Metagenomics analysis of the polymeric and monomeric phenolic dynamic changes related to the indigenous bacteria of black tea spontaneous fermentation

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1. Introduction

Black tea is the most widely consumed beverage in the world [1]. The active components of black tea, including phenolic compounds, are now being considered to fulfill the demand for functional beverages with potential health benefits [2,3]. Research on the health promoting compounds in tea has also been conducted, and recent studies have shown that potential phenolic compounds in black tea possess antiviral, antioxidant, anti-ageing, anti-obesity, and anticancer properties [4,5,6,7].

The quality of black tea is related to the production process and its constituent compounds, including caffeine, amino acids, and the four main catechins which include (-)-epigallocatechin-3-gallate (EGCG), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epicatechin (EC), and also pigments such as theaflavins (TFs), thearubigins (TRs), and theabrownins (TBs) [8–10]. Approximately 85% of catechins can be oxidised to TFs and TRs [11]. TFs, TRs, and TBs are formed due to extensive oxidation and catechin polymerisation [12,13]. Other phenolic compounds present in black tea include flavonols, their glycosides and phenolic acids. Flavonol glycosides (e.g., quercetin and kaempferol) are linked to astringency, bitterness, and colour [14,15]. While gallic acid (GA) and simple catechins are found in black tea and result from the degradation of catechin esters [16,17]. The production of black tea promotes the formation and conversion of sugars, the degradation of flavonoid glycosides, and the formation of TFs, TRs, amino acids, caffeine and theanine, which collectively are the compounds responsible for the colour, taste, and mouthfeel of tea [18]. Based on its constituent compounds, tea is an essential commodity fulfilling the demand for phenolic compounds with preferred characteristics.

The production of black tea involves several stages: plucking, withering, and rolling of the tea leaves using traditional, or crush, tear, curl methods, followed by spontaneous fermenting through oxygen exposure, and lastly drying of the tea to prolong shelf life [19,20]. The withering process takes between 12–18 h, then rolling and pressing take

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Abbreviations: TFs, Theaflavins; TRs, Thearubigins; TBs, Theabrownins; C, Catechin; EGCG, Epigallocatechin-3-gallate; PPO, Polyphenol Oxidase; POD, Peroxidase; FL, Fresh Leaves; SF, Spontaneous Fermentation; DL, Dry Leaves.
25–90 min. Next, spontaneous fermentation takes place for approximately 2–3 h, and finally, drying lasts 2 h at 100°C. Therefore, the total duration of black tea production is between 20–25 h [21,22,23,24]. Indigenous bacteria usually associated with plants, and those present in the environment could affect the production process, although their roles remain unknown. In contrast, post-fermented teas with longer fermentation times are usually studied to determine bacterial roles in the phenolic compounds found in tea [25]. Post-fermented teas (e.g., dark tea, Puerh tea, brick tea), like black tea, are produced through complete spontaneous fermentation, but are allowed to ferment for an extended period of time [26–28].

Changes in bacterial dynamics can modify the profile of phenolic compounds found in spontaneously fermented dark tea [29]. Bacteria involved in spontaneous fermentation, and their role in producing enzymes, are related to the degradation of polysaccharides and the metabolism of phenolic compounds which result in further changes to the contents of metabolites which affect the phenolic compounds of dark tea [30]. While black tea fermentation time is shorter than those of post-fermented teas, bacterial involvement in black tea production is not widely studied. Therefore, the role of bacteria in producing critical metabolites throughout the production stages of black tea remains unknown.

Spontaneous fermentation is a process that is essential to ensuring tea quality. The enzymatic reactions expressed during spontaneous fermentation are necessary to modify black tea’s taste, flavour, and colour [31]. Spontaneous fermentation changes the composition of tea leaves through enzymatic plant reactions (e.g., polyphenol oxidase (PPO) or peroxidase (POD) oxidation), chemical reactions (e.g., Maillard reaction), and also through enzymatic catalysis reactions of indigenous leaf microorganisms [2,3,9,32]. However, it has been widely assumed that indigenous bacteria present in tea leaves are not involved in the oxidation processes which occur during black tea production. Research of the spontaneous fermentation stage of black tea has often focused only on the process of spontaneous endo-oxidation resulting from the reactions of enzymes derived from tea plants, namely PPO and POD. However, limited information is available related to microbiological activity involving the dynamics of microorganisms in the black tea fermentation process.

