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Caffeine degradation by food microorganisms

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Abstract. *Purwoko T, Suranto, Setyaningsih R, Marliyana SD. 2023. Caffeine degradation by food microorganisms. Biodiversitas 24: 3495-3502.* Commercial coffee beans are dominated by robusta and arabica coffee beans. Caffeine is one of the important components in coffee beans. Caffeine has antimicrobial effect. Caffeine content in robusta beans was higher than in arabica beans. Caffeine content in coffee beans was affected by the coffee species, the coffee cultivation's altitude and the postharvest processing method. Microbial fermentation activity could reduce the caffeine content of coffee beans. *Lactobacillus casei, Leuconostoc mesenteroides, Rhizopus oryzae* and *Saccharomyces cerevisiae*, were able to reduce caffeine content of robusta beans. This study aimed to determine the degradation pathway of caffeine by food microorganisms, namely *L. casei, L. mesenteroides, R. oryzae* and *S. cerevisiae*. Caffeine content in NB-caffeine and PDB-caffeine media were reduced by *L. casei, L. mesenteroides, R. oryzae* and *S. cerevisiae*. Caffeine was transformed into dimethylxanthine and then into methylxanthine by *L. casei, L. mesenteroides, R. oryzae* and *S. cerevisiae*. They transformed more than 89% caffeine into paraxanthine, however, small amount of paraxanthine was transformed into methylxanthine. *L. casei* and *L. mesenteroides* transformed paraxanthine into 1-methylxanthine. However, *R. oryzae* and *S. cerevisiae* transformed into 7-methylxanthine. There were two patterns of degradation of caffeine into methylxanthine i.e., caffeine-paraxanthine-1-methylxanthine and caffeine-paraxanthine-7-methylxanthine. The first was shown by *L. casei* and *L. mesenteroides*, and the last by *R. oryzae* and *S. cerevisiae*.

Keywords: 1-methylxanthine, 7-methylxanthine, caffeine, paraxanthine

INTRODUCTION

Caffeine is often found in several plants, such as tea (*Camellia* spp.), coffee (*Coffea* spp.), guarana (*Paullinia cupana*) and cola (*Cola acuminata*) (Chaugule et al. 2019). However, the coffee plant was known as the main source of caffeine than other plants. Caffeine was found in the leaves, especially at upper leaves and seeds, but not in cotyledons (Patay et al. 2017). Biosynthesis of caffeine in leaves of *Coffea arabica* was from adenine that was converted into 7-methylxanthosine, then into 7-methylxanthine and theobromine and finally into caffeine (Ashihara et al. 1996).

Caffeine and chlorogenic acid are two important components in coffee beans. These components gave the taste of coffee brew. In coffee brew, the caffeine and chlorogenic acid give an astringent-bitter taste. In addition, there were more than 800 volatile compounds that contributed to the aroma of coffee beans and classify into furans (38-45%), pyrazines (25-30%), pyridines (3-7%) and pyrroles (2-3%) (Pinheiro et al. 2020). Commercial coffee beans are dominated by robusta and arabica beans, although there were 100 species of *Coffea* (Adepoju et al. 2017). The two coffee beans had different caffeine and chlorogenic acid content. Robusta beans contain higher content of caffeine and chlorogenic acid than arabica beans. This makes the taste of robusta coffee brew more bitter than arabica coffee brew. Therefore, robusta brew often added sugar to reduce its bitter taste. However, arabica roasted coffee brew was not sugar addition because the bitter taste was not strong. Therefore, economic value of robusta beans was lower than arabica beans.

Caffeine content of coffee beans was affected by several things such as the species and variety of coffee, the altitude of cultivation (Randriani et al. 2016) and the postharvest processing method (Worku et al. 2020). Several studies have shown that the higher cultivation altitude and longer spontaneous fermentation of coffee beans at the postharvest method, then the lower the caffeine content of coffee beans (Mubarok et al. 2014; Girma et al. 2020). Endogenous microorganisms decreased the caffeine content of coffee beans during spontaneous fermentation. The dominant endogenous microorganisms of coffee berries were yeast and lactic acid bacteria (Evangelista et al. 2015). Junqueira et al. (2019) reported that lactic acid bacteria in Colombian coffee berries were dominated by the genera *Leuconostoc* and *Lactobacillus*, whereas the yeasts were dominated by the genera *Pichia* and *Candida*. In addition, the temperature roasting of coffee beans could also affect the caffeine content of coffee beans.

