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The crucial role of yeasts in the wet fermentation of coffee beans and quality

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ABSTRACT

The objective of this study was to investigate the role of yeasts in the wet fermentation of coffee beans and their contribution to coffee quality using a novel approach. Natamycin (300 ppm) was added to the fermentation mass to suppress yeast growth and their metabolic activities, and the resultant microbial ecology, bean chemistry and sensory quality were analyzed and compared to non-treated spontaneous fermentation we reported previously. The yeast community was dominated by *Hanseniaspora uvarum* and *Pichia kudriavzevii* and grew to a maximum population of about 5.5 log CFU/g in the absence of Natamycin, while when Natamycin was added yeasts were suppressed. The major bacterial species in both the spontaneous and yeast-suppressed fermentations included the lactic acid bacteria *Leuconostoc mesenteroides* and *Lactococcus lactis*, the acetic acid bacteria *Gluconobacter cerinus* and *Acetobacter persici* and the *Enterobacteriaceae Enterobacter*, *Citrobacter* and *Erwinia*. For both fermentations, the mucilage layers were completely degraded by the end of the process and the absence of yeast activities had no significant impact on mucilage degradation. During fermentation, reducing sugars were consumed while lactic acid was accumulated inside the beans, and its concentration was significantly higher in the spontaneous fermentation (3 times) than that where yeasts were suppressed by Natamycin. Glycerol was detected with a concentration of 0.08% in the absence of Natamycin and was not identified when Natamycin was added. Green beans fermented with yeast growth contained a higher amount of isoamyl alcohol (21 times), ethanol (3.7 times), acetaldehyde (8 times), and ethyl acetate (25 times) compared to beans fermented in the absence of yeast activities, which remained higher in the former after roasting. Beans fermented without yeast activities had a mild fruity aroma, and lower sensory scores of fragrances (7.0), flavor (6.5), acidity (6.3), body (7.0) and overall score (6.5) compared to the former. These findings demonstrated the crucial roles of yeasts in wet fermentation of coffee beans and for producing high quality coffee.

1. Introduction

Coffee is one of the most widely consumed beverages worldwide and a main cash crop grown in over 50 countries including Brazil, Vietnam, Indonesia, Colombia and Mexico (International Coffee Organization, 2018). After harvest, coffee cherries are processed with one of the three main methods, dry, wet, semidry processes. In the dry process whole cherries are traditionally solar dried for up to 30 days to reach a moisture content of 12%, then mechanically crushed to separate the beans from the outer layers (Silva et al., 2008; Silva et al., 2000). In the wet process, coffee cherries are first pulped to remove the skin and most of the mucilage layers, leaving beans with some remaining mucilage attached. Subsequently, the beans were subjected to either wet fermentation or mechanical de-mucilage process to completely remove the mucilage and then dried to 12% moisture content. The dried coffee beans are further processed to remove the hull and then roasted (Brando and Brando, 2014; Silva et al., 2008). In the semidry process, harvested fruits are de-pulped like the wet processing and then subjected to sun drying for up to 15 days (Brando, 2010; Silva et al., 2000).

Over the last decades, studies have shown that the microbial ecology of the wet fermentation of coffee beans involves a diverse range of microbial groups including lactic acid bacteria (LAB), acetic acid bacteria (AAB), *Bacillus*, *Enterobacteriaceae*, yeasts and filamentous fungi. Microorganisms identified in fermentations conducted at different locations around the globe have both common species and region-specific ones (de Oliveira Junqueira et al., 2019; Elhalis et al., 2020; Masoud et al., 2004; Pereira et al., 2014; Silva et al., 2008; Vaughn et al., 1958; Vilela et al., 2010). The roles of each microbial group in coffee fermentations have been discussed in a recent review (Pereira et al., 2017). LABs and yeasts are believed to have the abilities to degrade macromolecules such as polysaccharides and are responsible for removing the mucilage layer surrounding the beans during the

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fermentation. Moreover, these microorganisms have been reported to produce secondary metabolites such as esters, alcohols, ketones and other aromatic compounds that might contribute to coffee flavor and aroma (Avallone et al., 2002; Avallone et al., 2001). Other identified microorganisms such as *Erwinia*, *Klebsiella*, *Bacillus*, and *Escherichia* were also reported to contribute to the mucilage degradation process in the wet fermentation (Agate and Bhat, 1966; Masoud and Jespersen, 2006; Pereira et al., 2014; Silva, 2014). However, the evidence that supports the contribution of these microorganisms to mucilage degradation and coffee flavor and aroma is not conclusive and direct evidence identifying the role of individual microbial groups is lacking. Overall, studies conducted to investigate the contribution of the different microbial groups to coffee fermentation and bean and coffee quality, especially coffee volatiles, are limited, and the contribution of groups such as yeast to coffee quality is poorly understood.

