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Chlorogenic acid and caffeine content of fermented robusta bean

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Abstract. *Purwoko T, Suranto, Setyaningsih R, Marliyana SD. 2022. Chlorogenic acid and caffeine content of fermented robusta bean. Biodiversitas 23: 902-906.* Robusta beans contain caffeine and chlorogenic acid higher than arabica beans, however, it has a lower economic value than arabica beans. Therefore, microorganisms in the robusta bean for the fermentation process are expected to reduce caffeine and chlorogenic acid contents. The objective of this research was to investigate the ability of fungus *Rhizopus oryzae*, yeast *Saccharomyces cerevisiae*, and bacteria *Lactobacillus casei* and *Leuconostoc mesenteroides* in degrading caffeine and chlorogenic acid content of robusta beans. Analysis of caffeine and chlorogenic acid content was carried out by spectrophotometer at OD_{275} and OD_{324}, respectively. The caffeine and chlorogenic acid content of unfermented robusta beans were 18.64 mg/g and 62.18 mg/g. *Saccharomyces cerevisiae, L. mesenteroides, L. casei*, and *R. oryzae* were able to degrade caffeine, and reduce the caffeine content of fermented robusta beans to 16.92, 16.71, 16.67, and 13.57 mg/g, respectively. The decrease of caffeine by *S. cerevisiae, L. mesenteroides* and *L. casei* were not significantly different ($p>0.05$) from control, however, the decrease of caffeine by *R. oryzae* was significant ($p<0.05$). *Saccharomyces cerevisiae, R. oryzae, L. mesenteroide,* and *L. casei* were able to degrade chlorogenic acid and reduce the chlorogenic acid content of fermented robusta beans to 44.54, 45.21, 45.79, and 47.31 mg/g. Chlorogenic acid was reduced significantly by *S. cerevisiae, R. oryzae, L. mesenteroides*, and *L. casei* ($p<0.05$). It can be concluded that *R. oryzae* was a potential microorganism to reduce caffeine and chlorogenic acid contents in robusta beans.

Keywords: Caffeine, chlorogenic acids, *Rhizopus oryzae*, robusta beans

INTRODUCTION

Coffee plants (*Coffea* spp.) originate from Africa, especially Ethiopia, then distributed to the world, including Indonesia. Coffee plants are members of *Rubiaceae*. There are 80 coffee plants species in the world including *Coffea arabica, Coffea canephora*, *Coffea liberica*, and *Coffea excelsa.* Coffee plants are annual plants and the coffee cherries are picked once or twice a year to obtain coffee beans. According to Baltazar and Buot (2019), the coffee plant is one of the most important commodity crops. *Coffea arabica* and *C. canephora* are grown at different elevations. *Coffea arabica* grows at altitudes above 1000 m asl, however, *C. canephora* grows at altitudes less than 1000 m asl.

Coffea arabica, C. canephora, C. liberica, and *C. excelsa* have economic value, however, the coffee market is dominated by *C. arabica* and *C. canephora*. The economic value of arabica beans was higher than robusta beans. However, Indonesian farmers prefer to cultivate *C. canephora* than *C. arabica*. In 2016, the cultivation of *C. canephora* was 81.9% compared to that of *C. arabica* was 18.04% of the cultivation area (Pusat Data dan Informasi Pertanian 2017). Therefore, coffee beans in Indonesia were dominated by robusta beans. Meanwhile, coffee beans production in the world is dominated by Brazil, Colombia, Vietnam, Indonesia, and Ethiopia.

The important chemical compounds in coffee beans are caffeine and chlorogenic acid. Caffeine is an alkaloid synthesized from xanthosine which is a purine derivative. Caffeine in coffee can reduce sodium reabsorption in the renal tubules, increase glomerular filtration and is a mild diuretic (Bistani et al. 2007). Chlorogenic acid is an ester of trans-cinnamic acid and quinic acid. Caffeine causes a bitter taste (Flament 2002) and chlorogenic acid causes an astringent taste (Wang 2012) in coffee. Farah (2012) showed that caffeine content in robusta and arabica beans were 1.5-2.5% and 0.9-1.3%, and chlorogenic acid content of robusta and arabica beans were 6.1-11.3% and 4.1-7.9%. Caffeine and chlorogenic acid content were influenced by internal and external factors. Internal factors that influence caffeine and chlorogenic content are species and variety of coffee plants, while the external factors are environmental conditions and cultivation techniques of coffee plants.