Microbial fermentation of coffee beans improved coffee beans' sensory characteristics, then increased coffee brew acceptance by consumers. Evangelista et al. (2014) used yeasts *Candida parapsilosis*, *Pichia guilliermondii* and

Saccharomyces cerevisiae to improve the aroma quality of roasted coffee beans, whereas, Afriliani et al. (2019) used kefir microorganisms. *Luwak* civet was able to ferment coffee beans. Normal flora that lives in *Luwak*'s intestines fermented coffee beans and produce coffee beans with a unique aroma and taste. This unique aroma and taste of coffee beans were of higher economic value.

Caffeine has been reported to have an antimicrobial effect at high doses and could affect normal intestinal flora growth (Nonthakaew et al. 2015). Nonetheless, some microbes had the ability to degrade caffeine into its derivative, therefore, reducing the negative effects of caffeine. Lactic acid bacteria *L. casei* and *L. mesenteroides* and fungi *S. cerevisiae* and *R. oryzae* can reduce the caffeine content of robusta beans (Purwoko et al. 2022).

Caffeine derivatives as the result of caffeine degradation were dimethylxanthines (paraxanthine, theobromine and theophylline) and methylxanthines (1-methylxanthine, 3-methylxanthine and 7-methylxanthine). *Pseudomonas monteilii* were able to degrade caffeine into paraxanthine and theophylline (Arimurti et al. 2018). Fungi *Aspergillus fumigatus* and *A. niger* were able to degrade caffeine into theophylline and then transform into 3-methylxanthine (Hakil et al. 1998). Tagliari et al. (2003) reported that fungus *Rhizopus delemar* was also able to degrade caffeine into theophylline, then into 3-methylxanthine and finally into xanthine.

Previous research showed that *L. casei, L. mesenteroides, R. oryzae* and *S. cerevisiae* reduced levels in coffee green beans (Purwoko et al. 2022). However, there was no followed to detect compounds as the result of caffeine degradation. Therefore, the aim of this study aim was to determine the degradation pathway of caffeine degradation by food microorganisms i.e., *L. casei, L. mesenteroides, R. oryzae* and *S. cerevisiae*.

MATERIALS AND METHODS

Materials

Caffeine, theobromine, theophylline, paraxanthine, 1-methylxanthine, 3-methylxanthine and 7-methylxanthine were obtained from Sigma Aldrich, Germany. Nutrient Broth (NB) and Potato Dextrose Broth (PDB) were obtained from Merck Germany. Acetic acid, water and methanol chromatography grades were obtained from Merck Germany. *L. casei* FNCC 0090, *L. mesenteroides* FNCC 0023, *R. oryzae* FNCC 6011 and *S. cerevisiae* FNCC 3049 were obtained from FNCC PAU-UGM Yogyakarta, Indonesia.

Microbial culture preparation

Lactobacillus casei and *L. mesenteroides* were cultured on NB, whereas *S. cerevisiae* and *R. oryzae* were cultured on PDB. All microbial cultures were incubated at 28°C for 5 days and stored in the refrigerator at 4°C. Prior to use, the cultures were warmed at 30°C for 1 hour.

Microbial cultivation on media containing caffeine

Five milliliters (± $2x10^6$ cfu/mL) of *L. casei* and *L. mesenteroides* cultures were respectively added to 50 mL NB-caffeine (NB with 1.25 g/l caffeine), whereas 5 mL (± $2x10^6$ cfu/mL) *R. oryzae* and *S. cerevisiae* cultures were respectively added 50 mL PDB-caffeine (PDB with 1.25 g/L caffeine). Bacterial and fungal cultures were incubated in a shaking water bath (60 rpm; 30°C) for 4 days. Microbial cultures at 1st, 2nd, 3rd and 4th days of incubation and control (media without microbial cultures) were sampled for further analysis.

Sample preparation for High Performance Liquid Chromatography (HPLC) analysis

Samples (2 mL) of microbial cultures on 1st, 2nd, 3rd and 4th days of incubation and control samples were centrifuged (12,000 g; 4°C) for 10 minutes and the filtrates were collected. The filtrates were then filtered twice with #42 Whatman paper. Standard caffeine compound and caffeine derivates compounds (theobromine, theophylline, paraxanthine, 1-methylxanthine, 3-methylxanthine and 7-methylxanthine) were dissolved in 50% methanol. Prior to HPLC analysis, all samples were filtered using HPLC filters.