Currently, the coffee fermentation process is still conducted worldwide in uncontrolled and spontaneous processes that produce coffee beans with inconsistent and unpredictable quality. To transfer coffee fermentation into a more controlled industrial process using a defined starter culture, it is essential to understand the contribution of individual microbial groups and species to the fermentation process and quality of coffee beans. In a previous study, we proved the essential contribution of overall microbial activities in the spontaneous wet fermentation of coffee beans to the production of coffee of high sensory quality (Elhalis et al., 2020). The objective of this study was to investigate the role of yeasts as a group in coffee wet fermentation and their contribution to coffee sensory quality. To achieve the objective, we used an innovative approach by adding Natamycin, a food-grade anti-fungal agent, to the fermentation to suppress the growth and activities of yeasts and comparing the resultant microbial metabolism, volatiles and coffee quality with those from uncontrolled spontaneous fermentation reported previously (Elhalis et al., 2020).

2. Materials and methods

2.1. 2.1Fermentation of coffee beans

Coffee cherries (*Coffea arabica* var. Bourbon, 40 kg) were harvested in October 2018 from the Kahawa Estate Coffee farm in Teven, NSW, Australia (latitude and longitude coordinates, −28.816667, 153.500000; altitude, 6.6 m above sea level) and immediately placed in iceboxes and airfreighted to the University of New South Wales in Sydney. Once arrived, the coffee cherries were de-pulped manually and subjected to wet fermentation underwater, with the whole process from harvest to the start of fermentation completed in 12 h. The de-pulping process was performed by packing about 200 g cherries in sterile plastic bags, which were squeezed to remove the skins; after that the beans were collected using sterile gloves. The de-pulped beans were placed into four boxes, each containing 3.5 kg of beans, and two of which were used to conduct spontaneous fermentation where the beans were immersed underwater for 36 h, as performed in the local practice as described in Elhalis et al. (2020). Another two boxes of beans were fermented in the same way except that Natamycin was added to a final concentration of 300 ppm to suppress yeast growth (Natamycin treated fermentation). Periodic mixings were performed every 6 h, and the environmental temperature was 25–30 °C and 10–15 °C during day and night, respectively, with an average of 18 °C. Mucilage degradation was monitored during fermentation by rubbing the parchments against each other between fingers and feeling texture changes from slippery to grittiness (Lin, 2010). Samples (100 g) were taken from the fermentation masses at the start and every 12 h and were analyzed immediately for viable microbial counts. Another 100 g was collected at each sampling time, immediately frozen (−20 °C) and later used for direct microbial DNA extraction and chemical analysis.

2.2. Drying, roasting and sensory analysis

After fermentation, the beans were washed three times and dried in a laboratory-scale air dryer at 45 °C with periodic mixing. Moisture content of the bean was monitored (Baggenstoss et al., 2008) during drying to reach 12% dry base. The dried beans were dehulled mechanically using a laboratory dehuller. Roasting was performed using a laboratory roaster (IKAWA, London, England) for 7 min at 225 °C and ground using a grinder (Mahlkonig's EK43, Revesby, Australia) individually. Sensory analysis was conducted by three expert coffee testers with Q Grader coffee certifications. The panel assessed the coffee brewed from the beans for fragrance, flavor, acidity, body, balance, sweetness, aftertaste, clean cup, uniformity, overall perception, which were added together to calculate the total score of the coffee.