Microorganisms can degrade caffeine and chlorogenic acid. Mazzafera (2002) reported that *Pseudomonas putida* degraded caffeine into xanthine and subsequently metabolized into uric acid. A study by Summers et al. (2015) showed that *Pseudomonas* sp. CBB1 can metabolize uric acid into glyoxylate then join the central metabolic pathway. *Aspergillus* and *Penicillium* also degraded caffeine to a methylxanthine (Hakil et al. 1998; Tagliari et al. 2003). The microflora degraded chlorogenic acid into various aromatic acids such as coumaric acid, benzoic acid

derivates and phenyl propionic acid. Gonthier et al. (2003) reported that rats given chlorogenic acid produced caffeic acid, ferulic acid, isoferulic acid, coumaric acid, hydroxyphenyl acids, hydroxybenzoic acid, hydroxyhipuric acid, and hippuric acid. Tomas-Barberan et al. (2014) showed that human microflora was able to metabolize chlorogenic acid into caffeine-glycerol intermediates and hydroxyphenyl acids. Kulik et al. (2017) reported that the fungus *Fusarium* spp. were also able to metabolize chlorogenic acid into caffeic acid and protocatechuic acid.

Previous research showed that bacteria and fungi can degrade caffeine and chlorogenic acid in arabica beans, therefore there was a potency of bacteria and fungi to degrade caffeine and chlorogenic acid in robusta beans. Robusta beans contain higher caffeine and chlorogenic acid than arabica beans. The objective of this research was to determine the ability of microbial cultures, namely the fungus *Rhizopus oryzae*, yeast *Saccharomyces cerevisiae*, and bacteria *Lactobacillus casei* and *Leuconostoc mesenteroides* in degrading caffeine and chlorogenic acid of robusta beans.

MATERIALS AND METHODS

Materials

Robusta coffee cherries (*Coffea canephora*) Tugusari variety were obtained from Gesing Kandangan, Temanggung, Central Java, Indonesia. Microorganisms, i.e., *Saccharomyces cerevisiae* FNCC 3210, *Rhizopus oryzae* FNCC 6010, *Lactobacillus casei* FNCC 090, and *Leuconostoc mesenteroides* FNCC 023 were obtained from UGM Yogyakarta. Caffeine and chlorogenic acid compounds were obtained from Sigma Aldrich. Yeast extracts Peptone Dextrose (YPD), Potato Dextrose (PD), and deMan Rogosa Sharpe (MRS) media were obtained from Oxoid.

Microbial culture preparation

Yeast *S. cerevisiae*, fungus *R. oryzae*, and bacteria *L. casei* and *L. mesenteroides* were cultured on YPD, PD, and MRS Agar, respectively. Yeast and fungal cultures were incubated under aerobic conditions for 5 days at 28°C and bacteria cultures were incubated under anaerobic conditions for 5 days at 28°C. All cultures were preserved in the refrigerator (4°C).

Coffee bean fermentation

Robusta cherries (1.5 kg) were soaked in water and the floating cherries were discharged. Robusta cherries are put in a coffee pulper to remove skin and pulp and obtain parchment beans. After mucilage removal, the parchment beans were soaked in water for 4 hours. The parchment and silver skin were removed to obtain robusta beans. Robusta beans were sterilized by autoclaving (121°C, 15 min). Four groups of robusta beans (@ 30 g) were put in Petri dishes and inoculated with ± 10^7 CFU/g *S. cerevisiae*, *R. oryzae*, *L. casei*, and *L. mesenteroides* respectively. Robusta beans fermented with *S. cerevisiae* and *R. oryzae* were aerobically incubated for 4 days at 28°C and robusta beans were fermented with. *L. casei* and *L. mesenteroides* were anaerobically incubated for 4 days at 28°C. Control and fermented coffee beans (1-, 2-, 3-, 4-day of fermentation) were sampled for extraction.