Diverse communities of naturally associated microorganisms have been found on the surfaces of tea plant leaves [33]. Most microorganisms associated with tea plant leaves are phylloplanes and endophytes [32,34,35]. However, no investigations into the activities of bacteria during black tea production have been conducted. Therefore, the present study attempted to investigate the role of similar bacteria in tea. Material origin may affect the phenolic compound dynamics during spontaneous black tea fermentation.

2. Materials and methods

2.1. Sample collections and preparations

The black tea samples used were of the tea-type *Camellia sinensis* var. Assamica, clone GMB-7. Six samples of each production stage, which included Fresh Leaves (FL), Spontaneous Fermentation (SF), and Dry Leaves (DL) stages, were collected from the black tea production at the Indonesia Research Institute for Tea and Cinchona, located at 7°08′36.8″S 107°30′58.4″E, at an altitude of 1330.85 m. Bacterial dynamics during the black tea production process were identified using a metagenomic approach in FL, SF, and DL samples (Fig. 1). The conditions during fermentation of conventionally produced teas included RH >90%, pH 4.5, and temperatures of 18°C–22°C. Fermentation was performed by stacking tea with a thickness of 12 cm and allowing the fermentation to last until the pile temperature reached 26°C within 3 h. The samples were then dried for 2 h at 100°C. These production conditions aimed to produce tea with ‘Leafy Grade’ quality.

2.2. DNA Genome extraction and amplification of 16S rRNA

DNA extraction from all samples was performed utilising the ZymoBIOMICS™ DNA Mini kit (Zymo Research, Irvine, CA, USA) with standard fabricated gDNA procedures. Amplification of 16S rRNA bacteria was performed using polymerase chain reaction (PCR) performed using MyTaq™ HS Red Mix (BIO-25044, Bioline, London, UK) on Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA) to analyse the taxonomic composition of bacterial communities, and universal primer pairs 341F (5′- CCTAYGGGRBGCASCAG-3′) and 806R (5′- GGACTACHVGGGTWTCTAAT-3′), which targeted the V3–V4 region of the bacterial 16S rRNA gene, selected for amplification and pyrosequencing of PCR products. The reaction conditions were as follows: initial denaturation at 90°C for 1 min, denaturation at 95°C for 15 s, annealing at 50°C for 10 s, and a final extension at 72°C for 10 s. The two-step PCR protocol of Illumina was used to create an amplicon library. The first stage aimed to produce PCR products using Nextera-style tag sequences (overhang sequences). The second stage used a specific sample of the Illumina Nextera XT double index (Nextera XT i7 and Nextera XT i5 indices). The final library product quality and quantity assessment was conducted using a TapeStation 4200 (Agilent Technologies, Inc., Santa Clara, CA, USA), a reagent of the ddPCR green HelixyteTM, and qPCR countered using the Jeteq Lo-Rox library quantification kit from Bioline (London, UK). Following the Illumina protocol, the final sequence of pairs was generated in a 2 × 300-bp format from the MiSeq platform using the fabricated standard instructions.

2.3. Bioinformatics data analysis

2.3.1. Processing of sequencing results data

Illumina sequencing generated raw data using system control and base calling devices through Real-Time Analysis software. First, BCL (Base Call) binary was converted to FASTQ using the Illumina bcl2fastq package. Next, the raw pair-end (FASTQ) read data were combined using VSEARCH, filtered from low-quality reads, and the primer was cut to produce a high-quality 16S rRNA reading [41].
2.3.2. Operational taxonomy units (OTUs) clustering
Taxonomic profiling was performed using the microbiome taxonomic profiling pipeline. First, non-redundant reads were extracted, with results identified taxonomically using the EzBioCloud database. Then chimeras were detected using UCHIME [42]. Next, OTU determination was carried out using an open reference method with species-level identification (97% cut-off). The ineligible sequences were then grouped using de novo clustering UCLUST (97% cut-off). Finally, species identified through these two stages were combined into the final set of OTUs [43].

2.3.3. Diversity analysis
Alpha diversity analysis was conducted to determine the complexity of diversity for samples through a diversity indices, including species richness (ACE, Chao1, and Jackknife) and evenness indices (Shannon, Simpson, and NPSHannon) [44,45].

2.3.4. Predictive functional analysis
Predictive functional analysis of bacterial communities using the 16S rRNA marker gene data was performed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) software [46]. Furthermore, the Kyoto Encyclopedia of Gene and Genome (KEGG) was used to identify the involved functional genes [47].