HPLC analysis

The HPLC instrument was the Waters Separations Module e2695 (USA) with a C-18 column (Reliant; 150x4.6mm; 5µm). The elution solvents were methanol, acetic acid and water (15:5:80, v/v). HPLC analysis was started by injecting 20 µL sample into the column and the isocratic elution was carried out for 10 minutes at speed of 0.7 mL/min. The compounds were detected by UV-Vis Waters 2489 detector (USA) at 272nm. The column was washed with methanol for 10 minutes before being reused for the next analysis. Retention time of caffeine and caffeine derivate compounds of samples were compared with the standard compounds. The concentration of caffeine and caffeine derivates were analyzed by comparing the peak area of the samples and the standard compounds.

Data analysis

Caffeine and caffeine derivates from samples of microbial cultures at 1st, 2nd, 3rd and 4th days of incubation were analyzed with One-way ANOVA to obtain caffeine degradation activities. Furthermore, microbial caffeine degradation pathways were reconstructed.

RESULTS AND DISCUSSION

Retention times of standard compounds were 2.571, 3.481, 5,126 and 6.866 minutes for caffeine, 7-methyl-xanthine, 1-methylxanthine and paraxanthine. However, retention time of sample compounds were 2.506-2.635, 3.454-3.562, 5.004-5.065 and 6.716-7.141 minutes for caffeine, 7-methylxanthine, 1-methylxanthine and para-xanthine. The differences in retention time between the standard compounds and the sample compounds were 2.5%, 1.9%, 2.4% and 4% for caffeine, 7-methylxanthine,

1-methylxanthine and paraxanthin, respectively. Dolan (2014) reported 2% retention time changes for 1°C change in column temperature. This research used room temperature to control column temperature of HPLC and samples analysis was carried out for several days. Therefore, retention time of caffeine derivates of samples differed from standard caffeine derivates.

This research showed that *L. casei, L. mesenteroides, S. cerevisiae* and *R. oryzae,* grew on media containing caffeine. Meanwhile, caffeine has antibacterial (Pruthviraj et al. 2011; Nonthakaew et al. 2015) and antifungal effects (Nonthakaew et al. 2015). Muslim and Dephinto (2019) added that extract of coffee leaves has antibacterial effect. Nonthakaew et al. (2015) reported that bacterial and fungal growth was affected by caffeine at 2 and 5 mg/mL, whereas caffeine in this research was 1.25 mg/mL. Therefore, in this research, all food microorganisms were not affected by caffeine and grew on media containing caffeine. The Intestinal microflora of mammals could degrade caffeine (Mukhtar et al. 2021). Lactic acid bacteria and yeasts are members of the mammalian intestinal microflora and were also used for production of fermented foods, including fermented coffee beans. This research showed that *L. casei, L. mesenteroides, S. cerevisiae* and *R. oryzae* can degrade caffeine on media containing caffeine.

Lactobacillus casei, L. mesenteroides, S. cerevisiae and *R. Oryzae,* were able to degrade caffeine into dimethyl-xanthine and then methylxanthine (Figure 1). There are 3 types of dimethylxanthine, i.e., paraxanthine, theobromine and theophylline. However, food microorganisms in this research mostly transformed caffeine into paraxanthine. Mazzafera et al. (1996) reported that bacterium *Serratia marcescens* could to degrade caffeine into paraxanthine and theobromine. Mills et al. (2021) reported there are two N-demethylase enzymes found in bacterium *Pseudomonas putida* CBB5 i.e., N-demethylase A and N-demethylase B, that removes methyl group at N-1 and N-3. Since *L. casei, L. mesenteroides, S. cerevisiae* and *R. oryzae* were able to degrade caffeine into paraxanthine, therefore, they might be producing caffeine demethylase (N-demethylase B) to remove methyl group at N-3 of caffeine and produce paraxanthine.

Lactobacillus casei, L. mesenteroides, S. cerevisiae and *R. Oryzae,* were quickly degrading caffeine into dimethyl-xanthine might be due to toxicity of caffeine. Szlapinski et al. 2023 reported that paraxanthine toxicity was lower than animal caffeine They also reported that high doses of paraxanthine did not cause death in animals. Meanwhile, Adamafio (2013) reported that livestock treated with theobromine died. However, preference of para-xanthine instead of theobromine and theophylline might be due to ability to synthesis a specific N-demethylase.