Fermented coffee beans extraction

Each fermented robusta beans (1-, 2-, 3-, and 4-days of fermentation) and control (unfermented beans) was dried at 40°C for 5 days. Dried robusta beans samples were grounded and extracted with 50 ml of water at 80°C for 1 hour. After filtering, the extracted samples were partitioned with 50 ml of dichloromethane. The dichloromethane and aqueous phases were collected and refrigerated (4°C) before spectrophotometer analysis.

Spectrophotometer analysis

Samples (2.5 ml) of dichloromethane and aqueous phase were analyzed by spectrophotometer (Hitachi UH5300) at OD_{275} and OD_{324} respectively. The absorbance values of OD_{275} and OD_{324} were converted into the concentration of caffeine and chlorogenic acid based on the curve of caffeine and chlorogenic acid standards.

Data analysis

Fermentation samples and control were carried out in 3 replications. Spectrophotometer Data on caffeine and chlorogenic acid content of control and fermented coffee beans were analyzed using One-Way ANOVA with Dunnett multiple comparisons (SPSS ver. 18).

RESULTS AND DISCUSSION

Caffeine concentration of fermented robusta beans

Caffeine is responsible for the bitter taste of the coffee brew. Caffeine has antioxidative activity (Pokorna et al. 2015) by scavenging free radicals mechanism (Vieira et al. 2020). Caffeine also has antimicrobial activity (Matan et al. 2015). The caffeine content of robusta beans was in the range of 15-30 mg/g (Farah 2012; Navarra 2017). The caffeine content of control (unfermented robusta beans) was 18.64 mg/g (Figure 1). Therefore, the caffeine content in this study was still within the range of previous studies.

Leuconostoc mesenteroides reduced the caffeine content of robusta beans to 18.05 mg/g, which was equivalent to 3.21% after 1-day of fermentation. The caffeine content of robusta beans fermented with *L. mesenteroides* at 2-, 3-, and 4- days of fermentation were 17.69, 17.27, and 16.71 mg/g after 2-, 3- and 4-day fermentation, which were equivalent to 5.09, 7.39, and 10.35%, respectively (Figure 1).

Lactobacillus casei also reduced the caffeine content of robusta beans to 17.80 mg/g after 1-day of fermentation, which was equivalent to 4.53%. The caffeine content of robusta beans fermented with *L. casei* reduced to 17.54, 16.99, and 16.67 mg/g, which were equivalent to 5.92, 8.88, and 10.59% after 2-, 3- and 4-days of fermentation, respectively (Figure 1).

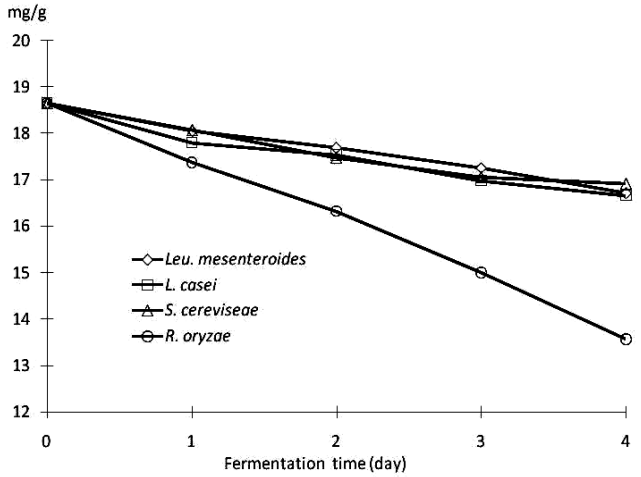

Figure 1. The caffeine content of robusta beans fermented with *Lactobacillus mesenteroides, L. casei, Saccharomyces cerevisiae,* and *Rhizopus oryzae* after four days of fermentation