2.3. Microbial ecology of coffee fermentation

2.3.1. Enumeration and identification by culture dependent method

Details of the culture-dependent method used for the identification and enumeration of the microorganisms in the coffee fermentation are given in Elhalis et al. (2020). Briefly, aerobic bacteria were enumerated using plate count agar containing cycloheximide (0.1%) to suppress fungal growth. Lactic acid bacteria were enumerated using de Man Rogosa Sharpe (MRS) and M17 agars with 0.2% of cycloheximide. Malt extract agar (MEA) containing oxytetracycline and chloramphenicol 50 mg/L each, to suppress bacteria growth, and Dichloran Rose Bengal Chloramphenicol Agar (DRBC) were used to enumerate fungi. All culture plates were incubated aerobically at 30 °C except for LAB where the plates were incubated anaerobically in candle jars. The agar plates were incubated at 30 °C for 48 h for bacteria and up to 7 days for yeasts and filamentous fungi. Three colonies from each type based on the colony and cell morphology were purified at least 4 times by streaking on their respective agar plates and used for identification by a combination of phenotypic and molecular-based methods. DNA extraction was conducted from the purified isolates following (Cocolin et al., 2002) and the concentration and purity of the extracted DNA were examined by a UV-1800 UV–Vis Spectrophotometer (Shimadzu Scientific Instruments) at UV 260 and 280 nm, respectively. The bacterial DNA was amplified using F338fgc (5′ GAC TCC TAC GGG AGG CAG CAG 3′) and R518 (5′ATT ACC GCG GCT GCT GG3′) primers targeting V3 region of the 16S rDNA gene (Ovreas et al., 1997). The yeast DNA was amplified using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers targeting the 5.8S-ITS rDNA gene region (Esteve-Zarzoso et al., 1999). The PCR products were assessed by electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. The respective forward primers (9.6 pmol) for each bacteria and yeasts were added to the PCR products and sent to the Ramaciotti Centre for Genomics at UNSW (Kensington, Sydney, NSW, Australia) for sequencing. The sequencing results were analyzed using the available data in Gen Bank of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/).

2.3.2. Analysis by culture-independent method

Details of the culture-independent method used were given in Elhalis et al. (2020). Briefly, direct extraction of DNA from the samples was done by a three-step procedure to improve the extraction efficiency and total recovery, which were enzyme lysis, chemical lysis and sonic breakage of cells, followed by protein digestion and purification. After measurement of DNA concentration, the extracted DNA was sent to the AGRF empowering Australian Genomic for amplicon profile diversity identification. 341F (5′-CCTACGGGNGGCWGCAG-3′) - 806R (5′-GGACTACHVGGGTWTCTAA T-3′) primers targeting V4 region of the 16SrRNA gene and ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) ITS2 (5′-GGCTGCGTTCTTCATCGATGC-3′) primers targeting ITS region were used for amplification of bacterial and yeast DNA, respectively. DNA extraction and amplification were performed in duplicate and the

results were reported as an average.

2.4. Chemical analysis

The pH of the fermentation liquid was measured using a portable pH meter (pH Cube, TPS Pty Ltd., Brisbane, QLD, Australia) every 12 h. The pH of ground coffee beans was determined following Angelucci et al. (1982). The concentration of sugars, organic acids, glycerol, and mannitol in the mucilage and endosperm of coffee bean were determined using the HPLC methods described in Elhalis et al. (2020). Each analysis was done in duplicate and average values were reported within a standard deviation of 0.05.

Volatiles were determined in fresh de-pulped beans, fermented green beans and roasted beans according to the solid phase micro extraction gas chromatography mass spectrometry (SPME-GC–MS) method described in Frank et al. (2017). Briefly, coffee bean samples (2 g) were placed in 15 ml hermetically sealed vials which were arranged into the autosampler injectors (AOC-5000, Shimadzu, Rydalmere, Australia). Equilibration and extraction were conducted at 40 °C for 40 min using a 1 cm 50/30 μm DVB/Carboxen/PDMS 23-gauge, 2 cm SPME fibers (Supelco, Sigma-Aldrich, Castle Hill, Australia). Subsequently, the fiber was desorbed into a 250 °C injector for 5 min in spitless mode. Volatile analysis was performed with a GC–MS system (QP-2010-Plus GC–MS, Shimadzu, Rydalmere, Australia) using a Sol-Gel Wax column (SGE, Ringwood, Australia, 30 m, 0.25 i.d, 0.25 μm film). The temperature program used was 35 °C for 5 min initially then heated to 250 °C (5 °C/min). Volatiles were detected by scanning the mass range of *m/z* 40–250 and 4-methylpentynol was used as internal standard. Linear retention indices were calculated using a standard alkane mix (C7-C40, Supleco, Sigma-Aldrich, Castle Hill, Australia). Identification of volatile compounds was performed by matching the mass spectra with those of reference compounds in the NIST mass spectral library and by comparing linear retention indices (LRI) with published values in the NIST and PubChem websites. In some cases, identities were confirmed using reference standards. The concentration of volatiles was calculated semi-quantitatively using the Shimadzu proprietary software "LabSolutions" (Version 2.53). The analysis was performed in triplicate for each sample.

2.5. Statistical analysis

One-way ANOVA was carried out to compare means between sample treatments and Tukey's HSD post-hoc test was used to separate the means. Differences were statistically significant at $p < 0.05$. Statistical analyses were performed with GenStat® (16th Edition, VSN International, Hemel Hempstead, UK).