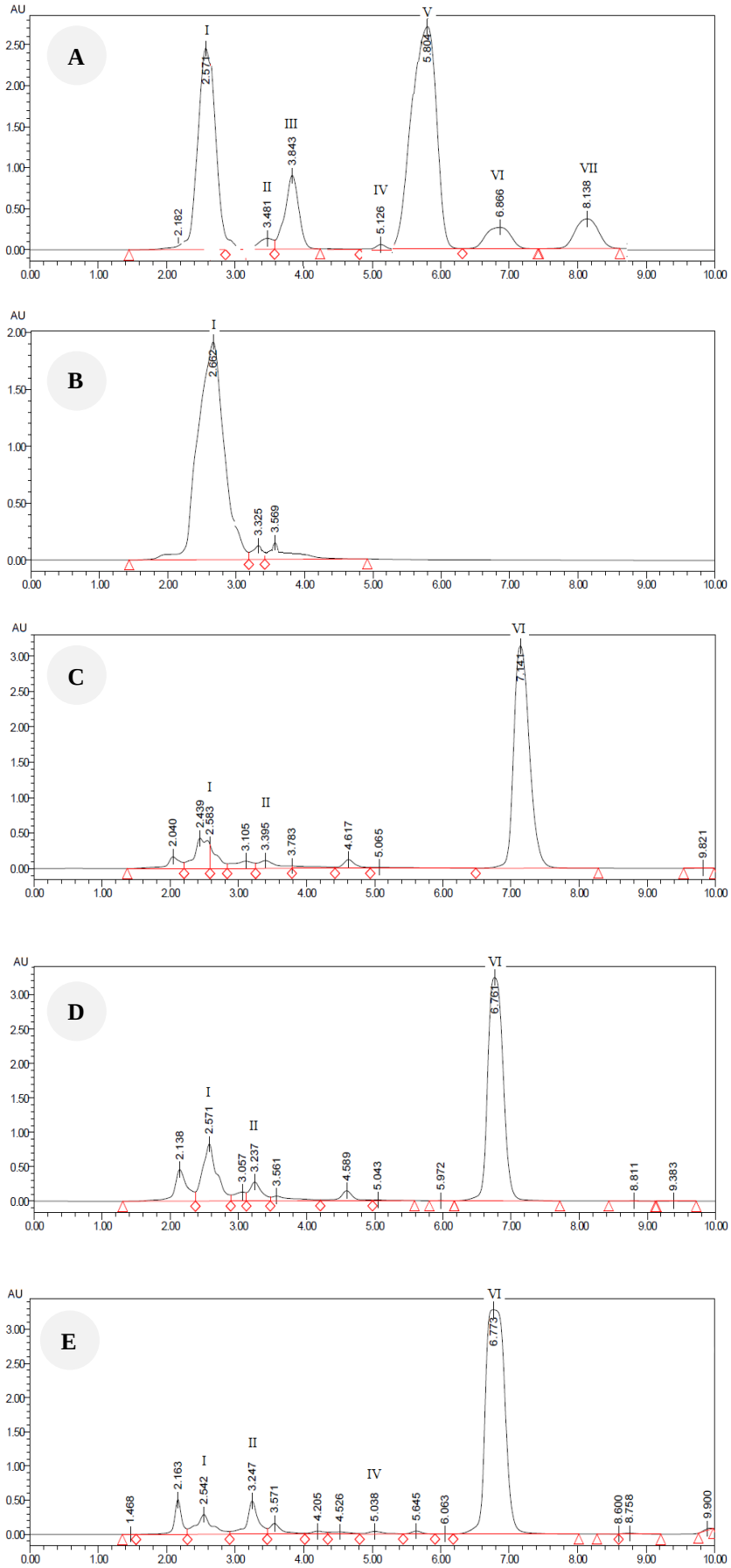
Paraxanthine was demethylated at N-7 and produced 1-methylxanthine, whereas demethylation at N-1 produced 7-methylxanthine. Previous studies reported that small amount of 1-methylxanthine and 7-methylxanthine were detected (Hakil et al. 1998). Our research also showed that *L. casei* and *L. mesenteroides* were able to degrade paraxanthine into 1-methylxanthine at small amounts as well as *R. oryzae* and *S. cerevisiae* were able to degrade paraxanthine into 7-methylxanthine at small amounts too. Small amounts of methylxanthines were probably due to the low toxicity of paraxanthine. Since paraxanthine has low toxicity, food microorganisms slightly degrade paraxanthine into 1-methylxanthine or 7-methylxanthine. It could be assumed that demethylation of caffeine by food microorganisms intended to reduced antimicrobial effect of caffeine. Antimicrobial effect of caffeine caused by inability to repair DNA damage when treated with caffeine (Alao et al. 2020) or inability to reduce stressor effect of oxidants, irradiation and toxic compounds when treated with caffeine (Chung 2021).

