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Traditionally produced tempeh harbors more diverse bacteria with more putative health-promoting properties than industrially produced tempeh

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ABSTRACT

In recent years, there has been a significant shift towards industrialization in food production, resulting in the implementation of higher hygiene standards globally. Our study focused on examining the impact of hygiene standards on tempeh, a popular *Rhizopus*-based fermented soybean product native to Indonesia, and now famous around the world. We observed that tempeh produced with standardized hygiene measures exhibited a microbiome with comparable bacterial abundances but a markedly different community structure and function than traditionally produced tempeh. In detail, we found a decreased bacterial abundance of lactobacilli and enterobacteria, bacterial diversity, different indicator taxa, and significantly changed community structure in industrial tempeh. A similar picture was found for functional analysis: the quantity of bacterial genes was similar but qualitative changes were found for genes associated with human health. The resistome of tempeh varied based on its microbiome composition. The higher number of antimicrobial resistance genes in tempeh produced without standardized hygiene measures mainly belong to multidrug efflux pumps known to occur in plant-based food. Our findings were confirmed by functional insights into genomes and metagenome-assembled genomes from the dominant bacteria, e.g. *Leuconostoc*, *Limosilactobacillus*, *Lactobacillus*, *Enterococcus*, *Paenibacillus*, *Azotobacter* and *Enterobacter*. They harboured an impressive spectrum of genes important for human health, e.g. for production of vitamin B_1, B_7, B_{12}, and K, iron and zinc transport systems and short chain fatty acid production. In conclusion, industrially produced tempeh harbours a less diverse microbiome than the traditional one. Although this ensures production at large scales as well as biosafety, in the long-term it can lead to potential effects for human gut health.

1. Introduction

In recent years, there has been a growing recognition of the importance of microbiomes, particularly in the field of human health. The gut microbiota, for example, play a vital role in developing the adaptive immune system, producing antimicrobial peptides, and regulating inflammatory responses (Donald & Finlay, 2023). Additionally, the gut microbiome influences nutrient absorption and metabolism, with anaerobic bacteria in the colon helping to maintain a healthy gut environment (De Vos et al., 2022). Studies have shown that a diet rich in fibre, including plant-based foods like vegetables, fruits, whole grains, legumes, nuts, and seeds, can enhance the diversity of the gut microbiota in humans (Armet et al., 2022; Puhlmann & de Vos, 2022; Tomova et al., 2019). By analysing more than 2,000 human stool metagenomes, a recent study indicated that bacteria associated with fruits and vegetables were consistently found in the human gut and contributed to gut microbial diversity (Wicaksono, Cernava, et al., 2023). Moreover, consuming bacteria through plant-based food such as kimchi, kefir, and unpasteurized sauerkraut can also contribute to a healthy gut microbiome (Marco et al., 2017). These findings underscore the importance of plant-based food microbiomes including fermented plant-based foods in shaping human gut health.

Fermented foods have been a significant part of the human diet since ancient times. The process of food fermentation offers benefits in terms

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of food preservation, safety, nutritional quality, and sensory attributes through microbial activity (McGovern et al., 2004). Beneficial bacteria in fermented foods can help maintain a healthy balance of gut microbiota, leading to improved digestion, nutrient absorption, and immune function (Lebeer et al., 2018; Marco et al., 2017). Moreover, the consumption of fermented foods has been linked to a reduced risk of gastrointestinal disorders like irritable bowel syndrome and inflammatory bowel diseases (Marco et al., 2017). The diverse lactic acid bacteria found in fermented foods play a crucial role in enhancing food safety by inhibiting the growth of spoilage-causing bacteria and foodborne pathogens i.e., *Bacillus cereus*, *L*isteria monocytogenes, and *Escherichia coli* (Fidan et al., 2022; Licandro et al., 2020). Understanding the interactions of native microbiota in fermented foods is essential for developing distinct nutrient profiles and ensuring food preservation. Indeed, food fermentation acts as pre-digestion of plant-based food and may therefore be an important step in the microbial food-gut axis. This axis is evidenced but less understood (Wicaksono, Cernava, et al., 2023), especially the functional interplay as well as their benefit for human health.

Tempeh, a fermented soybean product with origins in Indonesia, has attracted global attention and increased popularity in recent years due to its nutritional benefits and unique taste profile. In Indonesia, tempeh is typically produced by the cottage industry where the manufacturing of goods takes place mostly from home and the products are distributed locally within the community. This type of production is often lacking adequate control of fermentation processes and hygiene measures. Consumption of fermented foods that are contaminated with pathogenic bacteria, e.g. *Escherichia coli, Shigella* spp., *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes,* as well as microbe-derived toxins can present risks to human health. In addition, antimicrobial resistance genes (ARGs) mainly originated from *Enterobacteriaceae* have been identified in fermented foods (Leech et al., 2020; Yasir et al., 2022), which raises concerns about the potential transfer of ARGs into the gut microbiome through horizontal gene transfer and could lead to adverse health consequences (Groussin et al., 2021).

Tempeh is a significant food source in Indonesia, mass-produced and consumed on a large scale by the local population. With its status as a growing export product (Shanti et al., 2023), there is a need for improved production management and quality control processes to enhance product quality and address food safety concerns. Implementing hygienic practices in tempeh production can lead to a reduction in unwanted microorganisms present in the final product. This plays a crucial role in guaranteeing the safety and quality of fermented food, meeting the rising consumer expectations for a consistent and contamination-free product. In Indonesia, efforts have been made to establish standardized production practices for tempeh by the National Standardization Agency of Indonesia (Badan Standardisasi Nasional), coded as SNI 3144:2009. However, despite established standards for tempeh production, a significant number of tempeh products being sold lack proper product labeling.

In recent history, there have been notable shifts in our dietary patterns, largely attributed to the industrialization of food production, preservation, and processing connected to professional hygiene practices. While these changes are crucial in reducing outbreaks of foodborne pathogens in industrialized large-scale production, there is a growing recognition of their potential effects on gut microbiome-dependent host health and the increasing prevalence of non-communicable diseases (Gerasimidis et al., 2020; Levine et al., 2018). It is important to recognize the benefits of diverse microbiomes (including beneficial and pathogenic microorganisms) in strengthening the immune system and promoting overall health. Understanding the exposure to industrially produced food in the long term and the impact of the “edible plant microbiome” (Serrano & Bezrutcyzk, 2023; Soto-Giron et al., 2016) as well as the storage, processing, and industrial production is important for one health. Tempeh is an ideal model to study these drivers because diverse production facilities from home-made to industrial exist (Ahnan-Winarno et al., 2021; Astuti et al., 2000; Tamam et al., 2019). Tempeh is a plant-derived fermented food that is often consumed as a protein source, and alternative to meat. Additionally, tempeh serves as a natural source of vitamin B_{12} (Castanheira et al., 2020). It was already reported that certain bacterial taxa within the *Enterobacteriaceae* family, including the genera *Klebsiella* and *Citrobacter,* that are known as opportunistic pathogens, have been identified as key contributors to the production of vitamin B complex in tempeh (Denter & Bisping, 1994; Keuth & Bisping, 1994). Interestingly, *Klebsiella* which was found in almost all samples of tempeh from Indonesia may be harmless to humans due to the absence of *rmpA* and other virulence-associated genes (Cesrany et al., 2017).

The objective of this study is to assess structural and functional aspects of the tempe microbiota produced under different conditions – industrial vs. non-industrial – from a human health perspective. Using a comprehensive approach that integrates culture-dependent and independent methods, we aimed to address the following questions: 1) Is there variation in the microbiome composition and function of tempeh produced with and without standardized hygiene measures? 2) If discrepancies exist, which specific microbial groups show enrichment or depletion? This study aimed to shed light on the less explored aspects of tempeh production and offer a more comprehensive understanding of the factors that influence its microbial composition and function.