3. Results

3.1. Microbial ecology during coffee fermentation monitored by traditional plating method

The microbial ecology of the spontaneous fermentation was published previously by us in Elhalis et al. (2020). Key findings and results were sometimes mentioned here to facilitate comparison. In total, 17 bacterial and 6 yeast species were detected in the fermentation process (Figs. 1, 2, 3). The initial total aerobic bacterial count (TABC) was about 5 log CFU/g, which grew to 5.3 log CFU/g in 12 h and eventually 7.5 log CFU/g at the end of the spontaneous fermentation as described in Elhalis et al. (2020). No significant differences in the TABC were detected between the spontaneous and Natamycin treated fermentations. In the beginning, the dominant aerobic bacterial species were *Acinetobacter* and *Enterobacter* in both fermentations. *Acinetobacter lwoffii* (accession no. MF988732.1) count was 4.5 log CFU/g which declined during the fermentation process to < 2 log CFU/g at the end in both fermentations. *Enterobacter* (accession no. KY474547.1) population at the start of the fermentation was similar to that of *Acinetobacter lwoffii* and started to increase after 12 h, reaching about 5 log in both types of fermentation in 24 h, and then remained relatively unchanged in the absence of Natamycin, while slightly increased in the presence of Natamycin. *Citrobacter* (accession no. CP026709.1) increased during the whole fermentation process and became the dominant isolates after 12 h and then reached a maximum population of about 7.4 log CFU/g after 36 h, in both fermentations. The level of *Erwinia soli* (accession no. MH107054.1) increased from < 2 log CFU/g to a maximum population of 3.5 log CFU/g, in both fermentations. The levels of *Klebsiella pneumoniae* (accession no. CP020847.1) and *Pseudomonas* sp. (accession no. EU822884.1) were both low at the beginning of the fermentation at about 2 log CFU/g and remained relatively unchanged during the whole fermentation process, for both fermentations. There were two main isolates of acetic acid bacteria detected, which were *Gluconobacter cerinus* (accession no. MK049954.1) and *Acetobacter persici* (accession no. MH285266.1). The initial level of *Gluconobacter* was about 2 log CFU/g which grew to a maximum population of 4 log CFU/g in 24 h, and then progressively declined to about 1.8 log CFU/g in the absence of Natamycin. The growth of *Gluconobacter* in Natamycin treated fermentation reached a maximum population of 5.2 log within 24 h and then decreased slightly at the end. *Acetobacter* was present at lower counts than those of *Gluconobacter* and remained largely unchanged in the absence of Natamycin, while where Natamycin was added it increased slightly within 24 h to a maximum population of 3.2 log CFU/g, then subsequently declined to < 2 log CFU/g at the end. The total lactic acid bacteria count was about log 3.2 CFU/g at the start of the fermentation and increased at 12 h to reach 4.5 and 5 log CFU/g in the absence and presence of Natamycin, respectively. The population subsequently increased in both fermentations to a maximum of 5.3 and 5.8 log CFU/g within 24 h and then dropped to 5.2 and 5.4 log CFU/g in the absence and presence of Natamycin at the end, respectively. Two isolates of LAB were detected, namely *Leuconostoc mesenteroides* (accession no. MK290364.1) and *Lactococcus lactis* (accession no. MF623273.1) with an initial population of log 3.1 and 2.6 CFU/g, respectively. *Leuconostoc mesenteroides* grew to a maximum population of 5.6 and 5.1 log CFU/g within 24 h in the presence and absence of Natamycin, respectively, and then declined. Similarly, *Lactococcus lactis* grew to a maximum population of 5.1 and 4.8 within 24 h in the presence and absence of Natamycin, respectively, before dropped at the end (Fig. 2). Overall, the TABC in the spontaneous and Natamycin treated fermentations were not significantly different, demonstrating that Natamycin did not alter the bacterial ecology in the fermentation.

The initial total yeast count was approximately 4 log CFU/g which grew to reach 5.5 log CFU/g within 36 h in the absence of Natamycin. When Natamycin was added, the total yeast population was relatively unchanged during the first 24 h of the fermentation, then started to decline, reaching 3.5 log CFU/g by the end of the fermentation (Fig. 3). The predominate yeasts presented at the start of the fermentation was *Hanseniaspora uvarum* (accession no. MF574306.1) with an initial population of 3.6 log CFU/g, followed by *Pichia kudriavzevii* (accession no. CP021092.1) at 3.1 log CFU/g, and *Candida railenensis* (accession no. HQ438305.1) at 3.6 log CFU/g, in both fermentations, which subsequently grew or declined to (5.2, 2.8), (4.6, 2.7), and (3, 1.5) log CFU/g at the end, respectively, with the values in parenthesis corresponding to the yeast population in the absence and presence of Natamycin, respectively. *Candida xylopsoci* (accession no. KJ706861.1) was present with an initial population of 2 log CFU/g which increased slightly during the fermentation in the absence of Natamycin. *Pichia fermentans* (accession no. KM402059.1) and *Wickerhamomyces anomalus* (accession no. KY587120.1) were the remaining two yeasts with an initial population < 2 log and remained largely unchanged in the first 24 h and subsequently dropped at the end. The populations of these yeasts, however, declined during the fermentation progress to < 2 log CFU/g after 24 h in the presence of Natamycin.