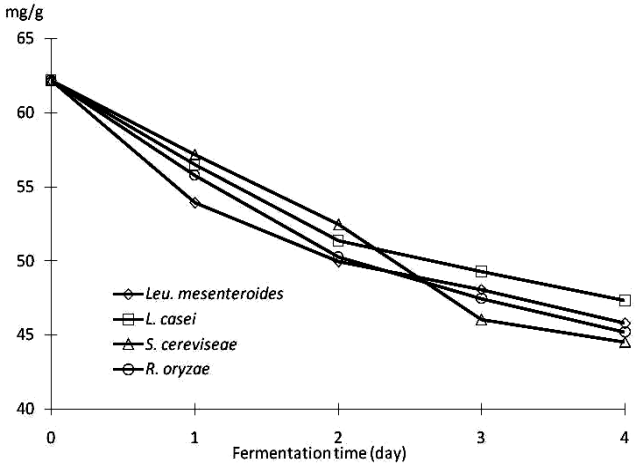

Figure 2. The chlorogenic acid content of robusta beans fermented with *Leu. mesenteroides, L. casei, S. cerevisiae,* and *R. oryzae* for 4-days of fermentation

Fermentation of robusta beans with *S. cerevisiae* also reduced the caffeine content of robusta beans to 18.07 mg/g, which was equivalent to 3.10% after 1-day fermentation. The caffeine content of robusta beans fermented with *S. cerevisiae* at 2-, 3-, 4- days of fermentation reduced to 17.48, 17.06, and 16.92 mg/g, which were equivalent to 6.26, 8.49, and 9.24% after 2-, 3- and 4-days of fermentation, respectively (Figure 1).

Fermentation of robusta beans with *R. oryzae* reduced the caffeine content to 17.37 mg/g, which was equivalent to 6.84% after 1-day fermentation. The caffeine content of robusta beans fermented with *R. oryzae* reduced to 16.33, 15.01, and 13.57 mg/g, which were equal to 12.41; 19.50, and 27.20% after 2-, 3- and 4-days of fermentation, respectively (Figure 1).

Fermentation of robusta beans with *S. cerevisiae, L. mesenteroides* and *L. casei* reduced caffeine content in the range of 9.24-10.59% after 4-days of fermentation. The average decrease of caffeine content in robusta beans was 2.31-2.65% per day. However, *R. oryzae* reduced caffeine content by 27.20% caffeine content after 4-days of fermentation, this was equal to 6.80% per day. Therefore, the ability of *R. oryzae* in reducing the caffeine content of robusta beans was around 2.5 times that of *S. cerevisiae, L. mesenteroides* and *L. casei.*

The content of chlorogenic acid of fermented robusta beans

Chlorogenic acid was responsible for the bitter and astringent taste of coffee brewing. Chlorogenic acid has antioxidative activity (Budryn et al. 2015), antimicrobial activity (Kabir et al. 2014), anticancer, antidiabetic, and neuroprotective activity (Roy 2019). Previous studies showed that the chlorogenic acid content of robusta beans was 59-151 mg/g (Farah 2012; Navarra 2017). Unfermented robusta beans (control) contained 62.18 mg/g of chlorogenic acid, which was within the range of previous studies.

Figure 2 showed the chlorogenic acid content of robusta beans fermented with *Leu. mesenteroides* after 1-, 2-, 3- and 4-days of fermentation were 53.94, 49.94, 48.08, and 45.79 mg/g, which were equal to a decrease of 13.25, 19.68, 22.67, and 26.35%, respectively. *Lactobacillus casei* also reduced the chlorogenic acid of robusta beans to 56.50, 52.37, 49.29, and 47.31 mg/g, which were equal to a decrease of 9.14, 17.39, 20.72, and 23.92% after 1-, 2-, 3- and 4-days of fermentation, respectively. *Saccharomyces cerevisiae* also reduced chlorogenic acid of robusta beans to 57.21, 52.47, 46.04, and 44.54 mg/g, which were equal to a decrease of 8.00, 15.62, 15.95, and 28.38% after 1-, 2-, 3- and 4-days of fermentation, respectively. The chlorogenic acid content of robusta beans fermented with *R. oryzae* after 1-, 2-, 3- and 4-days of fermentation were 55.80, 50.25, 47.47, and 45.21 mg/g, which were equal to a decrease of 10.27; 19.18; 23.65, and 27.29%, respectively.

