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Bacterial diversity in cheese wastewater using Next-Generation Sequencing (NGS)

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Abstract. *Estikomah SA, Suranto, Susilowati A, Masykuri M. 2024. Bacterial diversity in cheese wastewater using Next-Generation Sequencing (NGS). Biodiversitas 25: 482-490.* Cheese wastewater (whey) has a high content of organic substances, including lactose, protein, and fat. The lactose in whey wastewater can also be used as a bacterial growth medium. Bacterial community structure is an essential aspect of microbial water quality. The bacterial diversity data obtained can then be used to evaluate the existence of bacteria that might be useful for making microbiological products. This research employs Next Generation Sequencing (NGS) technology to determine the diversity and abundance of bacteria based on 16S rRNA gene amplicons for further processing of whey wastewater. The NGS-based technique overcomes the limitations of conventional bacterial culture techniques. The effectiveness and accuracy of microbial diversity analysis employing NGS technology are high. The research method includes the steps of sample preparation, DNA extraction using a ZymoBIOMICS DNA Microprep Kit (D4300), PCR amplification of the V3-V4 16S rRNA gene region, DNA sequencing, and a bioinformatics-statistical analysis. The results show that bacterial diversity in whey wastewater was found to have an average number of Operational Taxon Units (OTUs) of 259 tags. The metagenomics study of the microbial community in whey wastewater successfully detected the dominant genus of bacteria, which can benefit the management of whey wastewater. The presence of *Lactobacillus* and Acetobacter confirms that cheese whey wastewater exists in Yogyakarta Province. *Acetobacter* can oxidize ethanol to produce acetic acid, a pungent odor characteristic that causes environmental pollution. On the other hand, the presence of *Lactobacillus* and Acetobacter shows that whey wastewater can be reprocessed to produce fermented beverages, thereby improving their value and minimizing the impact of environmental pollution.

Keywords: Bacteria diversity, *Lactobacillus*, NGS technology, wastewater, whey

INTRODUCTION

Cheese wastewater (whey) is the main byproduct of the cheese industry (Fox PF et al. 2017). Cheese whey wastewater has a high lactose content, which contributes to the high Biochemical Oxygen Demand (BOD) (50,000 mg/L) and Chemical Oxygen Demand (COD) (80,000 mg/L) of polluted water bodies (Dullius et al. 2018). When discharged into bodies of water, the high BOD and COD levels in whey will decrease the dissolved oxygen level. Microorganisms use the dissolved oxygen in the water to degrade organic waste materials into a more volatile substance characterized by a foul odor (Ambarsari et al. 2023). These organic waste materials are degraded and converted into CO2 gas, water, and NH3 gas. The emergence of NH3 gas causes a foul odor in the polluted water body. If the dissolved oxygen level is extremely low, the aerobic bacteria will eventually die, and the anaerobic bacteria will then take over the degradation of waste materials in the water. These anaerobically degraded waste materials generally have an unpleasant odor (Gostelow et al. 2001; Burlingame et al. 2004). Unpleasant odors can have a detrimental effect on people's daily lives (Agus et al. 2012). Furthermore, a decrease in the dissolved oxygen concentration beyond a certain threshold will result in the death of other aquatic biota due to a lack of oxygen (Mahaffey 2023).

Cheese wastewater (whey) has a high content of organic substances, including lactose, protein, and fat, which can be processed into valuable products such as the functional beverage kefir, as described in the research by Weschenfelder et al. (2018). The lactose in whey wastewater can also be used as a bacterial growth medium (Khan et al. 2015), demonstrating how wastewater can be processed to produce other valuable products. According to Rama et al. (2019), cheese wastewater can be used as a lactic acid bacteria culture medium.

Cheese wastewater can be used as a growth medium for bacteria and yeast, including *Lactobacillus casei*, *Lactobacillus plantarum*, *Bacillus coagulans*, *Lactobacillus delbrueckii*, *Lactococcus lactis*, *Rhodovulum sulfidophilum*, *Haloferax mediterranei*, *Pseudomonas hydrogenovora*, *Pseudomonas taetrolens*, *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Kluyveromyces lactis*,

and *Yarrowia lipolytica* (Cutone et al. 2020; Nagarajan et al. 2020; Olszewska-Widdrat et al. 2020; Sahoo and Jayaraman 2019; Luongo et al. 2019). Cheese wastewater (whey) products can be produced by utilizing the components and biological activity in the waste, and for this purpose, it is necessary to evaluate the microbiological diversity. The bacterial diversity data obtained can then be used to evaluate the existence of bacteria that might be useful for making microbiological products such as probiotic bacteria-based products. This will improve the value of the processed cheese wastewater (whey) and help overcome the environmental pollution risk.