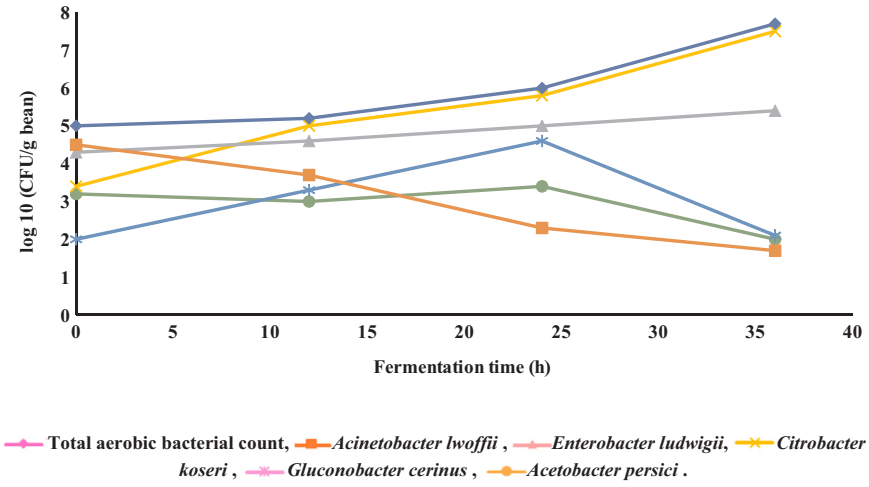

Fig. 1. Changes in the population of aerobic bacteria during Natamycin treated fermentations of coffee beans.

3.2. Evolution of microbial community during coffee fermentation analyzed by culture-independent methods

The average numbers of raw V3–V4 and internal transcribed spacer (ITS1) sequences per sample were about 350,000 and 200,000 sequences, respectively, with coverage ranged between 98.3 and 99.7%. At the start of the fermentation the bacterial operational taxonomic units (OTUs) mostly belonged to *Enterobacteriaceae* (such as *Serratia*, *Enterobacter* and *Erwinia*) and AAB (such as *Gluconobacter*, *Citrobacter* and *Swaminathania*). A small portion of the reads were LAB, such as *Leuconostoc*, *lactococus*, and *Enterococcus*. The remaining reads were mostly coffee DNA and soil associated bacteria.

During fermentation, the OUTs of *Enterobacteriaceae* decreased in relative abundance. In contrast, LAB increased in the relative abundance where *leuconostic* and *lactococus* were the predominant reads in the first 24 h, but their abundance decreased by the end of fermentation in the absence of Natamycin. The OUTs of LAB read showed an increase in relative abundance in the presence of Natamycin compared to spontaneous fermentation. AABs showed a similar increase in the first 24 h, then declined slightly by the end of fermentation in the absence of Natamycin. Similar pattern of change in AABs also occurred in the Natamycin treated fermentation; however, the abundance of AABs was higher compared to spontaneous fermentation. Other bacterial OTUs appeared mainly at the start of fermentation, and were sporadically detected with relatively low abundance during the whole fermentation process, such as *Paenibacillaceae* (*Saccharibacillus*), *Pseudomonadaceae* (especially *Pseudomonas*) *Nocardiaceae* (*Rhodococcus*) and *Methylobacteriaceae* (*Methylobacterium*), in both fermentations (Fig. 4).