Discussion

Tawali et al. (2018) reported that lactic acid bacteria from yogurt reduced the caffeine content of robusta beans after 2-days of fermentation. Our research findings also showed the caffeine content of robusta beans fermented with lactic acid bacteria, i.e., *L. mesenteroides,* and *L. casei* after 4-days of fermentation were also decreased. The caffeine content of unfermented robusta beans was 18.64 mg/g and reduced to 16.67-16.71 mg/g after 4-days of fermentation. Hatiningsih et al. (2018) reported that yeasts reduced the caffeine content of robusta beans after 16-hours of fermentation. Our research findings also showed the caffeine content of robusta beans fermented with yeast *S. cerevisiae* was decreased. The caffeine content of unfermented robusta beans was 18.64 mg/g and reduced to 16.92 mg/g after 4-days of fermentation.

Microorganisms decreased caffeine content by absorbing or degrading it. Microorganisms absorb caffeine because the structure of caffeine was similar to the purine molecule. Microorganisms degrade caffeine to obtain a

carbon source by demethylation of caffeine. Some bacteria such as *Pseudomonas, Alcaligenes, Serratia, Klebsiella, Rhodococcus, Brevibacterium,* and *Bacillus* were able to degrade caffeine (Ibrahim et al. 2014). Mills et al. (2015) reported that lactic acid bacteria could not degrade caffeine, and there was no research on caffeine degradation by lactic acid bacteria and yeasts previously. Caffeine had antibacterial and antifungal activity. Caffeine easily enters cells through porin channels (Mukhtar et al. 2021) or nonspecific transporters (Ruta and Farcasanu 2020). Substance efflux was a mechanism to reduce the deleterious effects of antibacterial compounds. However, a small amount of caffeine did not have a lethal effect on cells and was even beneficial for cellular metabolic processes. One of the benefits of caffeine in the metabolism of eukaryotic cells was to protect DNA from being damaged by a kinase (Ruta and Farcasanu 2020). Therefore, *S. cerevisiae* decreased caffeine levels by absorbing it into cells for cell metabolism and efflux the excess of caffeine. Bacteria *L. mesenteroides* and *L. casei* might also absorb caffeine as in *S. cerevisiae.*

The results of this study showed that *R. oryzae* could degrade caffeine up to 27.20% after 4-days of fermentation. This result was not different from the previous study. Lee et al. (2016) reported that *R. oligosporus* degraded the caffeine content of Arabica beans up to 30% after 5-days of fermentation. Several previous studies showed that some species of microorganisms can degrade caffeine, i.e., *Aspergillus* and *Penicillium* (Hakil et al. 1998; Ibrahim et al. 2014)), *Rhizopus delemar* (Tagliari et al. 2003), and *Phanerochaete* (Ibrahim et al. 2014). Fungi were able to degrade caffeine because they had N-methylase complex enzymes (Kim et al. 2019). Kobeticova et al. (2019) reported that caffeine affected fungal growth, however, caffeine-degraded compounds did not affect it. The mechanism of degradation by microorganisms was by neutralizing the antimicrobial effect of antimicrobial compounds. Our research showed *R. oryzae* were able to degrade caffeine, presumably through the mechanism of N-methylase complex enzymes. This caffeine degradation mechanism was by neutralizing caffeine's antifungal activity and obtaining a carbon source for its metabolism.

Statistical analysis showed caffeine content of robusta beans fermented with *L. mesenteroides, L. casei,* and *S. cerevisiae* after 1-, 2-, 3- and 4-days of fermentation were not significantly different from control ($p>0.05$). The caffeine content of robusta beans fermented with *R. oryzae* after 1-, 2-, and 3-days of fermentation was also not significantly different compared to control ($p>0.05$). However, the caffeine content of robusta beans fermented for 4 days with *R. oryzae* was reduced significantly compared to control ($p<0.05$). A nonsignificant decrease in caffeine content of robusta beans fermented with *L. mesenteroides, L. casei,* and *S. cerevisiae* up to 4 days of fermentation might show the presence of efflux mechanism by microorganisms or the use of small amounts of caffeine, however, there have been no studies of caffeine degradation by lactic acid bacteria and yeasts. Meanwhile, the significant decrease in caffeine content of robusta beans fermented with *R. oryzae* after 4-days of fermentation might show the ability of *R. oryzae* to degrade caffeine as a carbon source and maintain cell metabolism.