Research about bacterial diversity frequently uses conventional culture techniques and has recently included the metagenomics approach (Yeluri Jonnala et al. 2018; Almeida et al. 2014). Conventional techniques are often considered outdated because of their inefficiency and ineffectiveness. The greatest limitations of conventional techniques are time-consuming and error-prone (Chen 2020). The method is the metagenomics approach is based on an analysis of DNA that is extracted directly from the environment using NGS technology (Simamora et al. 2016).

The NGS technique is a revolutionary tool for microbiology researchers to explore microbial diversity. This technique can overcome the limitations of conventional bacterial culture techniques (Nalepa et al. 2020). NGS technology has been continuously developed into a newer, inexpensive, and better sequencing platform. The effectiveness and accuracy of microbial diversity analysis employing NGS technology are high-throughput and relatively rapid turnaround time (Palaniveloo et al. 2020). Garner et al. (2021) studied bacterial diversity in whey wastewater to evaluate water quality and whey wastewater. Similar research by da Silva Duarte et al. (2020) explored the microbial diversity of whey wastewater from four cheese industries in Italy. Numerous research studies have been conducted on microbial diversity, but no existing studies on microbial diversity in wastewater in Indonesia employ NGS technology.

Bacterial community structure is an essential aspect of microbial water quality. The bacterial diversity data obtained can then be used to evaluate the existence of bacteria that might be useful for making microbiological products such as probiotic bacteria-based products. This will improve the value of the processed cheese wastewater (whey) and help overcome the environmental pollution risk. Numerous research studies have been conducted on microbial diversity, but no existing studies on microbial diversity in cheese wastewater in Indonesia employ NGS technology. In the present study, the bacterial community diversity in cheese wastewater from Cancangan, Wukirsari Village, Cangkringan Sub-district, Sleman District, Yogyakarta, Indonesia.

MATERIALS AND METHODS

Study area

This exploratory and descriptive study was conducted in 2022. Samples were taken during the dry season. The whey wastewater samples were taken with two repetitions from the cheese industry in Cancangan, Wukirsari Village, Cangkringan Sub-district, Sleman District, Yogyakarta, Indonesia, to obtain samples 1 and 2.

Procedure

Stages of bacteria identification from cheese wastewater (whey) wastewater using the NGS Method:

DNA extraction

DNA extraction is the process of separating DNA from other cell components. The DNA was extracted from two whey wastewater samples using a ZymoBIOMICS DNA Microprep Kit (ZYMO Research, Inc., D4300). The DNA extraction stages in this research include: A sample of 250 µL of cheese wastewater (whey) was then put into a tube and added with 750 µL of ZymoBIOMICS™ Lysis Solution. The sample was put into a centrifugation bead beater for ≥5 minutes and then centrifuged at 10,000xg for 1 minute. Transfer 400 µL of supernatant to a filter tube and collection tube centrifuge 8,000x g 1 minute; next, 1,200 µL of genomic lysis buffer to the previous filtrate. Transfer 400 µL into the column tube and collection tab and centrifuged at 10,000 x g for 1 minute. Wash twice using 200 µL DNA prewash buffer and 500 µL DNA Wash buffer, centrifuged at 10,000xg for 1 minute. Move the spin column into a 1.5 mL microcentrifuge tab and add 100 µL DNA elution buffer to the column; next, centrifuge at 10,000 xg for 30 seconds. Place the filter tab into the collection tab and add 600 µL of prep solution centrifuged at 8,000xg for 3 minutes. The DNA filter results are ready for PCR or other downstream analysis.

16S rRNA gene amplification

The 16S rRNA gene, specifically in the V3-V4 region, was amplified using 314F (5'-CCTAYGGGRBGCAS CAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') primers. The PCR process was conducted using a 15 µL Phusion® High-Fidelity PCR Master Mix (New England Biolabs), with a PCR mix composition of 2 µM from each primer, 10 µL DNA template, and 2 µL ddH2O. The PCR condition was set in the pre-denaturation stage at 98°C for 1 minute, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30s, elongation at 72°C for 30s, and finally post-elongation at 72°C for 5 minutes.