Regarding the fugal community, members of both Ascomycota and Basidiomycota were dominant in the fermentation process. At the beginning of fermentation, a high relative abundance of unclassified *Nectriacea* was identified in the first 24 h, which then decreased with fermentation in the absence of Natamycin. Other members of Ascomycota such as *Penicillium*, *Aspergillus*, *Cladosporium*, *Pleosrales* and *Neodevriesia*, and Basidiomycota members such as *Rhodotorula*, *Leucospridiella*, *Sporidiobolus* and *Vishnicozyma* were observed only at the initial stage and then virtually disappeared by the end of fermentation in the absence of Natamycin. The abundance of these fungal members was dramatically decreased in the presence of Natamycin as shown in Fig. 4. OUTs yeast reads increased steadily with fermentation in the absence of Natamycin, with *H. uvarum* being the most prevalent, followed by *Candida* and *Wickerhamomyces* in the absence of Natamycin. A significant decrease in the yeast abundance was observed in the presence of Natamycin. *Debaryomyces* was also present with low abundance at the start and slightly increased within 24 h, but subsequently disappeared at the end of fermentation in the absence of Natamycin, while this yeast was only present at start of fermentation in the presence of Natamycin but disappeared after fermentation began (Fig. 4).

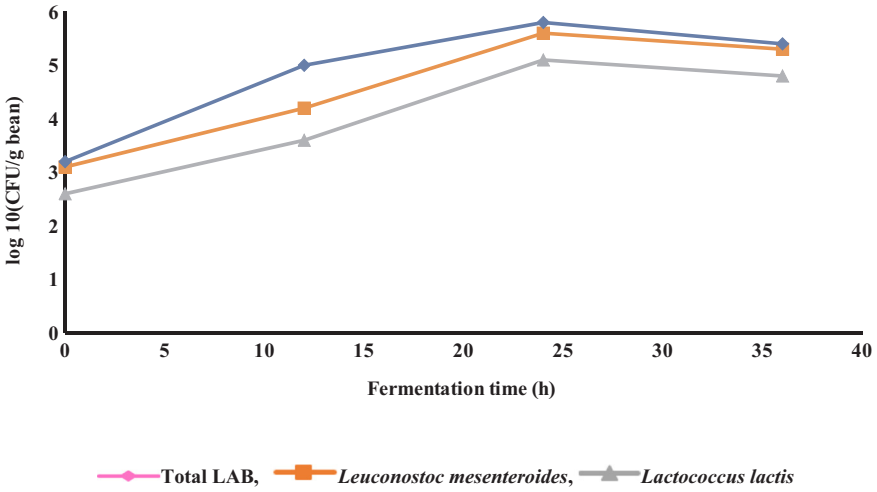

Fig. 2. Changes in the population of lactic acid bacteria during Natamycin treated fermentations of coffee beans.

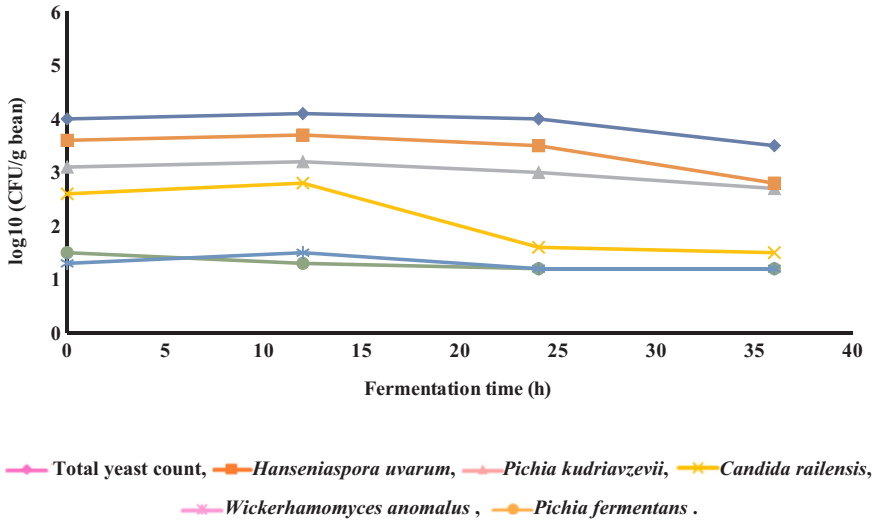

Fig. 3. Changes in the population of yeasts during Natamycin treated fermentations of coffee beans.