2. Material and methods

2.1. Experimental design and sample processing

In this study, we obtained four tempeh products from four different supermarkets that were produced with standardized hygiene measures, as labelled on the packaging with hazard analysis and critical control points (HACCP) certificate. These samples were further assigned to “H” samples. We also obtained four tempeh samples that were produced without standardized hygiene measures from four different traditional markets. These samples were further assigned to “NH” samples. In Indonesia, tempeh is primarily produced by small-scale cottage industries. The production process of tempeh includes soaking beans in tap water, dehulling, washing, boiling, draining, cooling, inoculating with starter, and incubating at room temperature under non-sterile conditions (Mulyowidarso et al., 1989). It is important to note that tap water in Indonesia is generally not suitable for drinking. Tempeh produced by smaller household industries may have lower hygiene standards and less controlled conditions. Additionally, these products lack HACCP certification and standardized production practices labeling on their packaging. Each tempeh sample was sliced into small pieces and stored in DNA/RNA Shield™ (Zymo Research, Freiburg, Germany) at a temperature of −20 °C. Following 10 days of storage under these conditions, DNA extraction was performed on all samples.

Approximately 10 g of tempeh samples were homogenized with 10 ml of sterile NaCl (0.85 %) solution using a Stomacher lab blender (BagMixer, Interscience, Saint-Nom-la-Bretèche, France) for three minutes before extracting the DNA. A total of 2 ml of the processed tempeh were separated by centrifugation at 16,000*g* for 20 min, and the resulting pellets were used for DNA extraction. DNA extraction was carried out using the FastDNA SPIN Kit for Soil and the FastPrep Instrument, following the manufacturer’s instructions. The extracted DNA was stored at 20 °C until qPCR and shotgun metagenome sequencing were performed.

2.2. Quantification of bacteria in tempeh

We used quantitative real-time PCR (qPCR) with SYBR Green fluorescence to determine the abundance of bacteria in the tempeh samples (copies of bacterial marker genes per gram). For the qPCR, we used 515f-806r primer sets (Caporaso et al., 2011) for quantification of total bacteria, F-lac/R-lac (Walter et al., 2001) for quantification of total

lactobacilli, and F-ent/R-ent (Castillo et al., 2006; Leser et al., 2002; Sghir et al., 2000) for quantification of total enterobacteria. The quantification of fluorescence was performed using the Rotor-Gene 6000 real-time rotary analyzer (Corbett Research, Sydney, Australia). The qPCR was set up using an initial denaturing step at 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 30 s, annealing at 54 °C (for total bacteria), 60 °C (for lactobacilli), or 63 °C (for enterobacteria) for 30 s, extension at 72 °C for 30 s, and finally, a melting curve analysis.

2.3. Analysing the taxonomic and functional diversity of bacteria in tempeh using shotgun metagenomic sequencing

The extracted DNA was sent to the sequencing provider Novogene (Cambridge, UK), Germany. The sequencing provider Novogene performed the DNA library preparations and sequencing on an Illumina NovaSeq 6000 system with 2 × 150 bp paired-end sequencing.

Prior to analysing the taxonomic and functional diversity of bacteria in tempeh, data preprocessing procedures were undertaken. This included conducting quality assessments using FastQC v0.12.1 (Andrews, 2010), eliminating Illumina sequencing adapters, and conducting initial quality filtration (removing low-quality reads with Phred < 20) on raw shotgun metagenomic reads using Trimmomatic v0.39 (Bolger et al., 2014) and VSEARCH v2.28.1 (Rognes et al., 2016). This process led to an average read depth of 38,296,493 high-quality reads per sample, with a range of 4,507,723–56,689,099 reads per sample.

For bacterial community structure and diversity analysis, we use Kraken2 v2.1.2 with the standard Kraken2 database, which includes genome references from archaeal, bacterial, viral, and human sources, for classifying shotgun metagenomic reads (Wood et al., 2019). Subsequently, we generated a table of bacterial abundance using Bracken v2.6.0 (Lu et al., 2017). The bacterial abundance table was collapsed to genus-level read counts. For bacterial gene profiling analysis, tempehthe metagenomic reads were assembled using the Megahit assembler v 1.2.9 (Li et al., 2015). Only contigs with a length exceeding 1 kb were retained for further analysis. Prodigal v2.6.3 (Hyatt et al., 2010) was used to predict open reading frames. To eliminate duplicate sequences, CD-HIT-EST v4.8.1 was used to cluster protein-coding gene sequences and create a non-redundant gene catalog with a nucleotide identity of 95 % similarity (Li & Godzik, 2006).

In order to identify a functionally relevant subsystem and bacterial genes relevant to human health, the non-redundant genes were then annotated utilizing the blast algorithm in DIAMOND in conjunction with eggNOG-mapper v.2.1.12 (Buchfink et al., 2015; Huerta-Cepas et al., 2017) and the eggNOG database v5.0 (Huerta-Cepas et al., 2019). All protein-coding gene sequences that were assigned to *Bacteria* based on eggNOG-mapper's taxonomic classification were retained for further analysis. To identify antimicrobial resistance genes in tempeh, the contigs were annotated using the deepARG antibiotic resistance gene database (Arango-Argoty et al., 2018) to generate antibiotic resistance gene profiles. To ensure accuracy, contigs with an E-value cutoff of 10^{-10} and a similarity of 35 % (as described by Looft et al. (2012) were identified as ARG-like contigs. The assembled contigs were utilized as input files for taxonomic profiling through MMseqs2 v14.7 (Mirdita et al., 2021).

To generate gene profiles from the samples, quality-filtered reads were mapped back to the generated non-redundant gene catalog using BWA v0.7.18 and SamTools v1.20 (Li & Durbin, 2010; Li et al., 2009). This analysis resulted in over 58 million reads classified as bacterial proteins according to eggNOG mapper. Additionally, 2.5 million were classified as ARG-like reads according to deepARG database.

2.4. Reconstruction of bacterial metagenome-assembled genomes

We used various binning methods, including Maxbin2 v2.2.7, MetaBAT2 v2.12.1, and CONCOCT v1.1.0, (Alneberg et al., 2014; Kang et al., 2019; Wu et al., 2016) to generate metagenome-assembled genomes (MAGs). DASTool v1.1.1 was used to select MAGs with the highest quality from all genome binners (Sieber et al., 2018). The quality of MAGs, in terms of completeness and contamination levels, was assessed using CheckM v1.0.13 (Parks et al., 2015). To facilitate a comparison of metabolic capabilities, only medium-quality MAGs with completeness above 50 % and contamination levels below 10 % were retained, following the current definition of the minimum information metagenome-assembled genome (MIMAG) standards (Bowers et al., 2017). Dereplication of metagenome-assembled bacterial genomes was accomplished using dRep v2.2.3 (Olm et al., 2017), resulting in a nonredundant set of genomes. The Genome Taxonomy Database Toolkit (GTDB-Tk) was employed to obtain taxonomical information for each MAG, and phylogenetic trees were constructed using PhyloPhlAn, incorporating closely related taxa from its database (Chaumeil et al., 2020). Gene annotations of the constructed MAGs were performed using DRAM v.1.4.6 (Distilled and Refined Annotation of Metabolism) (Shaffer et al., 2020). CoverM v0.4.0 (https://github.com/wwood/CoverM) with the option -m rpkm was utilized to calculate abundance profiles of each MAG. MAG abundance was determined as mapped reads per kilobase per million reads (RPKM) that is the counts of mapped reads divided by the MAG length and total number of reads in each metagenomic dataset (in millions of reads).