Quantification and qualification of PCR amplicons:

The purpose of quantifying the PCR amplicons was to measure the concentration and purity of the amplicon quantity using a Qubit fluorometer and bioanalyses. The qualification of the PCR amplicons was aimed at measuring the amplicon quality, including DNA band size, using 2% agarose gel electrophoresis. The electrophoresis was done by mixing the amplicons with loading dye and DNA staining (SYBR Green) in 2% agarose gel. Gel Documentation was then used to visualize the results to measure the DNA band size and quality (Woodman et al. 2016)

DNA library preparation and sequencing

The selected DNA amplicons from the quantification and qualification process were collected into a single pooled sample. Each DNA sequence was then prepared for the subsequent adapter and barcode ligation process to the sequences. The adapter sequence attached to the DNA amplicon sequence was required so the sequencer machine could recognize and read the DNA sequence. The barcode sequence served as an identity label for each sample in the pooled sample. The preparation process of adapter and barcode ligation involved three phases, namely (i) end-repair, (ii) A-tailing, and (iii) ligation. The end-repair phase was aimed to prepare the amplicon sequence end to accept the phosphate group donor in the 5' end later and poly-Adenil (A) nitrogen bases in the 3' end on the A-tailing phase. The adapter and barcode sequences could then be attached to both ends of the amplicon sequence (forward and reverse). This library preparation process for sequencing was synthesized with an NEB Next® Ultra™ II FS DNA PCR-free Library Prep Kit (New England Biolabs, USA, Catalog #: E7430L) following the manufacturer's manual. The library sequence was then quality control checked with Qubit, qPCR, and bioanalysis instruments. The sequencing reading process was then executed in the Illumina platform.

Data analysis

The data sequence was analyzed using UPARSE software (UPARSE v7.0.1001, http://drive5.com/uparse/) (Edgar 2013). Mothur (v1.35.1) was used to annotate the OTUs sequences based on SSUrRNA SILVA (threshold 0.8-1.0) (Quast et al. 2013) so that the taxonomic and bacterial diversity data at every classification level (kingdom, phylum, class, ordo, family and genus) could be analyzed. Before performing an alpha diversity analysis, the MUSCLE algorithm (v1.9.1) was used to align the sequences. The alpha diversity indices included Chao1, Shannon, Simpson, and ACE. The rarefaction and rank abundance curves were plotted using R software (v2.15.3).

RESULTS AND DISCUSSION

Analysis of bacterial community diversity in cheese wastewater

Cheese wastewater (whey) has a high content of organic substances, including lactose, protein, and fat (Weshenfelder et al. 2018). The lactose in whey wastewater can also be used as a bacterial growth medium (Khan et al. 2015). Bacterial community structure is an essential aspect of microbial water quality. The bacterial diversity data obtained can then be used to evaluate the existence of bacteria that might be useful for making microbiological products such as probiotic bacteria-based products. This will improve the value of the processed cheese wastewater (whey) and overcome the environmental pollution risk. Numerous research studies have been conducted on microbial diversity, but no existing studies on microbial diversity in cheese wastewater in Indonesia employ NGS technology. In the present study, the bacterial community diversity in cheese wastewater from Cancangan, Wukirsari Village, Cangkringan Sub-district, Sleman District, Yogyakarta, Indonesia. Richness and evenness are the two critical components of community diversity. The bacterial community diversity in whey wastewater was analyzed in sample 1 and sample 2, visualized in a rarefaction curve (Figure 1) and an alpha diversity indices table (Table 1). Rarefaction is a method that adjusts for differences in library sizes across samples to aid comparisons of alpha diversity. Rarefaction curves are widely used to indicate the biodiversity of a sample. The rarefaction curves can directly indicate the rationality of sequencing data volume and indirectly indicate the microbial community richness in the samples (Lundberg et al. 2013). The rarefaction curve (Table 1) provided the first indication of the quality of the metagenomics data. A rarefaction curve is created by randomly selecting a certain amount of sequencing data from a sample and then calculating the number of species it represents (i.e., the number of operational taxonomical units/OTUs). Rarefaction curves of bacterial diversity in whey wastewater are shown in Figure 1. The rarefaction curves in Figure 1 show that the sampling method was conducted adequately to represent the actual diversity of the samples.

In Figure 1, the X-axis shows the number of sequenced samples, and the Y-axis shows the number of identified species. The curves represent the rationality of sequencing data volume, which correlates to the richness of bacterial communities in the samples. Suppose the steep curve indicates that the sequenced data does not represent the actual number of OTUs in the sample. Vice versa, if the curve is flatter, it means that a credible number of sequenced data have been discovered to represent the actual OTU diversity in the sample sufficiently (the number of reads chosen for the normalized cut-off score, in this case, was 164.336). A specific pair of primers was successfully used to sequence 18.531 OTUs from the whey wastewater.