Bacteria and fungi able to degrade caffeine into paraxanthine. However, there were different degradation and production time of caffeine and paraxanthine respectively. Bacteria and fungi could degrade caffeine since 1st day of incubation (Figures 2 and 3). Bacteria *L. casei* and *L. mesenteroides* and yeast *S. cerevisiae* produced paraxanthine since 2nd day of incubation. However, fungus *R. oryzae* produced paraxanthine since 1st day of incubation (Figures 2 and 3). This suggested there was different caffeine adaptation between *R. oryzae* and *L. casei, L. mesenteroides* and *S. cerevisiae*. *R. oryzae* more adaptive by synthesized caffeine demethylase, earlier than *L. casei, L. mesenteroides* and *S. cerevisiae*. Therefore, *R. oryzae* produced paraxanthine earlier than *L. casei, L. mesenteroides* and *S. cerevisiae*.

Bacteria and fungi also showed different product of paraxanthine degradation. Bacteria. *L. casei* and *L. mesenteroides* degraded paraxanthine into 1-methylxanthine (Figures 1.C and 1.D), whereas fungi *R. oryzae* and *S. cerevisiae* degraded paraxanthine into 7-methylxanthine (Figures 1.E and 1.F). There was variation in selection of the methyl group by paraxanthine demethylase. Bacteria preferred to release N-7 methyl group to produced 1-methylxanthine, whereas fungi preferred to release N-1 methyl group to produce 7-methylxanthine. *L. casei, L. mesenteroides, S. cerevisiae* and *R. oryzae* were degrading most of caffeine into paraxanthine. However, they were degrading small amount of paraxanthine into methylxanthine. This was due to strain of microorganisms. *L. casei, L. mesenteroides, S. cerevisiae* and *R. oryzae* were type strains that usually use for food fermentation and they could not use caffeine as sole carbon and nitrogen source. Therefore, paraxanthine levels on media NB-caffeine and PDB-caffeine remained high.

Caffeine degradation by *Lactobacillus casei*

Lactobacillus casei was able to degrade caffeine into paraxanthine during 4th day of incubation on NB-caffeine. *L. casei* was able to degrade 15.8, 1150.5, 1195.8 and 1206 µg/mL caffeine on 1st, 2nd, 3rd and 4th days of incubation respectively (Figure 2). *Lactobacillus casei* was able to degrade 96.6% of caffeine during 4th day of incubation. *Lactobacillus casei* was degrading 1145.3 µg/mL caffeine during 2nd day of incubation. Caffeine content at 2nd, 3rd and 4th days of *L. casei* incubation on NB-caffeine were not significantly different ($p<0.05$). This showed that the caffeine degradation by *L. casei* on NB-caffeine reached a maximum on the 2nd day of incubation.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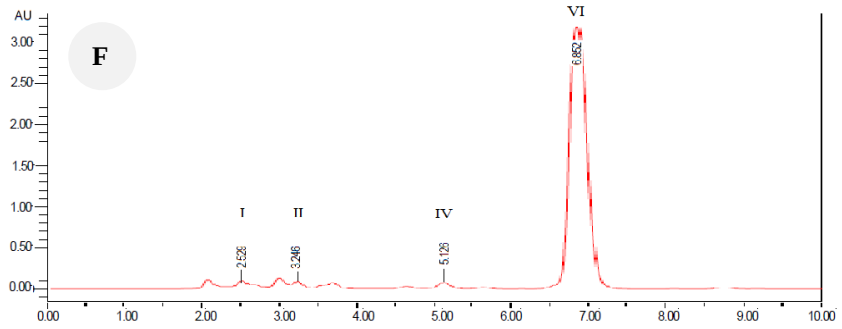

Figure 1. A. Chromatogram of standard, B. control, C. *L. casei* on NB-caffeine at 4th day of incubation, D. *L. mesenteroides* NB-caffeine at 4th day of incubation, E. *R. oryzae* on PDB-caffeine at 4th day of incubation, and F. *S. cerevisiae* on PDB-caffeine at 4th day of incubation samples and showed I. caffeine, II. 7-methylxanthine, III. 3-methylxanthine, IV. 1-methylxanthine, V. theobromine, VI. Paraxanthine, and VII. theophylline compounds