2.4. Phenolic compounds analysis

2.4.1. Monomeric phenolic compounds analysis
Monomeric phenolic compounds were identified using high-performance liquid chromatography (HPLC) following ISO 14502-2:2005 procedures adapted, with modifications, from Zhu et al. [48]. The preparation was carried out by weighing a sample (0.2 g) mixed with 2:2005 procedures adapted, with modifications, from Zhu et al. [48]. Standard curves were then constructed using linear regression formulas for calculating the pigment content are as follows:

\[ \text{TFs} \% = \frac{2.25 \times \text{Abs}^{-1}}{1 - M} \]

\[ \text{TRs} \% = \frac{7.06 \times (2\text{Abs}_2 + 2\text{Abs}_4 - 2\text{Abs}_2 - \text{Abs}_4)}{1 - M} \]

\[ \text{TBs} \% = \frac{7.06 \times 2\text{Abs}_4}{1 - M} \]

M is the water content of each sample. The results are expressed as the percentage (%) of each compound in black tea based on the dry weight.

2.5. Statistical analysis
Descriptive analyses were used to analyse bacterial and phenolic compound dynamics during black tea production. Data obtained from bacterial abundance and phenolic concentration analyses were analysed using Spearman correlation analysis to determine the relationship between data from bacterial and phenolic compound dynamics using OriginPro 2021 software (OriginLab, Northampton, MA, USA). The relationship between bacterial abundance and dynamic concentration of phenolic compounds was performed at the genera level using R software version 4.1.1 (R Foundation for Statistical Computing, Vienna, Austria), and the vegan package was used for redundancy analysis (RDA).
3. Results and discussion

3.1. Community and diversity of bacterial structures

Total reads of the samples were obtained at the sequencing stage. All chimeric reads were cleared to obtain clean reads for analysis (Table 1). Results from the α-rarefaction curve showed that the sequencing depth was sufficient to be utilised as a basis for analysis. Data analysis was conducted to obtain groupings of bacterial OTUs from each sample. In total, 12 phyla, 24 classes, 43 orders, 102 families and 237 genera were identified from all samples from the 16s rRNA amplicon data.

Proteobacteria were predominant in all samples, followed by Bacteroidetes and Actinobacteria (Fig. 2). These results are similar to those of a previous study on the identification of the 16s rRNA amplicon within the process of black tea production proposed by Tong et al., in which Proteobacteria was identified as the most abundant phylum (>85%), followed by Bacteroidetes (2.1%-10.6%). Another metagenomic analysis found that Proteobacteria (23.56%) and Actinobacteria (23.35%) were also involved in solid-state fermentation (SSF) which occurred during Puerh tea production [50]. Additionally, pyrosequencing analyses found that the dominant bacteria involved in the post-fermented tea process were Proteobacteria and Actinobacteria [33, 36]. The identification of different studies indicated that Proteobacteria and Actinobacteria may be the primary groups of bacteria involved in the SF of tea. However, additional samples from different regions and processes require further investigation to validate this hypothesis.

Observations were made of the OTU dynamics at the genera level to investigate the bacteria’s origins (Fig. 3). A total of 2,276 OTUs were simultaneously identified in the three samples. These results showed that most bacteria in the raw material continued to be naturally inoculated during fermentation until completely dry. Overall, at the genera level, bacterial dynamics were identified in all samples and were dominated by *Methylobacterium* (17.61%-37.80%), *Sphingomonas* (17.62%-34.16%), *Chryseobacterium* (0.39%-15.57%), *Pedobacter* (6.88%-10.21%), *Aureimonas* (4.55%-15.66%), *Rhizobium* (2.38%-3.79%), *Devosia* (0.11%-3.04%), *Hymenobacter* (1.27%-3.87%), and *Microbacterium* (0.41%-1.77%) (Fig. 4).

The number of OTUs which increased during the fermentation and drying stages have been thought to result from spontaneous environmental fermentation (room, air, or water) [51]. This finding supports the idea that the fermentation process could be characterised by growth and succession of various bacterial communities [29]. The abundance of *Methylobacterium* increased during the SF stage until drying, but in contrast, *Sphingomonas* decreased during SF until drying. Certain *Methylobacterium* and *Sphingomonas* bacteria were previously identified as endophytic bacteria which acted as nitrogen fixers and plant growth-promoting bacteria [35,52-54]. *Methylobacterium* has also been identified as the primary bacteria involved in the dark tea pile-fermentation process [36]. In contrast, *Chryseobacterium* increased during SF but decreased during drying. Some strains of *Chryseobacterium* can grow in temperatures ranging from 4°C-42°C and at pH 5-9.5 [55]. Drying at 100°C was shown to reduce the population of certain bacteria. Similarly, *Aureimonas* and *Pedobacter* were also detected to decrease during the fermentation and drying processes. *Aureimonas* were the primary bacteria involved in sweet tea fermentation [56]. While *Pedobacter* growth was positively correlated to pH and humidity [57]. Changes in pH and humidity during the withering, rolling, fermentation and drying processes affected the identified *Pedobacter* population.