2.5. Metabolomic analysis

We used liquid-chromatography tandem mass spectrometry (LC-MS^2) to determine the content of vitamin B_{12} in eight tempeh samples. The frozen samples were homogenized, and 100–4000 mg (depending on dry weight) were used for extraction in 500 µl ethyl acetate, acidified with two drops of 95 % H_2SO_4. We used the HPLC Ultimate 3000 hybrid quadrupole-orbitrap mass spectrometer Q Exactive (Thermo Scientific, Bremen, Germany) and a Waters Atlantis dC18 separation column with 2.1 mm diameter, 100 mm length and 3 µm particle size. The LC column was kept at 25 °C with a flow rate of 0.3 ml/min. A standard of 1 mg/l of cobalamin in aqueous solution was used to set the mass range and normalize peak areas. Two mobile phases, water and acetonitrile, were used (stepwise increasing from 5 % acetonitrile to 80 % in 35 min. Only positive ions were considered using an untargeted scan range of 150–2000 m/z. Cyanocobalamin was subsequently detected using single ion monitoring with a mass of 403.2313 m/z. The maximum accumulation time was 100 m/s, and the resolution was 70,000 with altering dd MS^2.

2.6. Functional characterization of cultivable tempeh bacteria

2.6.1. Isolation of bacteria from tempeh samples

A total of 200 g of each tempeh sample was mashed and suspended in 10 ml of NaCl. Suspensions were serially diluted 10-fold, and 100 µl of the dilutions were plated on both Reasoner's 2A (R2A) and Nutrient Broth II agar media in triplicates. The plates were then incubated at 25 °C for four days. Single colonies were picked and sub-cultured on Nutrient Broth II agar to ensure isolate purity. Representative bacterial colonies with distinct morphologies (shape and color) were chosen from each dilution to increase the number of unique isolates. These colonies were then transferred to 96-well plates containing Nutrient Broth II and 30 % glycerol for long-term storage. The plates were stored at −70 °C at the Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria. To prepare for the functionality assays, each isolate was grown in 200 µl of NB II medium in 96-well plates and subsequently incubated at 25 °C for 2 days. These liquid cultures were utilized as the bacterial suspensions for the functionality assays, as elaborated below.

2.6.2. Screening for biosurfactant-producing bacteria

A qualitative screening was conducted to identify biosurfactant-producing bacteria using a drop-collapsing assay, following the

established method (Bodour & Miller-Maier, 1998). In summary, the lid of a 96-well microtiter plate was prepared by applying 2 µl of mineral oil to each of the 96 wells and allowing it to equilibrate. Following this, 5 µl of suspension bacterial culture was added to the mineral oil, and the outcome was assessed after 1 min. Drops that retained their shape were defined as a negative result, while drops that collapsed were defined as a positive result.

2.6.3. Screening for protease-producing bacteria

For the qualitative screening of bacteria that produce protease, a 10 % skim milk agar was used, following a previously published method (Pailin et al., 2001). To prevent coagulation of the milk, sterilized milk was mixed with autoclaved LB medium. Five µl of suspension bacterial culture was then placed on the skim milk agar plates. After incubating the plates at 25 °C for 4 days, bacterial isolates with a clear zone surrounding the colony were identified as bacteria that produce protease.

2.6.4. Screening for bile salt tolerance bacteria

Bile salt tolerance testing was conducted following the method described in a previous study (Prete et al., 2020), with the exception that we utilized NB II medium instead of de Man, Rogosa, and Sharp (MRS) broth medium. Briefly, 5 µL of bacterial suspension was added to 200 µL of NB II medium containing varying concentrations of bile salts (0 %, 0.30 %, 1.8 %, and 3.6 % w/v; Thermo Fischer Scientific) in polystyrene 96-well microtiter plates. After a 24-hour incubation at 25 °C, bacterial growth was assessed by measuring the optical density (OD600) of each well using the Tecan microplate reader Infinite® 200 PRO (Tecan Austria GmbH, Grödig, Austria).

2.6.5. Antibiotic resistance profiling

Bacterial strains were evaluated for resistance to six different antibiotics using a standard agar plate method as previously described (D'Costa et al., 2006; Walsh & Duffy, 2013; Wicaksono, Reisenhofer-Graber, et al., 2023). Briefly, Mueller Hinton agar plates with a concentration of 20 µg/ml of each antibiotic (Supplementary Table S1) were inoculated with approximately 3 µl of bacterial culture using a multipoint inoculator and then placed in an incubator at 25 °C. Control plates without antibiotics were also set up. Bacterial isolates that exhibited growth after 4 days on agar plates containing antibiotics were identified as resistant strains.

2.6.6. Identification of selected bacterial isolates using Sanger sequencing of 16S rRNA gene

A total of 23 selected bacterial isolates were identified through Sanger sequencing of their 16S rRNA gene segments using the universal bacterial primer pair 27F and 1193R. The sequencing was performed at LGC genomics in Berlin, Germany. Raw sequencing reads were quality filtered with BioEdit to eliminate ambiguous sequences (Hall, 1999). The quality-filtered sequences were then compared against the NCBI standard database (nr/nt) utilizing the Basic Local Alignment Search Tool (BLAST) (Camacho et al., 2009).

2.7. Statistical analysis

Bacterial abundance, diversity, community composition, and gene abundance were analyzed using in R version 4.1.2, utilizing the R packages Phyloseq version 1.38.0 and vegan version 2.6-4 (Core Team, 2013; McMurdie & Holmes, 2013; Oksanen et al., 2007). For the alpha diversity analysis, the bacterial (genus-level read counts) and gene abundance tables were normalized by subsampling to the lowest number of reads among the samples. Alpha diversity was measured using the number of bacterial species, the number of observed genes, and the Shannon index. The statistical significance of observed differences was assessed using the non-parametric Kruskal Wallis test. The R package Deseq2 (Love et al., 2014) was utilized to normalize the bacterial taxa, bacterial genes and ARG dataset and conduct statistical analysis to identify bacterial taxa, genes and ARG that exhibit significant changes between tempeh produced with and without standardized hygiene measures. Beta diversity analysis utilized rarefied bacterial and gene abundance tables and used as input data to calculate distance matrix using Bray–Curtis dissimilarities. The dissimilarity matrix was further analyzed using Adonis to determine if there were significant effects between tempeh produced with and without standardized hygiene measures. The variance in MAG abundance between tempeh produced with and without standardized hygiene measures was analyzed using the non-parametric Kruskal Wallis test.

3. Results

3.1. Impact of hygiene standard on bacterial abundance, diversity, and community structure in tempeh

The implementation of hygiene standards during tempeh production significantly influenced bacterial abundance, diversity, and community structure in tempeh. Quantitative PCR revealed that the total bacterial abundance in tempeh produced with standardized hygiene measures (8.1×10^6 16S rRNA copies per gram) was lower, although the difference was not statistically significant when compared to tempeh produced without standardized hygiene measures (6.2×10^7 16S rRNA copies per gram, $P = 0.248$, Fig. 1A). Interestingly, we observed significantly higher abundance of lactobacilli (3.3×10^3 16S rRNA copies per gram, $P = 0.043$) and enterobacteria (4.6×10^1 16S rRNA copies per gram, $P = 0.013$) in the tempeh produced without standardized hygiene measures (lactobacilli – 2.6×10^3 16S rRNA copies per gram, enterobacteria – not detected). A higher number of bacterial genera was also observed in the tempeh produced without hygiene standard ($n_{genera} = 996$, $P = 0.043$, Fig. 1B) in comparison to in tempeh produced with standardized hygiene measures ($n_{genera} = 678$). The analysis of beta diversity using a Bray-Curtis dissimilarity matrix revealed distinct clustering between the tempeh produced with and without standardized hygiene measures (Fig. 1C). Results from PERMANOVA analysis indicated that the hygiene level had a significant impact on the bacterial community structure ($P = 0.032$), explaining 28.0 % of the variation in bacterial community composition.