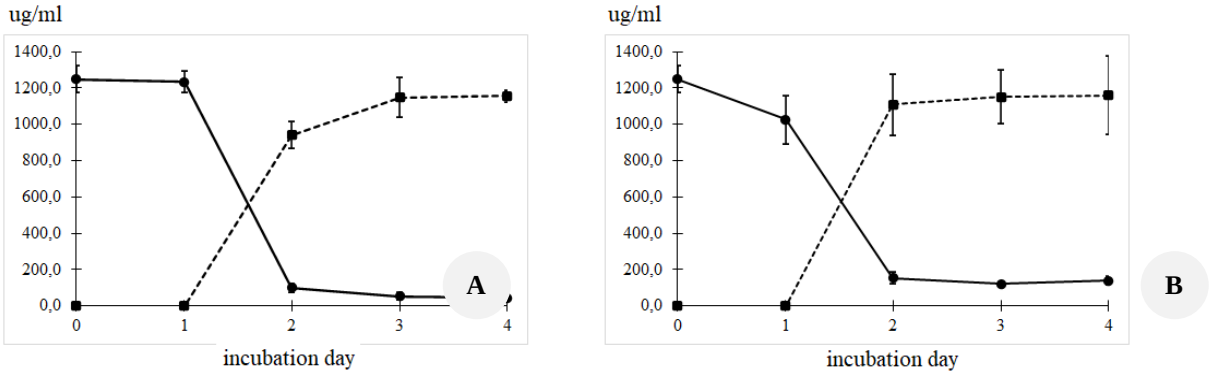

Figure 2. Caffeine degradation (−●−) into paraxanthine (--■--) by A. *L. casei* and B. *L. mesenteroides* on NB-caffeine

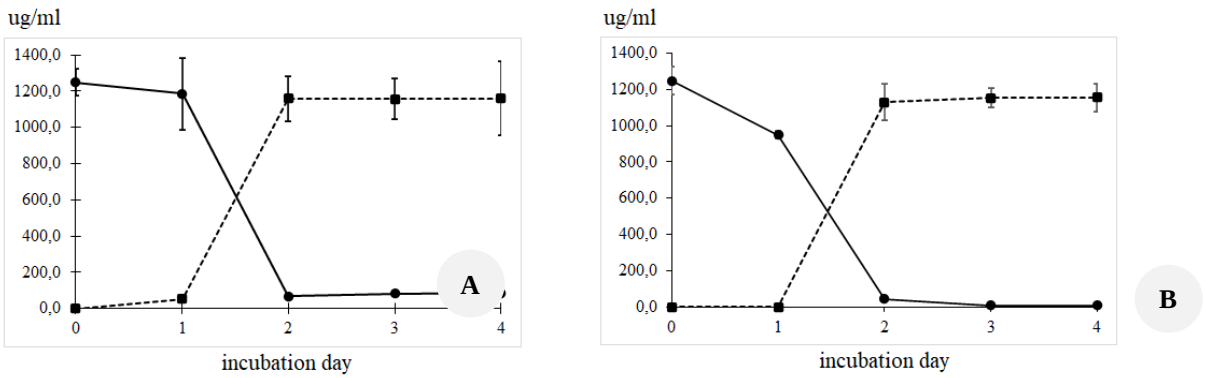

Figure 3. Caffeine degradation (−●−) into paraxanthine (--■--) by A. *Rhizopus oryzae* and B. *Saccharomyces cerevisiae* (right) on PDB-caffeine

Paraxanthine was not detected during 1st day of *L. casei* incubation, however at 2nd day of incubation was detected. This showed caffeine degradation by *L. casei* on 1st day of incubation was not directly produced by paraxanthine. *L. casei* produced 941.4, 1146.7 and 1155.6 µg/mL paraxanthine on 2nd, 3rd and 4th days of incubation respectively (Figure 2). Paraxanthine content at 2nd, 3rd and 4th days of *L. casei* incubation was not significantly different ($p<0.05$). This showed that paraxanthine production by *L. casei* on NB-caffeine reached maximum at 2nd day of incubation. Small amounts of paraxanthine were degraded by *L. casei* into 1-methylxanthine instead of 7-methylxanthine (Figure 1.C). The paraxanthine demethylase of *L. casei* preferred N-7 methyl group instead of N-1 methyl group to be released from paraxanthine. Therefore, 1-methylxanthine was detected in *L. casei* on NB-caffeine sample.