The diversity indices measured diversity based on the number and pattern of OTUs observed in the samples. The average diversity of Jackknife, Chao, and ACE observed in DL samples was highest among the different samples (Fig. 5a). In comparison, the α diversity of SF samples was higher than in FL samples. Similar to the evenness indices findings (NPShannon, Shannon, and Simpson), the DL samples had the highest average evenness compared with the other samples (Fig. 5b). The result of diversity indices showed that the diversity of bacteria increases after fermentation [32,36]. The DL sample was the highest compared with the other samples, and was identified by the larger values of NPShannon and Shannon, whereas Simpson’s value was smaller, indicating that evenness was greater. Overall, black tea production triggered an increase in the diversity and evenness of the bacterial community. This result demonstrated that changes in the diversity and function of the bacterial community are affected by the production process [58].

3.2. Phenolic compound dynamics

3.2.1. Monomeric phenolic compound dynamics

The phenolic compounds of tea plants are generally secondary metabolites synthesised through the shikimate, phenylpropanoid, and flavonoid pathways [59], and phenolic compounds and the chemical composition of tea leaves can be modulated by the tea production process. Black tea production is a complex process involving various enzymes and substrate types. These substrates and the reaction conditions affect enzymatic reactions and include reaction time, temperature, humidity, and pH [60]. Observations of the dynamics of catechin esters (ECCG), simple catechins (C), quercetin, kaempferol, GA, and caffeine were made during black tea production (Fig. 6).

The concentration of ECGG detected ranged from 5.28 mg/g to 44.90 mg/g. In comparison, the concentration of C ranged from 1.46 mg/g to 6.44 mg/g. The concentrations of ECGG and C in black tea were seen to gradually decrease during SF and drying. These findings are confirmed by previous studies, in which 74% of the total catechins decreased during the fermentation process [59]. This result was due to the enzymatic activity of plants that occurs during SF. These activities include catechin oxidation and then polymerisation due to enzymes such as PPO and POD, thus forming TFs and TRs [16,61].

Furthermore, from the flavonols group, the kaempferol concentrations detected throughout the entire process ranged from 0.13 mg/g to 0.20 mg/g, while quercetin concentrations ranged from 5.38 mg/g to 9.78 mg/g (Fig. 6). Flavonol glycosides are associated with a bitter taste and colour in tea, and flavonols in tea leaves are typically O-glycosides located at the C-3 position of aglycones, such as quercetin, kaempferol and myricetin. After the drying process, the an increase was seen in PPO and POD plant enzymes that actively catalysed the conversion of flavonol glycosides during the fermentation stage [60]. This result could be responsible for the increased concentration of flavonols observed in this study.

Moreover, GA was recognised as a member of the phenolic acid group. It was seen to range from 0.11 mg/g to 1.49 mg/g, and increased during the drying process. This result might be related to enzymatic conversion, Maillard reactions, and perhaps to microorganism activity that relates the degradation of catechin esters (ECC, CGC, and ECGG) to produce GA and simple catechins (C, GC, and GCG) [16,17]. However, GA is not a substrate for PPOs, although its quinone can be generated by reactions with quinones formed from particular catechins. Additionally, catechin quinones are the precursors to hundreds of volatile compounds found in the black tea aroma fraction [62]. Furthermore, reactions between GA and catechin quinones can initiate the formation of theaflavic acid.
acids which are structurally analogous to TFs [63].

During black tea production, caffeine detected ranged from 17.20 mg/g to 18.87 mg/g. Caffeine (1,3,7-trimethylxanthine) is one of the most common alkaloids in tea [64]. Because alkaloids and flavonoids have similar ontological genes, caffeine has been found alongside phenolic compounds [65]. In the present study the concentration of caffeine in each sample tended to be constant because caffeine is relatively stable during fermentation [66].

3.2.2. Polymeric phenolic compound dynamics

Polymeric phenolic compounds, such as TFs, TRs and TBs, are the products of catechin reactions that occur during black tea production [61]. The synthesis of these compounds could involve non-enzymatic oxidation reactions caused by oxygen exposure during fermentation, enzymatic oxidation from tea plants, and speculated enzymes secreted by indigenous tea leaf bacteria resulting in phenolic monomeric alterations [60,67]. The dynamics of polymeric phenolic compounds were observed during black tea production and are shown in Fig. 7.