This study classified the sequenced data into operational taxonomical units (OTUs), distance-based clusters of sequences initially constructed without a reference database (Wensel 2022). An OTU sequence identity greater than 97% (or with up to 3% dissimilarity) is typically estimated to define a species. In comparison, OTUs with sequence similarities of 95% and 80% are used to define genus and phylum, respectively. Only sequences with the highest frequency were selected as the representative sequences of OTUs and annotated using the Green Genes database (Xia 2018). Each sequenced sample was classified into species-level taxa. The bacterial diversity in whey wastewater had an average number of Operational Taxon Units (OTUs) of 259 tags, comprising 166.097 taxon tags (Figure 2). The number of unclassified tags recorded was categorized as small value, of which only approximately 1 OUTs, indicating that the data provided was reliable.

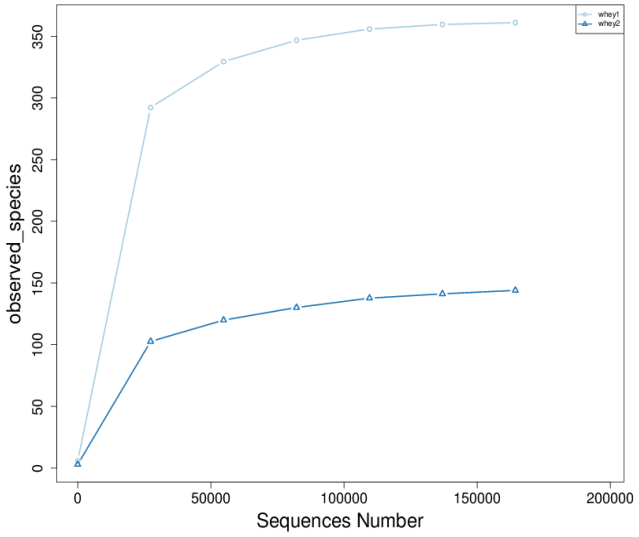

Figure 1. Rarefaction curves of bacterial diversity in whey wastewater. Note: Sample 1: dark blue; Sample 2: light blue

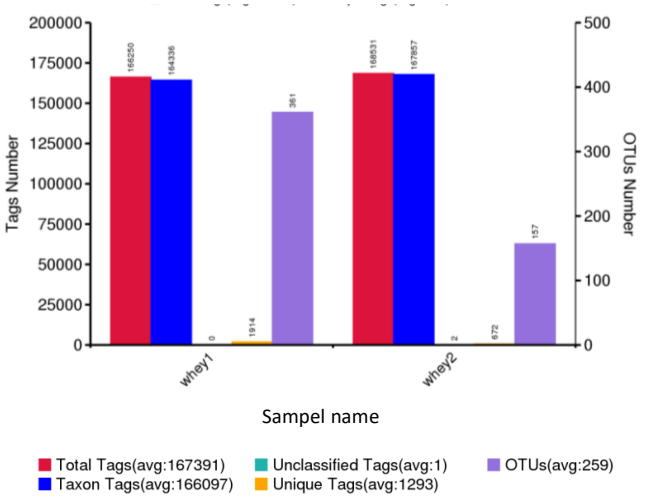

Figure 2. Statistics of taxonomical identification of sequenced data

Table 1. Alpha diversity indices of whey wastewater

Sample	Observed species	Shannon	Simpsons	Chao1	ACE
Whey1	361	3.482	0.67	361.158	362.210
Whey2	144	1.541	0.56	149.353	151.305

Alpha diversity analyzes the structure of an ecological community concerning the richness of taxonomical groups, the distribution of abundance of the groups, or both. The alpha diversity analysis of sequenced data is a method that can be used to measure the differences between groups; Alpha diversity indices of whey wastewater in Table 1.

The diversity index is an analysis to measure the diversity of a population in a sample or community. Table 1 calculated all the matrices based on a 97% degree of sequence similarity, a similarity degree ≥97% were identified as the same species. The Shannon and Simpson indices also describe community diversity (Kim et al. 2017). Shannon's diversity (H') is based on information theory that represents uncertainty about the identity of an unknown individual. In an evenly distributed and much diverse system, an unidentified individual could belong to any species, leading to uncertainty in its identification, but easier in a less diverse community of few species (Shannon 1948). According to Shannon-Weiner (1949), the classification of H'are as follows: low diversity(H' = 0-2,302), medium diversity (H' = 2,302-6,9078), and high diversity (H' >6,9078).