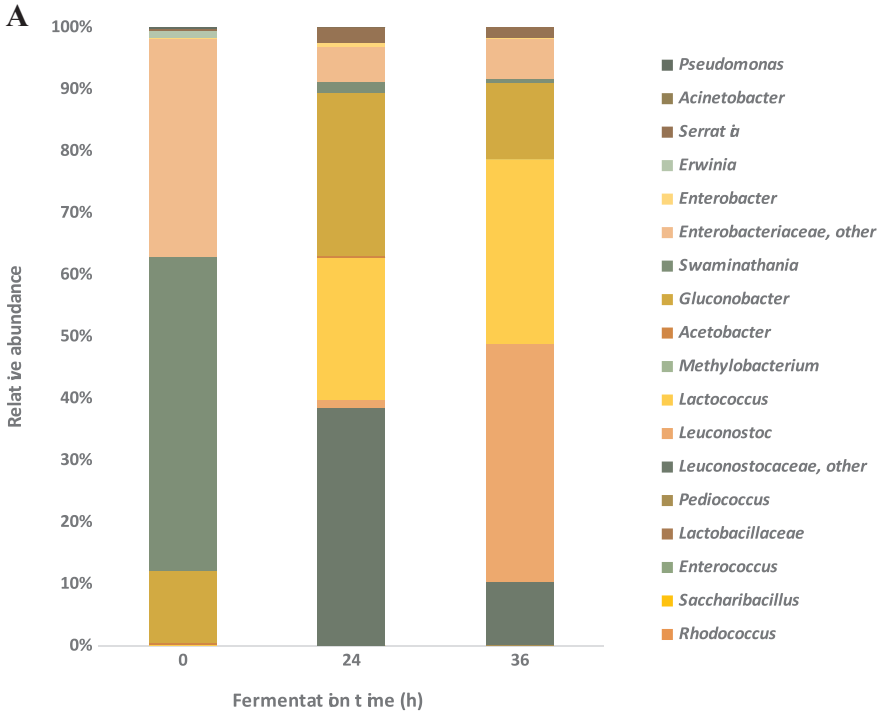

Fig. 4. a. Changes in the relative abundance of bacterial operational taxonomic units (OUTs) in coffee beans during Natamycin treated fermentations of coffee beans. b. Changes in the relative abundance of fungal operational taxonomic units (OUTs) in coffee beans during Natamycin treated fermentations of coffee beans.

3.3. Chemical changes during coffee fermentation

Changes in pH, sugars, organic acids and sugar alcohols during the spontaneous fermentation were published previously by us in Elhalis et al. (2020). Key findings and results were sometimes mentioned here to facilitate comparison.

3.3.1. pH

The initial pH of the fermentation mass was about 5.4 in both fermentations, which gradually dropped to 3.6 and 3.8 at the end of fermentation in the absence and presence of Natamycin, respectively. The pH value of unfermented beans was 6.5, which declined during fermentation to 4.8 and 5.1 in the absence and presence of Natamycin ($p < 0.05$), respectively.

3.3.2. Carbohydrate and organic acid metabolism

Fig. 5 shows the changes in the concentration of non-volatile compounds in both the mucilage and endosperm during the fermentation. The mucilage initially contained three sugars, fructose (30%), glucose (21%) and sucrose (9%), and their levels declined during fermentations, Fig. 5. Sucrose was totally consumed by the end of fermentations, while that of glucose and fructose dropped to 9% and 4%, respectively, in the absence of Natamycin. In comparison, the sugar utilization rates were much slower in the presence of Natamycin, where the levels of sucrose, fructose and glucose declined to 5%, 12%, and 13% ($p < 0.05$), respectively, Fig. 5A. Four organic acids were detected in the mucilage before fermentation, which were malic, quinic, succinic and citric acids at 0.9–2%. During fermentation, the concentration of malic and citric acids dropped slightly in the first 16 h and then increased slightly by the end of fermentation in both fermentations. The concentration of