The concentration of identified TFs ranged from 0.62% to 0.94%, while TRs levels ranged from 0.97% to 2.25%. The formation of TFs and TRs exhibited similar patterns in this study, and showed increases during the fermentation and drying stages due to oxidised monomeric phenolics, such as simple TFs formed from several catechin esters. TFs are catechin dimers formed from EGC and EC, theaflavin-3-gallate from EGCG and EC, theaflavin-3'-gallate from EGC and ECG, theaflavin-3,3'-digallate from EGCG and ECG, and also TRs [27]. At least 128 gene expressions from CsPOD and PPO were identified during various stages of tea production, with the CsAPX1 gene of tea thought to be capable of synthesising TFs using catechins as a substrate [61]. Apart from these reactions, bacteria may be related to converting phenolic compounds into polymeric phenolic compounds. Some active bacteria that produce PPO were successfully isolated from fermented black tea [68]. Furthermore, lower total phenolic levels and reduced bacterial populations were observed due to sterilisation, demonstrating the considerable influence of microorganisms [32].

The concentration of detected TBs increased from 5.42% to 8.93% during fermentation and then decreased during drying. TBs are essential factors for the taste and colour of tea, and are water-soluble polymeric compounds [25,49]. Previous studies have shown that the formation of TBs in dark tea is caused by interactions between microorganisms and PPO activity, which result in lower brightness values (L value) of the tea solution [69]. Similarly, the phenolic compounds of tea oxidise further and polymerise with polysaccharides, proteins, lipids, and other compounds to form TBs during pile fermentation [12,13]. This reaction resulted in TBs having large molecular weights with complex chemical structures [69,70]. Nevertheless, in this study, the concentration of TBs decreased during the drying stage. According to Wang et al., TB concentration increased with decreasing TR levels because TBs may be generated from TRs with the assistance of the enzymes produced by the microorganisms during SSF of Puerh tea [69]. These processes indicate that phenolic compounds may oxidise to TRs, and further oxidise and polymerise to TBs, which explains the inverse relationship between the concentrations of TBs and TRs. Similarly, the concentrations of TRs in this study remained constant during the drying stage since TRs can oxidise and polymerise into TBs.
3.3. Correlation analysis of bacterial and phenolic compounds dynamics

Black tea production affects the abundance and diversity of bacteria, and is accompanied by changes in the concentration of phenolic compounds detected at each stage of black tea production. For example, the drying stage at 100 °C for 2 hours contributed to changes in the dynamics of the bacteria and identified phenolic compounds. Heat

![Fig. 4.](image)

Fig. 4. The relative abundance of 20 dominant taxa at the genera level found in each sample of black tea production.

![Fig. 5.](image)

Fig. 5. Bacterial diversity (a) richness indices and (b) evenness indices found in each sample of black tea production.

![Fig. 6.](image)

Fig. 6. Monomeric phenolic dynamics during black tea production. Values are the means of three replicates.

![Fig. 7.](image)

Fig. 7. Polymeric phenolic dynamics during black tea production. Values are the means of three replicates.
treatment reduced the populations of some genera of bacteria that were not resistant to heating, including *Sphingomonas* and *Chryseobacterium*. In contrast, the concentrations of some phenolic compounds tended to increase during this stage. Drying aims to stop the enzymatic oxidation process by exposure to heat which causes enzymes to denature [23]. However, the activity of bacteria could contribute to the increase in phenolic compounds in the stationary phase that produces metabolites in response to heating stress [71]. This could be related to an increase in the concentration of phenolic compounds. Each black tea production stage results in biochemical reactions resulting from non-enzymatic and enzymatic activities. Enzymes and metabolites derived from tea plants and possibly bacteria would be involved in every stage of black tea production. This finding allowed for a relationship between bacterial and detected phenolic compound dynamics.

The linear relationship between the dynamics of the highest abundance bacteria and the phenolic compounds of tea was studied using Spearman’s correlation (Fig. 8). Acidobacteria and Deinococcus-Thermus phyla were positively correlated with TFs (p<0.05), TRs (p<0.01), and kaempferol (p<0.05). Actinobacteria and Armatimonadetes phyla showed a significant positive correlation with TFs (p<0.05). Firmicutes and Saccharibacteria phyla had a significant positive correlation with quercetin (p<0.01). In comparison, the phylum Proteobacteria showed a significant positive correlation with EGC and C (p<0.05). However, they had a significant negative correlation with the formation of TBs (p<0.05). In contrast, no bacterial phyla were significantly correlated with caffeine and GA formation.