In Table 1, the Shannon index is over 6,9078, which means that diversity and community stability are high. The Shannon index shows the degree of domination. The higher the degree of domination, the lower the level of diversity. Simpson's Diversity Index is a measure of diversity that considers the number of species present and the relative abundance of each species. As species richness and evenness increase, so does diversity increases.

Simpson's index developed by Simpson (1949) measures the probability of two randomly selected species to be the same in a less diverse system. Simpson's index represents the probability that two randomly chosen individuals belong to different species (McCune and Grace 2002). The range is between 0-1, where the larger the value of Simpson's index the lower the diversity. Simpson's index estimated in whey 1 (0.67) and whey 2 (0.56) indicating a diverse system. The index represents the probability that two individuals randomly selected from a sample will belong to different species and ranges from 0-1. The higher the value the greater the diversity. The Simpson index is 0.67-0.56, considered high because it is around the estimation score of 0.61-1.0 and has a high distribution level.

The Chao1 and ACE indices are based on species abundance in a community (Chao et al. 2000; Colwell 2013). The Chao1 index can estimate the minimum number of OTUs in a sample as a richness estimator. ACE Abundance-based coverage estimator of species richness (Chao and Lee 1992). The total number of OTUs for the bacterial community of whey as estimated by Chao1 and ACE was similar to the observed OTUs, indicating the 100% coverage, all bacterial phylotypes presented in the sample had been covered. Based on the ACE measurements, the percentage of bacterial species sampled from the whey wastewater was (100%). The higher the diversity value of an area, the more stable the community (Ouyang et al. 2021).

Bacterial diversity of whey wastewater

The bacterial diversity of whey wastewater, based on the phylum annotations of all the OTUs, was classified according to the corresponding bacterial domains. Figure 3 shows the bacterial groupings are based on phylum taxa. The level of diversity in whey wastewater, at the phylum level, shows that Firmicutes, Proteobacteria, Bacteriodota, and Actinobacteria are the dominant phyla. This research is

in line with the research of Wang et al. (2018), which found that Firmicutes, Proteobacteria, Bacteriodota, and Actinobacteria were the dominant phyla in a diverse study of acidic whey in Yunnan Province, China. Mayo et al. (2021) report that Proteobacteria and Firmicutes were found to be dominant in cheese products. This research also aligns with Mazorra-Manzano et al. (2022), which explains that Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria were dominant in cheese whey fermented at different temperatures. The Firmicutes phylum is identical to the general phylum that is frequently found in fermented foods, for example, in microbiological studies of lactic acid bacteria in kefir seeds (Chen et al. 2022), homemade yogurt (Demirci et al. 2022), and fish sauce fermentation (Korena et al. 2023).

The bacterial diversity of whey wastewater at the genus level is shown in Figure 4. Figure 4 shows that *Lactobacillus* (91,488 OTUs representing 55% of the total bacteria in sample 1 and 65,814 OTUs representing 40% of the total bacteria in sample whey 2) and *Acetobacter* (12,403 OTUs representing 7% of the bacteria in sample whey1 and 87,025 representing 52% of the bacteria in sample 2) were the dominant genera in whey wastewater from Yogyakarta Province. *Lactobacillus* and *Acetobacter* are usually associated with naturally fermented dairy products (Li et al. 2017; Sun et al. 2013).

Lactobacillus is the main genus because this bacteria group was used as the cheese inoculum. This genus is a probiotic with specific health benefits, such as a countermeasure for diarrhea, stimulating the immune system, lowering cholesterol levels, and preventing colon cancer and atopic dermatitis in children. *Acetobacter*, the second most dominant genus, can oxidize ethanol to produce acetic acid, with a strong foul odor that causes environmental pollution. The presence of Acetobacter bacteria in cheese liquid waste has the potential to be used as a raw material for various vinegars, an organic acid chemical compound known for with sour taste; vinegar acid is also known as acetic acid.