After observing changes in the bacterial community composition, our goal was to study specific bacterial taxa that were significantly impacted by the implementation of hygiene standards during tempeh production. The dominant bacterial order identified was *Lactobacillales* accounting for an average of 49.1 % and 50.1 % of the total 16S rRNA sequence reads in both the tempeh produced with and without standardized hygiene measures, respectively (Fig. 2A). Although *Lactobacillales* were found to be prevalent in both types of tempeh, further analysis of bacterial genera revealed variations. *Lentilactobacillus* and *Leuconostoc* were the dominant genera in tempeh produced with standardized hygiene measures, while *Lactobacillus* and *Enterococcus* dominated in tempeh produced without standardized hygiene measures (Fig. 2B). *Rhodospirillales* and *Enterobacterales* were detected in high abundance in tempeh produced without hygiene standard e.g., 14.3 % and 12.2 %, respectively. In contrast, these taxa were present at lower abundances in the tempeh produced with standardized hygiene measures. In addition, other bacterial taxa such as *Pseudomonadales* (11.1 %), and *Bacillales* (7.9 %) showed a similar pattern. Differential abundance analysis in genus level indicated that several bacterial genera ($n = 43$) were identified as enriched in the tempeh produced with standardized hygiene measures belonging to bacterial order *Pirellulales*, *Flavobacteriales*, *Sphingobacteriales*, *Pasteurellales*, and *Thermotogales* (Fig. 2C). Additionally, a total of 57 bacterial genera were found to be enriched in the tempeh produced without standardized hygiene measuress. These bacterial genera belonged to *Rhodobacterales*, *Sphingomonadales*, *Hyphomicrobiales*, *Rhodospirillales*, *Enterobacterales*, and *Burkholderiales.* Our study found a bacterial signature that is linked to hygiene standards in tempeh production.

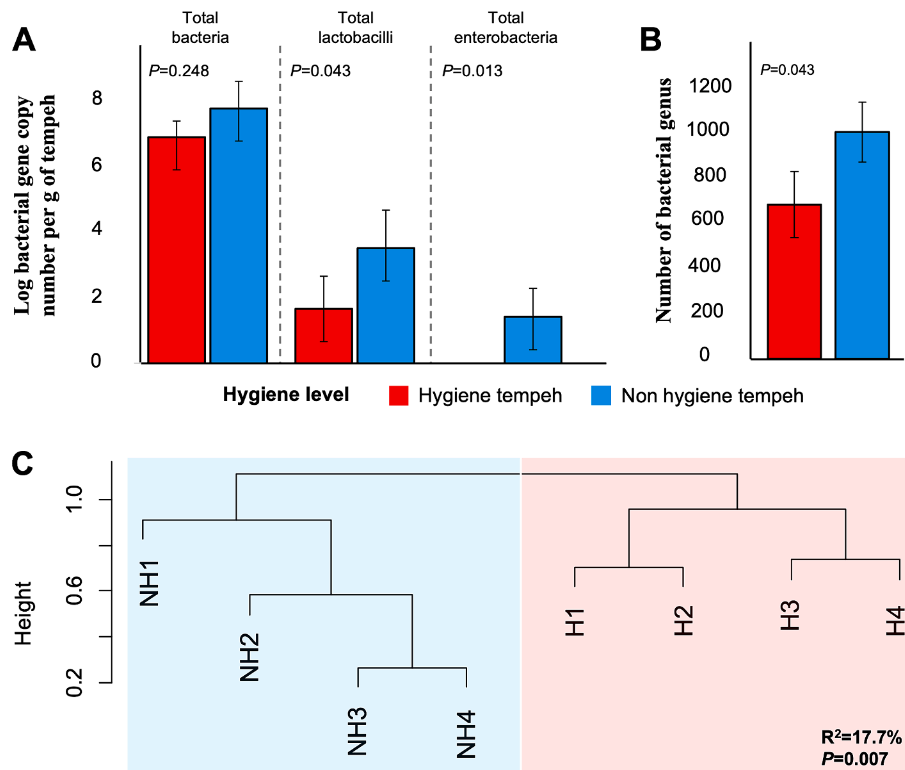

Fig. 1. Bacterial abundance, diversity, and community structure associated with tempeh produced with and without standardized hygiene measures. Total bacterial, lactobacilli, and enterobacteria abundances were quantified utilizing a qPCR-based approach and converted into logarithmic values (A). The number of bacterial genera that were detected in the tempeh samples (B). The hierarchical clustering analysis was performed on the genus-level bacterial community structures present in the tempeh that was produced with ("H") and without standardized hygiene measures ("NH") (C).

3.2. Gene-centric analysis revealed the impact of hygiene standards on bacterial genes linked to human health

Following the observed changes in microbial community structure, it was anticipated that changes in community structure would be accompanied by corresponding shifts in microbial functioning. Surprisingly, alpha and beta diversity analysis revealed no significant differences in the number of bacterial genes ($P = 0.149$) and bacterial gene compositions based on eggNOG annotation ($P = 0.088$) between tempeh produced with and without standardized hygiene measures. We then shifted our focus to bacterial genes that are associated with human health, such as those involved in vitamin production and short-chain fatty acids. Differential abundance analysis using DESeq2 indicated that a small number of specific genes exhibited variations in abundance between tempeh produced with and without standardized hygiene measures. Genes that involved in cobalamin (vitamin B_{12}) biosynthesis and transport systems were more abundant in tempeh without standardized hygiene measures in comparison to tempeh produced with hygiene standards. In contrast, genes that involved in vitamin B_2 and K biosynthesis as well as production of butyrate showed the opposite pattern (Supplementary Table S2).

A high impact of hygiene standards was observed on the antibiotic resistance gene (ARG) profiles. We observed a higher number of ARGs in tempeh produced without standardized hygiene measures ($n_{ARG} = 591$, $P = 0.020$, Fig. 3A) in comparison to tempeh produced with standardized hygiene measures ($n_{ARG} = 490$). Our analysis revealed a significant correlation between bacterial richness and ARG richness ($r = 0.83$; $P = 0.015$). The examination of ARGs based on the Bray-Curtis dissimilarity index revealed distinct clustering, with tempeh produced without standardized hygiene measures showing a tendency to group together (Fig. 3B). PERMANOVA analysis results indicated that the hygiene level significantly influenced the ARG composition ($P = 0.001$), accounting for 33.5 % of the variation in ARG composition. Our study indicated that hygiene standard during production of tempeh had an influence on the richness and composition of ARGs.

Additionally, the findings from the analysis using DESeq2 revealed a significant increase in the number of differentially abundant ARGs. Specifically, there were 65 enriched ARGs in tempeh produced with hygienic standards compared to tempeh produced without standardized hygiene measures, where only 29 enriched ARGs were observed (Fig. 3C). Most ARGs that were enriched in tempeh produced without standardized hygiene measures belong to multidrug classes with efflux pump resistance mechanism (13 of 29 ARGs) (Fig. 3C and D). Only 14 of 65 ARGs were identified as multidrug classes with efflux pump resistance mechanisms enriched in tempeh produced with standardized hygiene measures. Other ARGs that enriched in tempeh produced with standardized hygiene measures belong to ARGs that confer resistance to glycopeptide ($n = 12$), tetracycline ($n = 8$), aminoglycoside ($n = 4$) and chloramphenicol ($n = 4$). Moreover, a notable distinction was observed by a higher number of ARG with antibiotic inactivation mechanism and antibiotic target alterations in tempeh produced with standardized hygiene measures (Fig. 3D).