The results of the Spearman correlation analysis showed that some bacterial phyla were significantly correlated with black tea phenolic compounds. Proteobacteria, Bacteroidetes and Actinobacteria have been suggested to be the most abundant bacterial phyla in tea plants. These three phyla are among the endophytic bacteria which live and multiply in plants without damaging their hosts [34]. Analysis of the predictors of bacteria identified in dark tea showed that the Actino-terriodetes group was a predictor of caffeine, and poly saccharide concentrations [72]. In contrast, the Bacte-roidetes group was a predictor of caffeic acid concentration, and the Proteobacteria group was a predictor of GCG, EGC, and ECG concentrations [72]. These results suggest that the three bacterial phyla observed in this study may be associated with several phenolic compounds produced during the SF of tea. However, the precise mechanisms of action remain unknown.

At the genera level, the Spearman correlation identified the relationships between the 20 most abundant bacterial dynamics involved in black tea production and the concentrations of phenolic compounds (Fig. 8b). The genera *Bosea* had a significant positive correlation with TRs (p<0.05), and is an endophytic bacteria in tea plants that promotes plant growth [35]. Nevertheless, the interaction mechanisms associated with the phenolic compounds remain unknown.

The dominant genera level relationships of bacterial dynamics with monomeric phenolic compounds (EGCG and C) and caffeine tended to be negatively correlated. However, bacterial dynamics were positively correlated with polymeric phenolic compounds (TFs, TRs and TBs) and flavonols (quercetin and kaempferol). Concurrently, the relationships between bacterial dynamics and GA have yielded divergent results. Some bacteria showed a positive correlation with GA, while others showed a negative correlation. The degradation of phenolic compounds could explain the implication of negative correlation resulting from SF [73]. While the positive correlation between flavonols and polymeric phenolics may imply increased concentrations along with increased populations and bacterial diversity due to enzymatic activity in SF [60, 73], SF can cause changes in the monomeric and polymeric structures of tea phenolic compounds [74, 75]. These results were related to monomeric phenolic compound modifications in the formation of polymeric phenolics, and other compounds produced may also be correlated with bacterial activity during each stage of black tea production.

Redundancy analysis (RDA) was used to identify non-symmetrical relationships at the genera level of bacterial dynamics on the dynamics of the phenolic compound concentrations (Fig. 9). The RDA results showed that the relationship between bacterial and phenolic compound dynamics was invariant in the first two axes of the RDA (>90%), indicating that bacteria were statistically correlated with several phenolic compounds. The bacterial dynamics of the *Methyllobacterium* and *Devasia* genera were associated with GA and quercetin. In contrast, the dynamics of bacteria of the genera *Pedobacter*, *Sphingomonas*, *Chryseobacterium* and *Aureimonas* were correlated with kaempferol. Moreover, the genera *Sphingomonas*, *Chryseobacterium* and *Aureimonas* were associated with TFs, TRs and TBs.

According to the RDA, the bacterial dynamics of *Methyllobacterium*, *Devasia* and unidentified genera were statistically associated with GA and quercetin. *Methyllobacterium* was the dominant phylloplane bacteria

![Graph](image_url)
living on tea leaf surfaces [29,76], and was also the most abundant bacteria identified in the production of black tea [32]. Methylobacterium has also been identified as a functional genus in the dark tea process responsible for the formation of amino acids, dissolved sugars, PPOs, cellulase, and pectinase [29]. Data also revealed a significant positive correlation between Methylobacterium and total phenolics, EGC, C, EGCG, EC, ECG and flavonoids formed in dark tea-type Liupao. Meanwhile, Devosia is a bacteria which plays a nitrogen fixation role in tea plants [76]. However, its role in phenolic compounds remains unknown. Similarly, the role of the genera of unidentified bacteria belonging to the family Hymenobacteraceae relative to phenolic compounds remains unknown.

The dynamics of bacteria of the genera Pedobacter, Sphingomonas, Chryseobacterium, Aureimonas and an unidentified bacteria were correlated with kaempferol. The genus Pedobacter was confirmed to have the ability to enhance antioxidant compounds, and this type of antioxidant activity may result from phenolic compounds [77]. Pedobacter is closely related to Flavobacterium, a flavonoid-producer bacteria [78]. Furthermore, Sphingomonas, Chryseobacterium, Aureimonas, and an unidentified bacteria might also be related to polymeric phenolic compounds which were produced by the SF of black tea.