Degradation of caffeine by *Leuconostoc mesenteroides*

Leuconostoc mesenteroides were also able to degrade caffeine to paraxanthine during 4th day of incubation on NB-caffeine. *L. mesenteroides* was able to degrade 224.1, 1094.5, 1129.2 and 1111.3 µg/mL caffeine at 1st 2nd, 3rd and 4th days of incubation (Figure 2). *Leuconostoc mesenteroides* were able to degrade 89.0% of caffeine during 4 days incubation. *Leuconostoc mesenteroides* were degrading 870.4 µg/mL caffeine during 2nd day of incubation. Caffeine content at 2nd, 3rd and 4th days *L. mesenteroides* incubation on NB-caffeine were also not significantly different ($p<0.05$). This also showed that caffeine degradation by *L. mesenteroides* on NB-caffeine reached maximum at 2nd day of incubation.

Paraxanthine was not detected during 1st day of *L. mesenteroides* incubation, however at 2nd day of incubation was detected. *L. mesenteroides* produced 1107.8, 1151.3 and 1159.4 µg/mL paraxanthine at 2nd, 3rd and 4th days of incubation respectively (Figure 2). Paraxanthine content at 2nd, 3rd and 4th days of *L. mesenteroides* incubation were not significantly different ($p<0.05$). This also showed that *L. mesenteroides* on NB-caffeine reached maximum at 2nd day of incubation.

Small amounts of paraxanthine were degraded into 1-methylxanthine by *L. mesenteroides* (Figure 1.D). This also showed paraxanthine demethylase of *L. mesenteroides* preferred N-7 methyl group instead of N-1 methyl group to be released from paraxanthine. Therefore, 7-methylxanthine was not detected in *L. mesenteroides* on NB-caffeine sample.

Caffeine degradation by *Rhizopus oryzae*

Rhizopus oryzae was able to degrade caffeine into paraxanthine during 4 days of incubation on PDB-caffeine. *R. oryzae* degraded 63.1, 1180.4, 1167.9 and 1163.7 µg/mL caffeine at 1st, 2nd, 3rd and 4th day of incubation on PDB-caffeine (Figure 3). During 4th day of incubation, *R. oryzae* was able to degrade 93.2% of caffeine. *R. oryzae* was degrading 1117.3 µg/mL caffeine during 2nd day of incubation. Caffeine content at 2nd, 3rd and 4th days of *R. oryzae* incubation on PDB-caffeine were not significantly different ($p<0.05$). This also showed that caffeine degradation by *R. oryzae* on PDB-caffeine reached maximum at 2nd days of incubation.

Paraxanthine was detected at 1st day of *R. oryzae* incubation on PDB-caffeine. Caffeine demethylase of *R. oryzae* might be more active than caffeine demethylase of other food microorganisms. *R. oryzae* on PDB-caffeine, produced 52.4, 1158.8, 1157.2 and 1159.3 µg/mL paraxanthine at 1st, 2nd, 3rd and 4th days of incubation (Figure 3). Paraxanthine content at 2nd, 3rd and 4th days of *R. oryzae* incubation on PDB-caffeine were not significantly different ($p<0.05$). This showed paraxanthine production by *R. oryzae* was maximum at 2nd day of incubation on PDB-caffeine.

Small amounts of paraxanthine were degraded by *R. oryzae* into 7-methylxanthine instead of 1-methylxanthine (Figure 1.E). This showed that the paraxanthine demethylase of *R. oryzae* preferred N-1 methyl group instead of N-7 methyl group to be released from paraxanthine, therefore 7-methylxanthine was detected in *R. oryzae* on PDB-caffeine sample.

Caffeine degradation by *Saccharomyces cerevisiae*

Saccharomyces cerevisiae was able to degrade caffeine into paraxanthine during 4th day incubation on PDB-caffeine. *Saccharomyces cerevisiae* degraded 298.9, 1205.6, 1241.5 and 1241.9 µg/mL caffeine at 1st, 2nd, 3rd and 4th days of incubation on PDB-caffeine respectively (Figure 3). During 4th day of incubation, *S. cerevisiae* was able to degrade 99.4% of caffeine and this was highest compared to other food microorganisms. S. *cerevisiae* was degrading 906.7 µg/mL caffeine during 2nd day of incubation. Caffeine content on PDB-caffeine at 2nd, 3rd and 4th days of *S. cerevisiae* incubation were not significantly different ($p<0.05$). This also showed that caffeine degradation by *S. cerevisiae* on PDB-caffeine also reached maximum at 2nd day of incubation.