3.3. Members of the Bacilli and Gammaproteobacteria play a significant role in health-related factors and the determination of antibiotic resistance

In total, 66 MAGs were assembled with at least 50 % completeness and less than 10 % contamination (Supplementary Table S3). Tempeh produced without and with standardized hygiene measures yielded 39 and 27 MAGs, respectively. Furthermore, a total of 34 non-redundant MAGs were successfully assembled. Most metagenome-assembled genomes (MAGs) were identified as *Bacilli* ($n = 21$), *Alphaproteobacteria* ($n = 5$) and *Gammaproteobacteria* ($n = 4$). Twenty-two MAGs were classified as high-quality MAGs, with at least 90 % completeness and less than

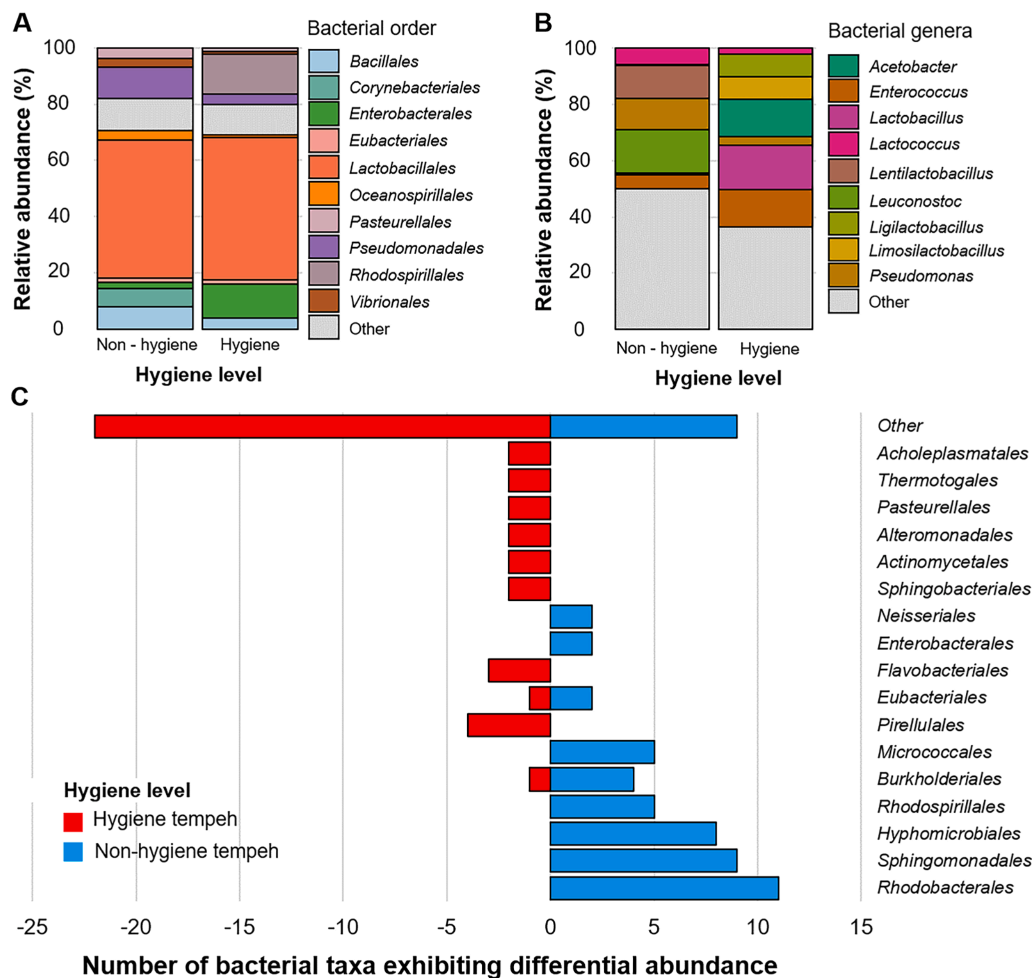

Fig. 2. Bacterial community composition and identification of enriched bacterial genera with differential abundance between tempeh produced with and without standardized hygiene measures. The relative abundance of distinct taxa in bacterial communities were visualized on order (A) and genus (B) level. The number of differentially abundant bacterial genera in tempeh produced using standardized hygiene practices was compared to tempeh produced without such measures and categorized by order level (C). Negative values indicate number of bacterial genera based on DeSeq2 anaylsis, with results categorized by order level, that enrich in tempeh produced with standardized hygiene measures in comparison tempeh produced with standardized hygiene measures. Positive values indicate number of bacterial genera that enrich in tempeh produced without standardized hygiene measures in comparison tempeh produced with standardized hygiene measures.

5 % contamination.

Genome-centric analyses using representative bacterial MAGs enabled in-depth evaluations of their potential role in health-related factors (Fig. 4A). A total of 26 MAGs contained genes responsible for production SCFA namely acetate. Many of these MAGs were assigned to genera belonging to *Bacilli* i.e., *Leuconostoc*, *Limosilactobacillus*, *Lactobacillus*, *Enterococcus* and *Paenibacillus* as well as genera belonging to *Gammaproteobacteria* i.e., *Azotobacter* and *Enterobacter*. Genes that were involved in thiamine (vitamin B_1), iron and zinc transport systems were also detected in the latter taxa. Additionally, 13 MAGs harbored a gene set, *menB*, *menC*, *menD*, *menE* and *menH*, involved in menaquinone (vitamin K) biosynthesis, with most of them assigned to *Leuconostoc*, *Weissella*, *Enterococcus* (*Bacilli*) and *Enterobacter* (*Gammaproteobacteria*). Interestingly, genes involved in cobalamin (vitamin B_{12}) biosynthesis i.e., *cobCDQUP* and *cbiB* were consistently detected in MAGs that belonged to *Azotobacter* and *Acetobacter*.

A total of 22 different antibiotic resistance gene classes were identified within the MAGs. MAGs that belonged to *Enterobacter* harboured the highest number of ARGs. The predominant ARGs found in MAGs were glycopeptide, macrolide-lincosamide-streptogramin, bacitracin, beta-lactam, tetracycline, and multidrug resistance genes (Fig. 4B). MAGs that belonged to *Bacilli* exhibit a high prevalence of ARGs that confer resistance to macrolide-lincosamide-streptogramin and glycopeptides. Conversely, MAGs that belonged to *Gammaproteobacteria* exhibited a high prevalence of genes that provide resistance to aminoglycoside and fluoroquinolone antibiotics. These results show that both taxa played a role in the overall resistome of tempeh.

Next, we investigated if the prevalence of a specific MAG is associated with specific produced tempeh. A total of 11 MAGs were identified as being differentially abundant between tempeh produced with and without hygienic standards. MAGs that belonged to *Leuconostoc* were more prevalent in tempeh produced with hygiene standards whereas *Lactobacillus* and *Limosilactobacillus* showed the opposite pattern (Fig. 4C and D). Furthermore, we observed an enrichment of MAGs associated with genera belonging to *Gammaproteobacteria* i.e., *Azotobacter* and *Enterobacter* in tempeh produced without standardized hygiene measures. This reinforces our findings that there are distinct differences in bacterial composition between tempeh produced with and without standardized hygiene measures.

3.4. Bacteria isolated from tempeh harbour beneficial properties

In total, we isolated 634 isolates from tempeh samples. A total of 378 isolates were recovered from tempeh produced with standardized hygiene measures (H1: 122 isolates, H2: 124 isolates, H3: 119 isolates, and H4: 13 isolates). Furthermore, we recovered 256 isolates from tempeh