The RDA plot showed vectors of TFs, TRs and TBs in the same quadrant which forecasted a positive correlation with several identified bacteria. Based on RDA, bacteria from the genera Sphingomonas, Chryseobacterium and Aureimonas were related to TFs, TRs and TBs. Previous research has confirmed that the genus Sphingomonas had a significant positive correlation (p<0.05) with phenolic compounds, and that Chryseobacterium was positively correlated with EGC, C, EGCG, EC, and ECG (p<0.05) in the fermentation of dark tea [57]. Furthermore, several strains of Chryseobacterium isolated from green tea revealed catalase and oxidase activity [55]. These results suggest that Sphingomonas and Chryseobacterium might be associated with TF, TR, and TB formation through production of monomeric phenolic-degrading (EGC, C, EGCG, EC, and ECG) enzymes to form polymeric phenolics. Concurrently, Aureimonas has been a critical bacteria with a strong association with non-volatile compounds and flavour in Chinese sweet tea fermentation [56]. However, its role relative to phenolic compounds remains unknown. While unknown bacteria from the family Beijerinckiaceae have been identified, to this day there has been insufficient evidence that they have a relationship with phenolic compounds.

The increase in populations of Chryseobacterium during SF was considered to be related to another mechanism of increasing phenolic

Fig. 9. (a) RDA plot of dominant bacterial genera correlated with monomeric phenolic compounds dynamics. (b) RDA plot of dominant bacterial genera correlated with polymeric phenolic compounds dynamics during black tea production.
compounds. Simultaneously, changes in phenolic compounds also occurred due to fermentation, such as decreased concentrations of EGCG and catechins, and increased levels of TFs, TRs and TBs. During the SF stage, pH decreases due to the accumulation of the resulting acid oxidation products, such as theaflavic acid, propepitheaflagallin, and TRs [79]. This condition may be related to acid-producing endophyte bacteria [71]. For example, Chrysobacterium can produce acids through simple sugar conversion [80], and under acidic conditions, cellulose can be randomly hydrolysed [81]. This condition allows bounded phenolics to be released. This result suggests that bacterial activity generated metabolites are related to increasing concentrations of several phenolic compounds.

3.4. Predictive functional analysis of bacterial dynamics related to phenolic compounds

Further identification regarding the prediction of several enzyme contributions related to the bioconversion of phenolic compounds in black tea production was carried out using the metagenomic prediction software PICRUSt2. This study utilised the KEGG database. Thus, KEGG-based PICRUSt2 analyses were conducted to provide more comprehensive information concerning bacterial gene function expression. At the KEGG pathway level, the flavonoid biosynthesis (ko00941) pathway abundance was associated with the increased bioconversion of phenolic compounds during the SF stage (Fig. S1). This result suggested that bacteria had contributions related to flavonoid biosynthesis.

Furthermore, several predicted functional bacterial genes related to the bioconversion of phenolic compounds were found, such as for the oxidation, hydrolysis and degradation of phenolic compounds (Fig. 10). The abundance of all related functional genes increased relatively during the black tea SF stage. The highest abundances of genes identified in this predicted functional analysis related to phenolic compounds were for yfiH and katG, which could be predicted in bacteria during black tea production.

Several reactions that may be related to the function of these genes are shown in Fig. S2. katG (catalase-peroxidase: K03782) and yfiH (polyphenol oxidase: K05810) had the greatest gene function abundances related to the oxidation of black tea phenolic compounds. The abundance of these genes increased during the fermentation and drying stages. Catalase-peroxidases were secreted enzymes that may catalyse the oxidation of catechins during the SSF of Puerh tea [82]. Furthermore, several bacterial isolates have been identified with polyphenol oxidase activity responsible for forming the primary quality compounds of SSF brick tea [83]. The katG and yfiH genes present in bacteria of black tea SF suggest they could contribute to oxidising phenolic compounds, as well as having roles as PPO and POD enzymes found in the tea plant [3,16,39,59]. Predictions for these genes’ presence forecasted the potential contribution of bacteria in secreting enzymes involved in the oxidation process of phenolic compounds in black tea production. This is in addition to enzymatic reactions from tea plants and chemical reactions such as the Maillard reaction.

Furthermore, genes related to the hydrolysis of phenolic compounds were also found in bacteria involved in this process. The genes aguA (α-glucuronidase: K01235), uidA/GUSB (β-glucuronidase: K01195), and FAEB (feruloyl esterase: K09252) were predicted to be associated with bacterial activity in hydrolysing phenolic compounds. The α-glucuronidase enzyme hydrolyses 1,2-linked glucuronic acid from the terminal, non-reducing xylose of xylooligosaccharides [84]. Furthermore, in Puerh tea, glucuronidase was suggested to be involved in the metabolism of glucosides and glucopyranosides, capable of degrading phenolic glycosides [30]. Subsequently, feruloyl esterases or ferulic acid esterases, also called tannases, represent a diverse group of hydrolases which catalyse the cleavage and formation of ester bonds between plant cell wall polysaccharides and phenolic acids which can be produced by microorganisms [85]. Feruloyl esterases are enzymes involved in the release of phenolic compounds, including caffeic, ferulic, p-coumaric, and syringic acids, from plant cell walls, and are often used in the food industry [86,87]. In the tea fermentation process, this enzyme is associated with its ability to hydrolyse gallates and release GA, which results in changes in the concentrations of these compounds [30].