Many reports comprehended that whey could be used for vinegar, such as research by Lustrato et al. (2013). Production of vinegar from kashar cheese whey and volatile component profile, antibacterial effect, and antioxidant potential of whey vinegar. Vinegar is commonly used in vinaigrettes, mayonnaise, processed canned foods, cooked meat and fish, and whey has been used to produce acetic acid. In related studies, Mazorra et al. (2022), the LAB genera are related to lactic acid fermentation during spontaneous whey fermentation. Lustrato et al. (2013) also revealed that acetic acid bacterium were used as inocula, in cheese whey for alcoholic and lactic acid fermentation, respectively. The domination of *Lactobacillus* and *Acetobacter* in whey wastewater has previously been reported by Y. Wang et al. (2018), who found this two genus to be dominant in whey samples in a study of bacterial diversity in traditional acidic whey from Yunnan province, China. *Lactobacillus* is the dominant genus because this bacteria group was used as the cheese inoculum.

Lactic Acid Bacteria (LAB) in cheese waterwaste

Next-Generation Sequencing (NGS) for metagenomics is a method that allows the identification of existing microbial communities without culturing. NGS technology can generate more sequences per sample, thus enabling the analysis of many detailed taxonomic profiles. NGS is a DNA sequencing technique that identifies sequences through parallel sequencing of multiple small fragments of DNA. The 16S ribosomal RNA (rRNA) is part of the small 30S ribosomal subunit in prokaryotic cells. Sequence variation in the 16S gene is widely used to characterize diverse microbial communities (Rizzo et al. 2012). Consequently, this study aimed to identify by NGS was employed to perform 16S rRNA metagenomic analysis to identify bacterial diversity in cheese wastewater (whey), especially lactic acid bacteria with potential commercial applications.