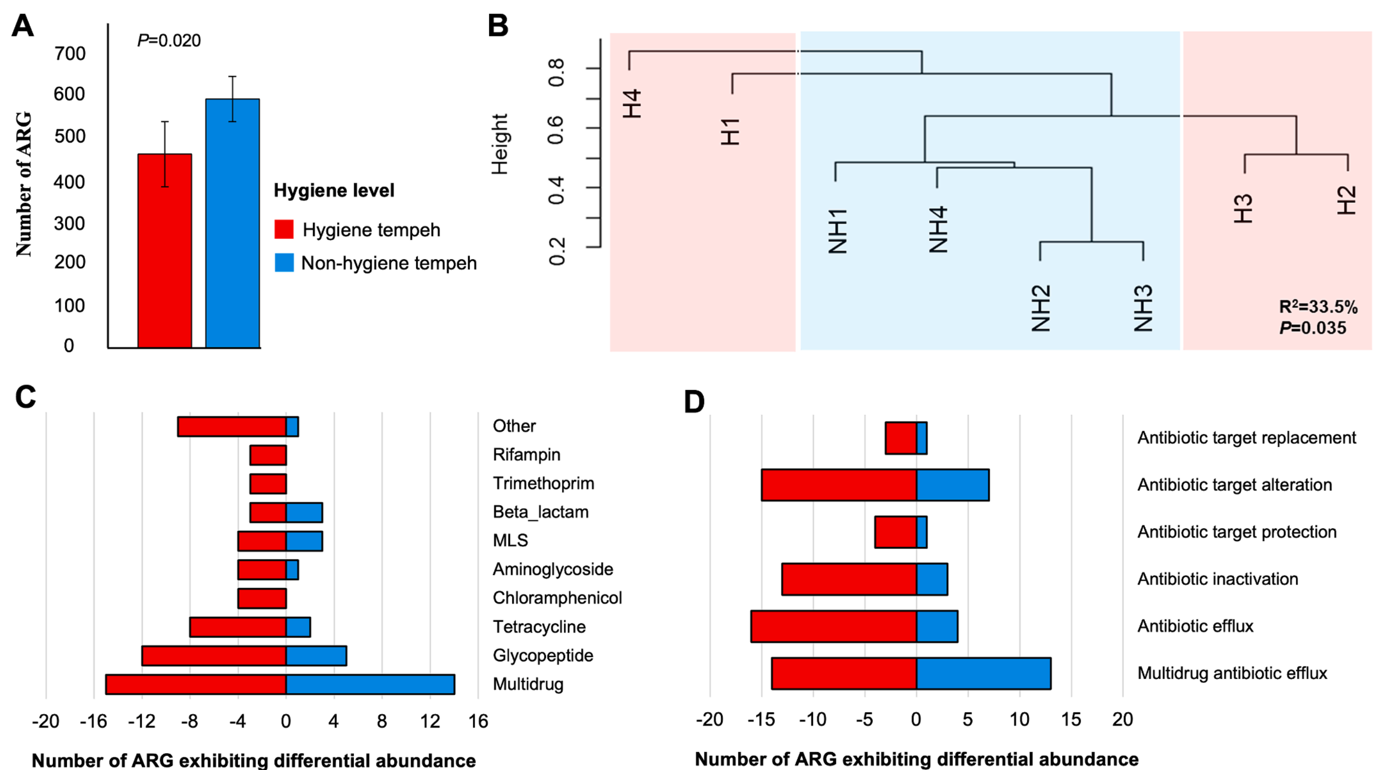

Fig. 3. Analysis of antimicrobial resistance genes (ARGs) in the tempeh microbiome. Alpha diversity was determined by calculating the number of ARG in each metagenome sample (A). Beta diversity was assessed with a Bray-Curtis distance matrix of ARG profiles and visualized using the hierarchical clustering analysis (B). Differentially abundant ARGs following antimicrobial were identified using DeSeq2 analyses and categorized them according to antibiotic classes (C) and resistance mechanisms (D). H – tempeh produced with and standardized hygiene measures and NH – tempeh produced without standardized hygiene measures.

produced without standardized hygiene measures (NH1: 56 isolates, NH2: 56 isolates, NH3: 64 isolates, and NH4: 80 isolates.

A total of 38 isolates, out of the 634 total isolates, exhibited ability to produce biosurfactants. A higher proportion of those isolates were recovered from tempeh produced without standardized hygiene measures (average = 8.4 %, min = 6.3 %, max = 10.7 %, Fig. 5A) compared to tempeh produced with standardized hygiene measures (average = 3.5 %, min = 0 %, max = 7.3 %). Our result also indicated that protease activity was more prevalent in tempeh produced without standardized hygiene measures (average = 14.8 %, min = 7.8 %, max = 26.8 %, Fig. 5B) compared to tempeh produced with standardized hygiene measures (average = 8.7 %, min = 0 %, max = 15.9 %). Bacteria that could grow in media with 3.6 % bile salt were present in similar proportion in both tempeh categories (16.7 % of isolates from tempeh produced without standardized hygiene measures, and 16.9 % of isolates from tempeh produced with standardized hygiene measures, Fig. 5C). Interestingly, in one tempeh sample, produced with standardized hygiene measures, one third of total isolates grew in media with 3.6 % bile salt.

Cultivation techniques confirmed the presence of bacteria exhibiting resistance to multiple antibiotics. A high number of isolates (n = 504) demonstrated resistance to at least one antibiotic, with 193 isolates showing resistance to three or more antibiotic classes and 94 isolates displaying resistance to all antibiotics tested. The most observed resistances were against ampicillin (63 % of total isolates) and vancomycin (55 % of total isolates). We further compared the prevalence of resistant isolates that recovered from tempeh produced with and without standardized hygiene measures. Isolates with resistance to tetracycline, ciprofloxacin, rifampicin, and gentamycin were more prevalent in tempeh produced with standardized hygiene measures (44.4 %, 22.4 %, 42.9 % and 36.0 %, respectively) compared to tempeh produced without standardized hygiene measures (24.7 %, <1.0 %, <1.0 % and 0.0 %, respectively, Fig. 5D). The proportion of isolates that were resistant to ampicillin was higher in tempeh produced without standardized hygiene measures (72.8 %) compared to tempeh produced with hygienic standards (53.6 %). Furthermore, we observed that 30.1 % of isolates recovered from tempeh produced without hygienic standards were resistance to at least 3 tested antibiotics whereas 56.6 % of multi resistance isolates were recovered from tempeh produced with standardized hygiene measures.

The taxonomical information of 23 representative isolates with bioactive properties was evaluated using Sanger sequencing (Supplementary Table S4). These isolates were primarily from the genera *Enterobacter*, *Pantoea*, *Bacillus*, *Stenotrophomonas*, and *Xanthomonas*. Among the isolates, six demonstrated the ability to produce biosurfactants and proteases, belonging to *Enterobacter*, *Bacillus*, *Paenibacillus*, *Lactiplantibacillus*, and *Limosilactobacillus*. It was observed that most isolates showing multi-antibiotic resistance were identified as *Enterobacter*. Additionally, other taxa exhibiting multi-resistance included *Sphingomonas*, *Erwinia*, *Bacillus*, *Lactiplantibacillus*, and *Limosilactobacillus*.

4. Discussion

The examination of microbial ecosystems in fermented foods has attracted considerable interest due to its potential implications for food safety, quality, and nutritional characteristics. The inherent fermentation process presents difficulties in ensuring consistent food quality which can be improved by the implementation of standard hygiene protocols. Nevertheless, hygiene protocols may unintentionally diminish the diversity of beneficial bacteria. By utilizing a comprehensive methodology incorporating high-throughput sequencing, qPCR, and functionality assays, this study showed that the bacterial composition and function in tempeh were notably impacted by hygiene standards. This can be attributed to changes in the composition of two dominant members, *Bacilli* and *Gammaproteobacteria*, which are key