Several other functional genes associated with the degradation of phenolic compounds were also found in bacteria involved in black tea production. The genes cate/ dmpB (catechol 2,3-dioxygenase: K07104, K00446), cata (catechol 1,2-dioxygenase: K03381), and the gene that encodes phenol 2-monoxygenase (K03380) were identified in bacteria involved in black tea production. These genes may contribute to the degradation of phenolic compounds, as shown in Fig. 10. It is noteworthy that catechol 2,3-dioxygenase is a key enzyme in the biodegradation of aromatic hydrocarbons [88]. This gene is also involved in the reaction of salicylate 1-monoxygenase oxidises salicylate to form catechol, and the reactions of catechol 2,3-dioxygenases and catechol 1, 2-dioxygenase degrade catechol to form cis-cis-muconic acid or

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**Fig. 10.** Classification of predicted functional genes related to phenolic compounds dynamics during black tea production.
2-hydroxymuconate semialdehyde in Puerh tea fermentation [30].

Further investigations were conducted to identify the bacterial genera predicted to contribute to the SF process (Tables S1 and S2). The investigation was carried out on the functional genes yfiH and katG, the genes with the highest abundance found in this predictive functional analysis related to phenolic compounds. The yfiH gene was predicted in several bacteria involved in each stage of black tea production. The abundance of yfiH genes increased as the abundance of bacteria detected during fermentation and drying increased. Several dominant genera were predicted to possess yfiH functional genes, including *Methyllobacterium*, *Sphingomonas* and *Aureimonas*. According to Chai et al., the yfiH gene, through its laccase-like protein, was identified in melanogenic *Aeromonas* strains of bacteria, and *CatA* possesses PPO activity [89]. The substrate specificity of laccase is relatively broader. For instance, it can oxidise p-diphenols, triphenols, and even lignin [90]. This is similar to the substrate specificity of yfiH.

These functional predictions forecasted several bacteria related to phenolic compounds, including *Methyllobacterium*, *Sphingomonas*, *Aureimonas* and *Devesia*. These results led to the hypothesis that this may be related to their potency in secreting several enzymes. To our knowledge, this is the first study examining the prediction of bacterial-produced enzymes involved in the metabolism of phenolic compounds in the SF of black tea. Thus, the level of bacterial contribution to the oxidation processes of phenolic compounds, besides enzymatic (e.g., oxidation by the PPO of plants) and chemical reactions (e.g., Maillard reaction), still requires further investigation. Future studies are required to verify the expression of these genes using complementary techniques, such as qPCR or RT-qPCR.

4. Conclusions

The dynamics of bacteria identified in the SF of black tea revealed that several groups of bacteria were significantly correlated to phenolic compounds involved in the SF of black tea. At the genera level, *Methyllobacterium* and *Devesia* correlated with GA and quercetin. The genera *Sphingomonas*, *Chryseobacterium* and *Aureimonas* correlated with kaempferol, TPs, TRs and TBs. Predicted functional genes of bacteria linked to phenolic compound bioconversion, such as oxidation, hydrolysis and degradation, were forecasted: *yfiH* and *katG* were the dominant genes related to the bioconversion of phenolic compounds found through this predictive functional analysis. Thus, these findings suggest that indigenous bacteria are associated with the bioconversion of phenolic compounds in black tea production, and could contribute to the quality of the black tea produced, thereby providing a baseline for further study. Future research on endophytic microorganisms might involve axenic plants, and could also utilise a process conducted in a sterile environment to determine the involvement of these indigenous bacteria.

Credit Authorship Contribution Statement

Siti Nurmila et al. investigation, data curation, formal analysis, visualisation, and writing-original draft. Yana Cahyana: supervision and review. Gemilang Lara Utama: conceptualisation, methodology, supervision, writing, review, editing, and validation. The authors have given their final approval for the final version to be published, and all authors have read and approved the final manuscript.

Declaration of competing interest

The authors declare no conflicts of interest regarding the publication of this paper.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2022.e00774.

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