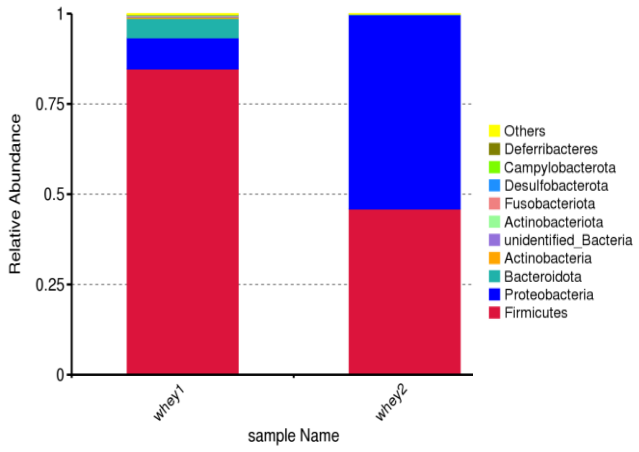

Figure 3. Bacterial diversity at phylum level in whey wastewater samples

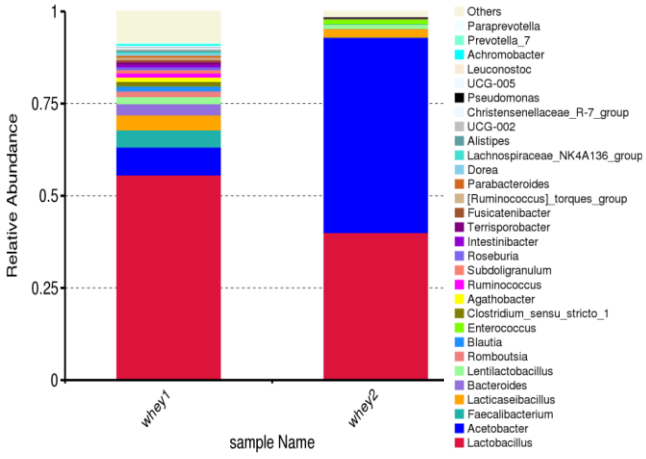

Figure 4. Bacterial diversity at genus level in whey wastewater samples

Lactic Acid Bacteria (LAB) are bacteria often used in the food industry to produce functional food. Lactic acid bacteria are bacteria that can ferment carbohydrates to produce lactic acid, bacteria that are commonly used as probiotics. The benefits of probiotics range from the relief of gastrointestinal disorders to the treatment of allergies, depression, obesity, bacterial vaginosis, and the improvement of the gastrointestinal tract (Vera et al. 2023). This bacteria is nonpathogenic, nontoxigenic, gram-positive, anaerobic, and does not produce spores. Lactic acid bacteria are 'generally recognized as safe (GRAS). Since LABs are considered GRAS organisms, they are used for producing industrially important compounds (Mukherjee et al. 2023). This GRAS status underlines their increasing use in traditional foods and an expanding range of novel foods and products designed to have specific nutritional or other health-enhancing benefits (nutriceuticals, prebiotics, probiotics, etc). The critical property in defining LAB is that these bacteria produce lactic acid as the primary or sole fermentation product. Lactic acid-producing bacteria are produced from lactose from whey cheese wastewater. Cheese whey still has a relatively high nutritional composition: protein 2.75%, lipid 0.054%, and lactose 4.1 (Estikomah et al. 2023). Lactose levels can be used as an energy source for the growth of LAB. The availability of carbohydrate reservoir of lactose in whey and other essential nutrients for the growth of microorganisms makes whey one of the potential substrates for producing different bio-products through biotechnological means (Sar 2022). The diversity of lactic acid bacteria in this research on liquid cheese waste can be seen in Table 2.In order to study microbial community composition in each sample, Operation Taxonomic Units (OTUs) were obtained by clustering with 97% identity on In the Effective Tags of samples, and then identified. According to OTUs annotations results and sample characteristic tables, species abundance tables at the level of the kingdom, phyla, class, order, family, genus, and species are obtained. These abundance tables with annotation information are the core content of amplification analysis. The taxa below were sequencing the NGS as they have similar nucleotide sequences in the large subunit of the gene (rbcl gene) segments in the mitochondria DNA OTUs order.

The 16S rRNA gene sequencing showed the presence of *Lactobacillus sakei*, *Lactobacillus harbinensis*, *Lactobacillus parafarraginis*, *Lactobacillus murinus*, *Lactobacillus mucosae*, *Lactobacillus plantarum*, and *Lactobacillus reuteri*. *Enterococcus faecium* and *Enterococcus faecium*. *Lactobacillus plantarum* in the whey cheese likely originated from the raw milk. Previous studies Yunita et al. (2018), *Lactobacillus plantarum* has an important role in flavor cheese development and has been shown to inhibit Listeria monocyte-genes in a smear-surface soft cheese and *Staphylococcus aureus* as well as *Salmonella typhimurium* in Montasio cheese. *Lactobacillus plantarum* is used as an additional culture for ripening cheese in cheese making (Gemechu, T.2015). *Lactobacillus* plantarum is beneficial for the health of the human body. The presence of *Lactobacillus plantarum* in both the core and the rind of the mature cheese could constitute an important biocontrol aspect, particularly in raw milk cheese where pasteurization does not eliminate pathogens. Enterococcus faecium is mostly found in cheese flora and is important in developing cheese taste, flavor, and aroma. Enterococcus sp, has been used in the starter culture combination in Europe, cheeses such as feta, mozzarella, and Cebreiro. The enterococci are part of the natural flora in cheese manufactured from raw milk and are believed to contribute to ripening and are used to develop flavor and aroma. Besides this, their presence in dairy samples is often associated with fecal contamination during the milking and storing (Avnİ et al. 2016). Moreover, in previous studies on the microbiota of São Jorge cheese, the genus Enterococcus accounted for 62% of isolates in the curd and 30-37% in the cheese (Coelho et al. 2023).

Table 2. Species of Lactic Acid Bacteria (LAB) in cheese wastewater (whey)

Taxon name	Count (OTU) whey 1	Count (OTU) whey 2	Taxa detail
Lactobacillus parafarraginis	3184	1912	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__ Lactobacillaceae;g__Lenti*Lactobacillus*;s__*Lactobacillus_parafarraginis;*
Lactobacillus murinus	243	0	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__ Lactobacillaceae;g__Ligi*Lactobacillus*;s__*Lactobacillus_murinus*;
Lactobacillus harbinensis	40	30	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__ Lactobacillaceae;g__Schleiferi*Lactobacillus*;s__*Lactobacillus*_harbinensis;
Lactobacillus mucosae	34	0	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__ Lactobacillaceae;g__Limosi*Lactobacillus*;s__*Lactobacillus*_mucosae;
Lactobacillus sakei	2	0	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__ Lactobacillaceae;g__Lati*Lactobacillus*;s__*Lactobacillus*_sakei;
Lactobacillus plantarum	0	5	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__ Lactobacillaceae;g__Lactiplantibacillus;s__*Lactobacillus*_plantarum;
Enterococcus faecium	2	2089	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__ Enterococcaceae;g__Enterococcus;s__Enterococcus_faecium;

The genus *Lactobacillus* represents the largest group of rod-shaped organisms within the lactic acid bacteria. *Lactobacillus murinus*, *Lactobacillus parafarraginis*, and *Lactobacillus plantarum* were identified cheese whey in this research. *Lactobacillus mucosae* were found in liquid cheese waste, possibly because *Lactobacillus mucosae* was used in making low-fat cheese, as in research conducted by Ryan et al. (2015). *Lactobacillus parafarraginis* is a heterofermentative bacteria, produces lactic acid, and carbon dioxide (Endo and Okada 2007). *Lactobacillus parafarraginis* was also found in research strain-level multi-omics analysis revealing significant variations in cheeses from different regions conducted by Yang (2021).