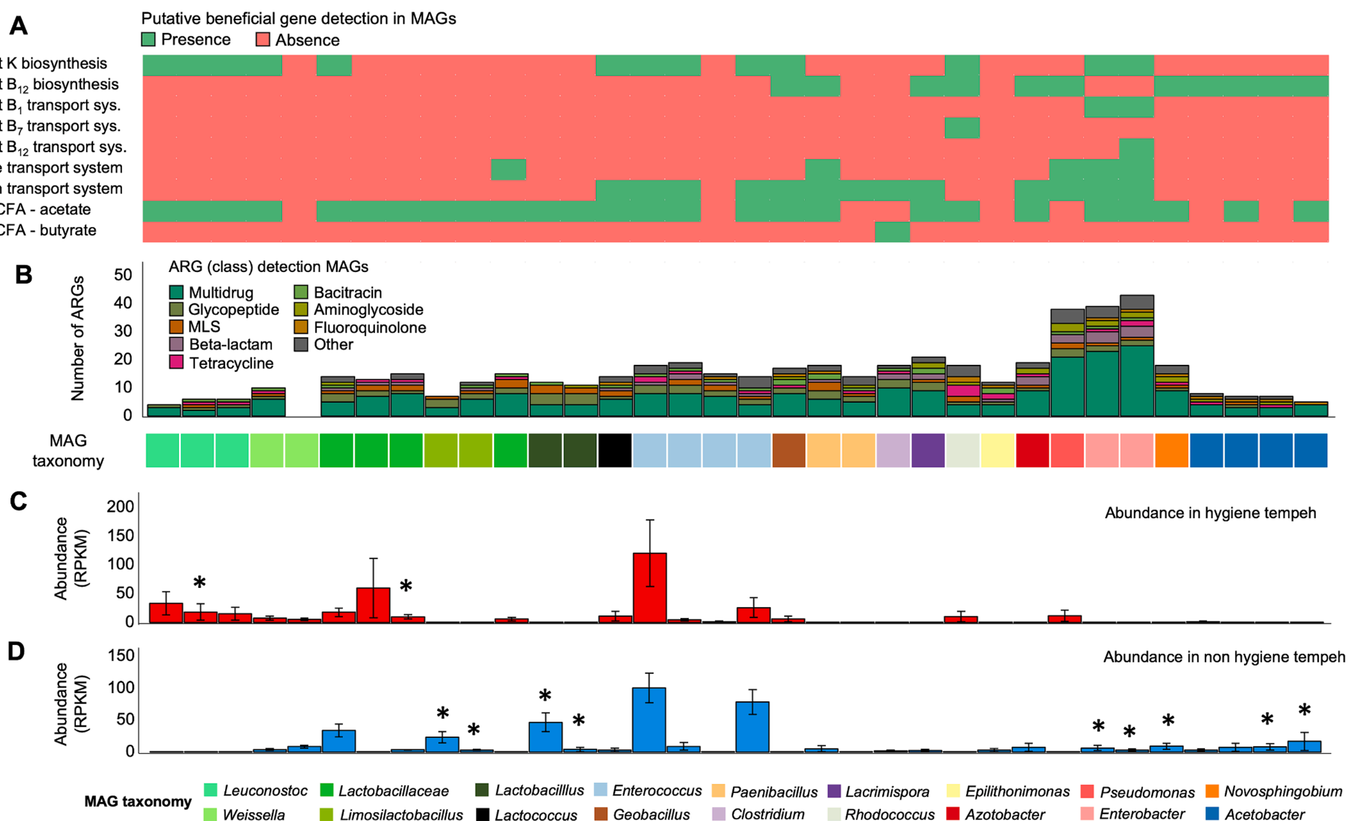

Fig. 4. Gene profiles for selected functions in metagenome-assembled genomes (MAGs) constructed from the tempeh metagenome. Visualization of gene presence/absence through color-coded plots, showcasing profiles of selected genes within the MAGs from the tempeh samples. ARG profile of each MAG was visualized using plot bars (B). Assessment of MAG abundances between tempeh produced with (C) and without standardized hygiene measures (D) based on reads per kilobase per million reads (RPKM).

contributors to factors affecting health and antibiotic resistance.

The hygiene standard resulted in a reduction in enterobacteria and lactobacilli levels, as well as a decrease in bacterial richness. The lack of detection of enterobacteria in tempeh produced in hygiene standard facilities was not unexpected, as members of this group of bacteria is typically considered hygiene indicator organisms (Tortorello, 2003). Moreover, we also showed that *Enterobacter*, a member of enterobacteria, harbored the highest number of ARG which could pose an additional risk for consumers, especially when combined with virulence mechanisms. However, analyzing metagenome contigs and MAGs that belong to enterobacteria indicated that they are involved in vitamin B_1, vitamin B_7, vitamin B_{12}, and vitamin K (menaquinone). Thus, a question that arises is whether enterobacteria could be an essential component of the tempeh microbiome that plays a significant role in health-related factors. Although members of this bacterial group are recognized as opportunistic pathogens, they have the potential to provide benefits such as allergy protection and immunomodulation (Sokolowska et al., 2018). Moreover, lipopolysaccharide from *E. coli* can elicit endotoxin tolerance *in vivo* in NOD mice and also decrease the incidence of diabetes in these mice (Vatanen et al., 2016). These studies indicated that early exposure to enterobacteria can positively impact immune system development, leading to protection against allergic and autoimmune diseases. A higher abundance of lactobacilli compared to enterobacteria in the tempeh produced with hygiene standards, indicating their resilience to implemented hygiene standards. *Lactobacillales* are involved in the fermentation, production of lactic acid, and vitamins as well as preventing the growth of spoilage microorganisms (Bao et al., 2019; Brownlie et al., 2022; Kwaw et al., 2018). Therefore, a decline in these taxa may also influence the nutritional properties of tempeh.

Hygiene standards in tempeh production have a substantial effect on bacterial composition. Our analysis revealed a clear clustering pattern suggesting a substantial overlap in bacterial community profiles among tempeh samples with varying hygiene standards. This was observed despite the diverse origins of the samples, including different producers and locations. In Indonesia, tempehh production has traditionally involved the use of water from natural sources, which may not always meet hygiene standards. Bacterial taxa that are enriched in tempeh produced without standardized hygiene measures i.e., *Rhodobacterales*, *Sphingomonadales, Burkholderiales*, and *Enterobacterales* were likely originated from the soybean or water utilized during the soaking process and then persisted during the production stages (Ilham et al., 2021; Radita et al., 2017). In contrast, the production of hygiene products requires treated water and sanitary facilities to minimize the proliferation of the mentioned microorganisms. Therefore, various variables play a role in the production of hygienic tempeh, leading to variations in bacterial diversity and community structure in comparison to tempeh produced without standardized hygiene measures. We argue that non-hygiene tempeh production environments may offer a more diverse range of microhabitats and nutrient sources, facilitating the colonization and proliferation of a broader spectrum of bacterial taxa.

Hygiene standards play a key role in influencing health-related factors and determining antibiotic resistance in tempeh. The higher abundance of genes involved in vitamin B_{12} biosynthesis and transport systems in tempeh without standardized hygiene measures may be attributed to the higher abundance of enterobacteria as well as *Acetobacter*, known as a producer of these compounds (Bernhardt et al., 2019; Denter & Bisping, 1994). LC-MS analysis confirmed a higher content of vitamin B_{12} in tempeh without standardized hygiene compared to in tempeh with standardized hygiene (Supplementary Fig. S1). In contrast, the enrichment of taxa that belong to *Flavobacteriales* and *Sphingobacteriales* could be linked to a higher abundance of genes involved in vitamin B_2 and K biosynthesis in tempeh with hygiene standards as they