Paraxanthine was not detected during 1st day of *S. cerevisiae* incubation, however detected at 2nd day of *S. cerevisiae* incubation on PDB-caffeine. *S. cerevisiae* produced 1130.3, 1154.3 1154.8 µg/mL paraxanthine at 2nd, 3rd and 4th days of incubation on PDB-caffeine respectively (Figure 3). Paraxanthine content on PDB-caffeine at 2nd, 3rd and 4th days of *S. cerevisiae* incubation was not significantly different ($p<0.05$). This showed that paraxanthine production by *S. cerevisiae* reached maximum at 2nd days of incubation on PDB-caffeine. `

Small amounts of paraxanthine were degraded by *S. cerevisiae* into 7-methylxanthine instead of 1-methylxanthine (Figure 1.E). This showed the paraxanthine demethylase of *S. cerevisiae* preferred N-1 methyl group instead of N-7 methyl group to be released from paraxanthine, therefore 1-methylxanthine was not detected in *S. cerevisiae* on PDB-caffeine sample.

Caffeine degradation pathway

There are two modes of caffeine degradation by microorganisms, i.e., N-demethylation and C8-oxidation. N-demethylation mode produced dimethylxanthine whereas C8-oxidation mode produced trimethyl uric acid (Summers et al. 2015). Previous research reported that bacteria and fungi degraded caffeine in N-demethylation mode and produced dimethylxanthines (Mazzafera et al. 1996; Hakil et al. 1998). Bacteria degraded caffeine into theophylline and paraxanthine (Mazzafera 2004; Ibrahim et al. 2014) or into theobromine and paraxanthine (Summers et al. 2015). However, this research showed bacteria *L. casei* and *L. mesenteroides* were degrading caffeine into paraxanthine. Previous research showed fungi *Aspergillus* spp. and *Penicillium* spp. degraded caffeine into theophylline (Hakil et al. 1998; Zhou et al. 2018). However, this research showed that fungi *R. oryzae* and *S. cerevisiae* were degrading caffeine into paraxanthine. This research showed that *L. casei, L. mesenteroides, R. oryzae* and *S. cerevisiae* were degrading caffeine into paraxanthine instead of theobromine or theophylline in N-demethylation mode. Since they produced paraxanthine, they synthesized caffeine demethylase for caffeine degradation.

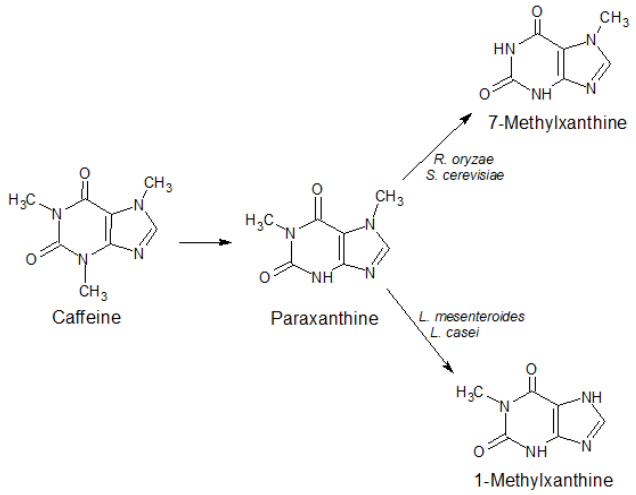

Figure 4. Caffeine degradation pathway by *Lactobacillus casei*, *Leuconostoc mesenteroides*, *Rhizopus oryzae* and *Saccharomyces cerevisiae*

Lactobacillus casei, *L. mesenteroides*, *R. oryzae* and *S. cerevisiae* were degrading paraxanthine into small amounts methylxanthine. The degradation of paraxanthine into methylxanthine was carried out by paraxanthine demethylase. Previous research reported that bacteria demethylated paraxanthine into 7-methylxanthine (Mazzafera 2004), whereas fungi demethylated paraxanthine into 1-methylxanthine (Hakil et al. 1998). However, our research showed that bacteria *L. casei* and *L. mesenteroides* demethylated paraxanthine into 1-methylxanthine, whereas fungi *R. oryzae* and *S. cerevisiae* demethylated paraxanthine into 7-methylxanthine. Therefore, we proposed a caffeine degradation pathway by *L. casei*, *L. mesenteroides*, *R. oryzae* and *S. cerevisiae* (Figure 4). Bacteria *L. casei* and *L. mesenteroides* degraded caffeine into paraxanthine, then into 1-methylxanthine. Whereas, fungi *R. oryzae* and *S. cerevisiae* degraded caffeine into paraxanthine, then into 7-methylxanthine.

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