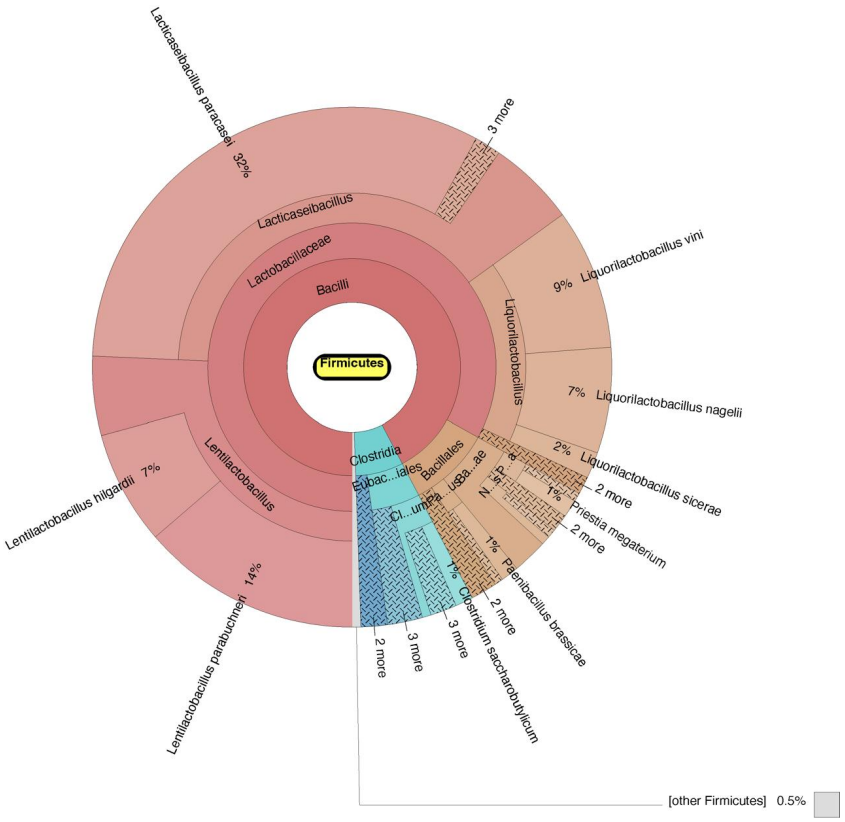



**FIGURE 5** Bacterial composition of family Lactobacilaceae in pigeon pea soaked for 24 h.

remains unclear (Gulitz et al. 2011).

Metagenomic analysis results showed the presence of diverse bacterial families and genera in pigeon pea soaked in water. During fermentation, certain microorganims with metabolic activity were able to synthesize vitamins, increase the nutritional content, and produce metabolites that affect the taste and texture of the products. Some lactic acid bacteria were reported in this study, suggesting their importance for pigeon pea fermentation. These species can be isolated to assess their capability as starter cultures for developing pigeon pea-based functional foods or drinks.

## 4. Conclusions

The metagenomic analysis of pigeon pea soaked in water for 12 and 24 h shows the diversity of bacterial families and genera, mainly from phylum Proteobacteria. The main bacterial families observed and detected were Enterobacteriaceae, Erwiniaceae, Yersiniaceae, Peptobacteriaceae, and Lactobacillaceae. The pigeon pea samples collected after 24 h of soaking showed higher bacterial diversity and richness than those collected after 12 h. The presence of *Lacticaseibacillus*, *Lentilactobacillus*, and *Secundilactobacillus* is interesting because of their importance as starter cultures for the development of fermented pigeon pea beverages for lactose-intolerant individuals. These three lactic acid bacteria genera are potential for improving nutritional content, flavor characteristics, and health-promoting effects of soy beverage products. These products may prevent malnutrition (low lysine, etc. in pulses), and for reducing antinutritional and flatulence factors.

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## Authors' contributions

Conceptualization, WW, DW, YAP., and YS; methodology, YS, WW, DW, YAP, and BHL; validation, YS, WW, DW, YAP, and BHL; formal analysis, YS, WW, DW, and YAP; investigation, YS, WW, DW, and YAP; resources, YS, and WW; data curation, YS, WW, YAP, DW, and BHL; writing—original draft preparation, YS, DW, YAP, DW, HRP, and BHL; supervision, WW, DW, and YAP; funding acquisition, YS, and WW. All authors have read and agreed to the published version of the manuscript.

## Competing interests

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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