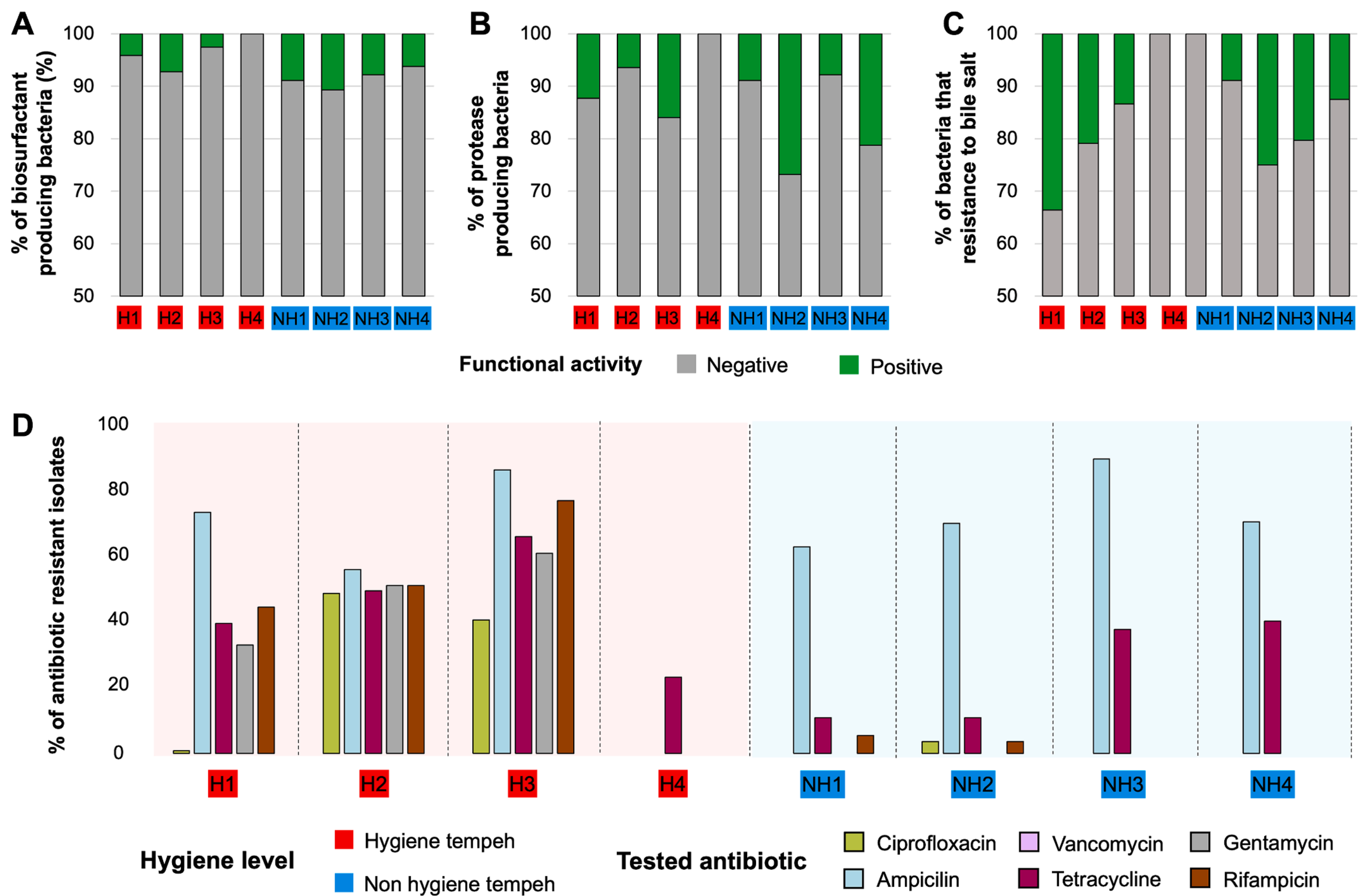

Fig. 5. Functional and antibiotic resistance profiling of bacterial isolates from tempeh. The percentage of bacterial isolates demonstrating biosurfactant production (A), protease activity (B) and resistance to bile salt (C). The percentage of bacterial isolates demonstrating resistance to each tested antibiotic. (D).

have been linked to other systems, such as lichens (Cernava et al., 2017) and insects (Béchade et al., 2022; Kinjo et al., 2022).

A higher number of isolates from tempeh without standardized hygiene exhibiting protease activity, biosurfactant production, and bile salt tolerance compared to tempeh with standardized hygiene could have significant implications for the quality and safety of the final product. The higher number of protease-producing bacteria in tempeh without standardized hygiene could potentially lead to more efficient protein breakdown, impacting the digestibility and nutritional value of tempehh (Yarlina et al., 2023). While the presence of biosurfactants in tempeh without standardized hygiene may be beneficial as this trait was found to inhibit the formation of biofilms by opportunistic pathogens (Nitschke & Costa, 2007; Wicaksono, Reisenhofer-Graber, et al., 2023), it is important to carefully assess and characterize any potential antimicrobial properties of the biosurfactant which was not carried out in this study. Furthermore, quantifying the concentration of biosurfactant produced by bacterial isolates is essential. Bile salt tolerance is another crucial trait for bacteria to survive in the gastrointestinal tract, potentially affecting the safety and probiotic potential of tempeh (Ruiz et al., 2013). A higher bacterial richness and diversity observed in tempeh without standardized hygiene may indicate a broader spectrum of metabolic activities and potential functional capabilities within the microbial community of tempehh. It is important to assess whether this increased diversity contributes to tangible improvements in tempehh quality or other attributes.

A strong impact of hygiene standards was observed on resistome profiles. Various studies have observed a correlation between a wide variety of resistance genes and high bacterial taxonomic richness in environments (Obermeier et al., 2021; Pal et al., 2016; Wicaksono et al., 2021). This likely explains a higher diversity of ARG in tempeh produced without hygiene standards in comparison to tempeh produced with hygiene standards. *Enterobacter*, *Azotobacter*, and *Pseudomonas* are significant contributors of antibiotic resistance genes, particularly those associated with multidrug efflux pumps, in tempeh produced without hygiene standards. These bacteria are also commonly found to be associated with plant hosts (Ferreira et al., 2019; Ilham et al., 2021; Soussi et al., 2016). The presence of multidrug efflux pump genes, which lack specificity (Alcalde-Rico et al., 2016; Martinez et al., 2009; Wicaksono et al., 2022), is likely a component of bacterial adaptation in natural environments including plant–microbe and microbe-microbe interactions. Although the impact of hygiene standards on the risk of antibiotic resistance gene transfer cannot be definitively determined from the data presented, it is important to highlight that hygiene standards influence the overall bacterial community composition that eventually leads to changes in resistome profiles in tempeh, potentially favoring certain taxa with specific resistance i.e., antibiotic inactivation and target, as seen in both built and clinical environments as well as natural settings (Mahnert et al., 2019; Raymond et al., 2016; Wicaksono et al., 2021). Ensuring a high bacterial diversity in traditional fermented food production is critical to mitigate the risk of harmful bacteria with resistance to multiple antibiotics.

In conclusion, hygiene practices in tempeh play a critical role in shaping the microbial richness and abundance in tempeh and lead to shifts in microbial functioning and resistome profiles. The hygiene standards in food production are designed to reduce bacterial contamination, prevent the growth of harmful pathogens, and extend the shelf life of the final products. Our study has shown that while adherence to these standards can eliminate unwanted bacteria, it may also

inadvertently diminish beneficial bacterial taxa with potential health benefits. A decline in microbial diversity in our food system may also have implications for non-communicable diseases such as asthma, obesity, and diabetes. Therefore, it is crucial to acknowledge the importance of a diverse microbiome in enhancing the immune system and supporting overall health. Particularly, the microbiome that is natively associated to edible plants might play a beneficial role for human health; a component that has been largely overlooked until now (Wicaksono, Cernava, et al., 2023). This research highlights the importance of developing guidelines for best practices in food hygiene that not only enhance food safety but also preserve the beneficial bacteria present in indigenous fermented foods. Such guidelines may involve the use of clean water, yet less aggressive sterilization methods to maintain a diverse edible plant microbiome. Future studies could explore the mechanisms underlying the observed microbial dynamics and develop strategies to utilize microbial functionality for enhancing tempeh fermentation processes and nutritional values.

Author contribution

GB, AS, and WAW designed the study. WAW and OEA conducted the sampling and performed all microbiological and molecular works. WAW and OEA analysed the data. WAW, SB, BW, AS, and GB interpreted the data and wrote the manuscript. All authors critically read the final draft.

CRediT authorship contribution statement

Wisnu Adi Wicaksono: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Oluwakemi Elizabeth Akinyemi:** Writing – original draft, Visualization, Investigation, Formal analysis. **Birgit Wassermann:** Writing – review & editing, Visualization, Data curation. **Samuel Bickel:** Writing – review & editing, Visualization, Data curation. **Antonius Suwanto:** Writing – review & editing, Conceptualization. **Gabriele Berg:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The sequencing data has been deposited in the European Nucleotide Archive (ENA) database under the study number PRJEB75611.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.115030.

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