LAB is classified as a beneficial bacteria because it has an essential role in food and health. In the cheese industry, LAB is crucial to improving food taste (Gumechu 2015); LAB also plays a significant role in cheese making as a starter culture, determining the quality characteristics of cheese. As a starter culture produces a fresh sour taste, LAB helps coagulate rennet and forms specific textural characteristics in cheese. Besides producing lactic acid as a final product, LAB produces organic substances that contribute to taste, texture, and aroma, resulting in unique organoleptic properties. The production or degradation of exopolysaccharides, lipids, and proteins leading to the production of nutritional components such as vitamins make LAB a functional culture, so it can provide therapeutic effects and be used as a probiotic agent. LAB contained in liquid cheese wastewater can be used in the health sector as a functional food in the form of probiotics. LAB has antibacterial properties, and it can stop the growth of bacteria that cause disease in the digestive system. As a source of probiotics, LAB contains short-chain amino acids, so it can reduce blood pressure, improve the immune system, and reduce cholesterol in the body (Okfrianti et al. 2019).

Potential of whey wastewater as a source of probiotic bacteria

Cheese wastewater (whey) is major of product of product dairy industry. Cheese wastewater can be used as a carbon source for microbes because it contains high lactose (Perez et al. 2021). The lactose in whey wastewater can be used as a growth medium for bacteria. Most of the whey generated is mainly disposed to the environment without any resource recovery practice. The lactose in whey wastewater potential utilization by microorganisms for converting into valuable products reduces the requirement for expensive nutrient-rich media and helps in waste management. After being analyzed with NGS technology, the microorganisms in whey wastewater included the *Lactobacillus*, as the most dominant genus (Figure 4). In the cheese whey wastewater samples, the *Lactobacillus* genus included *Lactobacillus sakei*, *Lactobacillus harbinensis*, *Lactobacillus parafarraginis*, *Lactobacillus murinus*, *Lactobacillus mucosae*, *Lactobacillus plantarum*, and *Lactobacillus reuteri*.

According to Kumar et al. (2016), the *Lactobacillus* genus has the potential to be used as a probiotic source. Probiotics are usually dietary supplements containing viable nonpathogenic microorganisms that confer health benefits to the host (FAO/WHO 2001). This bacteria can lower cholesterol levels, as research by Ji et al. (2019) and Nguyen et al. (2022) shows that *Lactobacillus sakei* can lower cholesterol in rats. Madani et al. (2013) show that *Lactobacillus* can lower cholesterol levels. According to research by Zhang et al. (2022), *Lactobacillus reuteri* can reduce hypercholesterolemia in white rats. Jiang et al. (2020) show that *Lactobacillus mucosae* can lower cholesterol. In the research of Shen et al. (2023), *Lactobacillus murinus* is identified as having the ability to lower cholesterol levels.

In conclusion, the metagenomics study of the microbial community in cheese wastewater successfully detected the dominant genus of bacteria. The presence of *Lactobacillus* and Acetobacter confirms that cheese whey wastewater in the Yogyakarta Province. *Lactobacillus* is an essential source of promising lactic acid bacterial strains with a potential probiotic and biotechnological profile. Whey can be processed into probiotics in fermented beverages, as has been researched by Skryplonek (2019). Probiotic whey beverages stimulate the immune system and serum cholesterol levels, reduce blood pressure, and minimize the risk of various cancers (Pino 2020). Besides its health-promoting properties, whey fermentation leads to other advantages, such as a decrease in lactose value, partial hydrolysis of whey protein (which may cause allergies), increase in shelf life because of lactic acid, and production of aroma compounds that improve sensory features (Chavan et al. 2015). *Acetobacter*, the second most dominant genus, can oxidize ethanol to produce acetic acid, a strong foul odor that causes environmental pollution. The presence of Acetobacter bacteria in cheese wastewater has the potential to be used as a raw material for various vinegars. The presence of *Lactobacillus* and *Acetobacter* shows that whey wastewater can be reprocessed to produce fermented beverages, thereby improving the value of cheese wastewater and minimizing the impact of environmental pollution.

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