Lafay et al. (2006) showed microflora of the rat intestine reduced chlorogenic acid to around 10% chlorogenic acids in 24 hours, however, they did not isolate and identify the microflora. Our research results showed that Lactic Acid Bacteria, i.e., *L. mesenteroides* and *L. casei* reduced chlorogenic acid of robusta beans in 24 hours up to 9.14 and 13.25%, respectively. *Leuconostoc mesenteroides* and *L. casei* decreased chlorogenic acid by 26.35 and 23.92% in robusta beans after 4-days of fermentation. Couteau et al. (2001) reported that *Escherichia coli, Bifidobacterium lactis,* and *Lactobacillus gasseri* were able to degrade chlorogenic acid into hydroxycinnamic acid and ferulic acid. Lafay et al. (2006) reported that caffeic acid and ferulic acid were the product of chlorogenic acid degradation. Therefore, *L. mesenteroides* and *L. casei* might absorb and degrade chlorogenic acid of robusta beans into chlorogenic acid derivates and use it as a carbon source during fermentation. *S. cerevisiae* decreased chlorogenic acid by 28.38% in robusta beans after 4-days of fermentation. *Saccharomyces cerevisiae* is also one of the intestine microflora, that might degrade chlorogenic as lactic acid bacteria did.

Lee et al. (2016) reported that *R. oligosporus* reduced 16% chlorogenic acid in arabica beans after 5-days of fermentation. Our research results also showed that *R. oryzae* reduced 27.29% chlorogenic acid of robusta beans after 4-days of fermentation. Schobel and Pollmann (1980) has isolated chlorogenase from the fungus *Aspergillus niger* which can break down chlorogenic acid. Gauthier et al. (2016) reported that the fungus *Fusarium graminearum* could degrade chlorogenic acid into caffeic acid and ferulic acid. Therefore, *R. oryzae* might degrade the chlorogenic acid of robusta beans into its derivative products to obtain carbon sources during fermentation.

Statistical analysis of chlorogenic acid content of robusta beans fermented with *L. casei* and *S. cerevisiae* after 3- and 4-days of fermentation were significantly lower than control ($p<0.05$), whereas chlorogenic acid content of robusta beans fermented with *L. mesenteroides* and *R. oryzae* were significantly lower than control after 2-, 3-, and 4- days of fermentation ($p<0.05$). It showed that *L. mesenteroides* and *R. oryzae* can degrade chlorogenic acid faster than *L. casei* and *S. cerevisiae. Lactobacillus mesenteroides* and *R. oryzae* were able to significantly reduce chlorogenic acid after 2-days of fermentation, whereas *L. casei* and *S. cerevisiae* decreased chlorogenic acid after 3-days of fermentation. All microorganisms used in this study were able to degrade chlorogenic acid in robusta beans so that all these microorganisms were potential as a chlorogenic acid-reducing agent in robusta beans.

Our research results showed that *S. cerevisiae, L. mesenteroides,* and *L. casei* were able to degrade chlorogenic acid significantly, however, they could not degrade significantly caffeine of robusta beans. Our research results also showed that *R. oryzae* was able to degrade both chlorogenic acid and caffeine significantly. Previous studies showed lactic acid bacteria could degrade

chlorogenic acid significantly (Couteau et al. 2001; Szwajgier and Jakubczyk 2010) and there was no previous study reported on caffeine degradation by lactic acid bacteria and yeasts. However, previous studies showed that fungi can degrade caffeine and chlorogenic acid (Hakil et al. 1998; Gauthier et al. 2016; Kulik et al. 2017). Therefore, the fungus *R. oryzae* could be applied to degrade robusta beans' caffeine and chlorogenic acid. Nevertheless, the mechanism of caffeine and chlorogenic acid degradation by *R. oryzae* should be investigated in future studies.

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