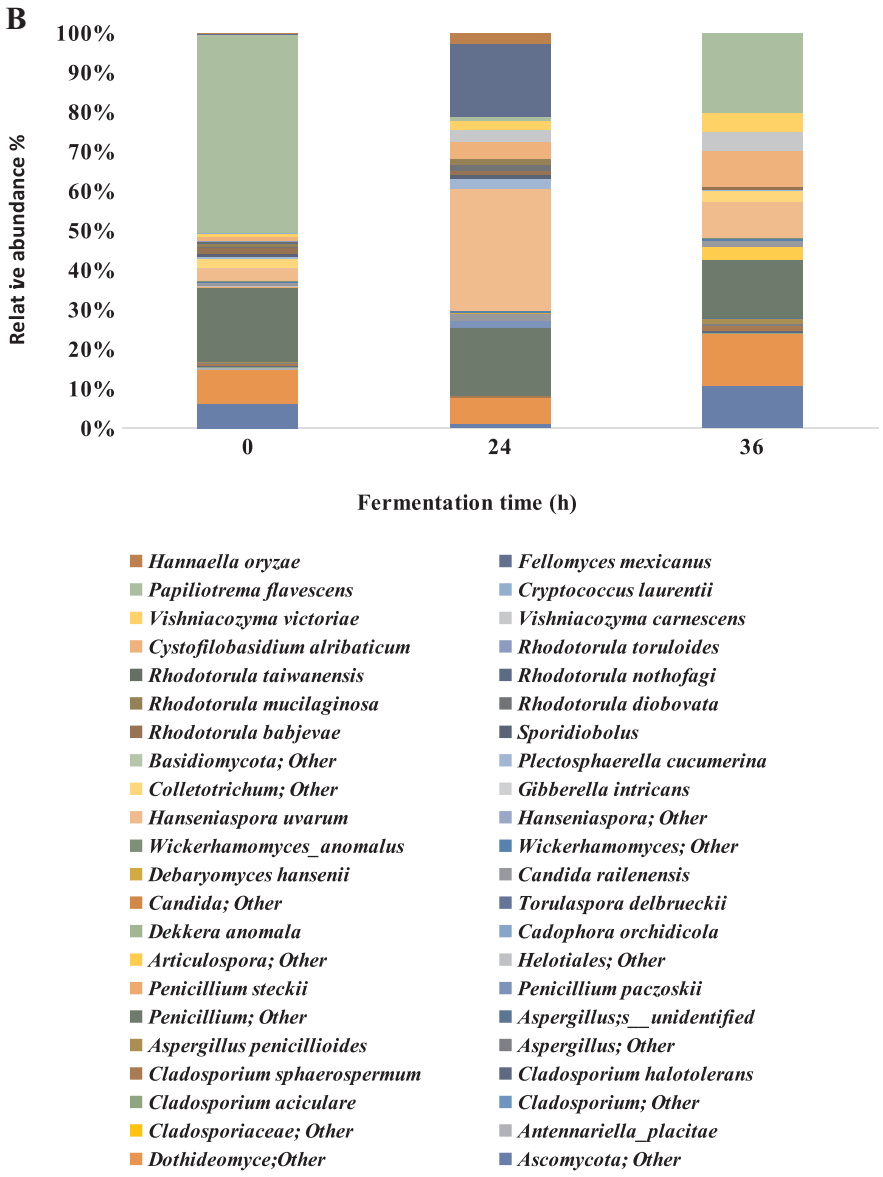

Fig. 4. (*continued*)

succinic and quinic acids also showed small variations during fermentation, but no significant differences were observed between the spontaneous and Natamycin treated fermentations ($p > 0.05$). Lactic acid was detected at 16 h and its level increased slowly, reaching 0.4% within 36 h in the absence of Natamycin, while it was detected only after 24 h when Natamycin was added with a maximum concentration of 0.2% ($p < 0.05$), Fig. 6A. Glycerol was detected at 24 h with a concentration of 0.9%, and then increased slowly to 1.2% at the end of fermentation in the absence of Natamycin, while it was not detected at any stage during fermentation in the presence of Natamycin. A low initial concentration of mannitol (0.5%) was found in the mucilage, which subsequently increased during the fermentation to a maximum concentration of 0.8% and 1.7% in the absence and presence of Natamycin ($p < 0.05$), respectively.

The endosperm showed much less changes in chemical composition compared to the mucilage layer. The initial levels of sugars in the endosperm were sucrose (8.6%), fructose (1.3%) and glucose (0.9%). During fermentation, the level of sucrose dropped to 5.7%, while fructose and glucose declined to about 0.8% in absence of Natamycin. These sugar concentrations were slightly higher in the Natamycin treated beans ($p < 0.05$) as shown in Fig. 5B. The endosperm contained, at the beginning, citric (1.4%), quinic (0.5%), malic (0.4%), and succinic acids (0.2%). During fermentation, the concentrations of citric acid increased to 1.8%, while malic acid dropped to 0.2%. The levels of succinic and quinic acids remained relatively stable during fermentation. The concentration of these organic acids and the magnitudes of changes during fermentation were not significantly different ($p > 0.05$) between beans undergoing spontaneous and Natamycin treated fermentations. Lactic acid was not detected in the endosperm at the start of fermentation, but it was detected during fermentation to reach 0.3% and 0.1% at the end in the absence and presence of Natamycin, respectively, Fig. 6B. The endosperm did not contain glycerol or mannitol initially, but they were detected as the fermentation progressed, reaching a maximum concentration of 0.08% and 0.1% in the absence of Natamycin, respectively. In the presence of Natamycin, glycerol was not detected, while mannitol was detected at 0.6 g/100 g at the end of the fermentation.

3.3.3. Volatile compounds

Over 79 volatiles were identified in coffee beans (Tables 1a & 1b). Volatiles were grouped, based on their chemical class, into alcohols, esters, aldehydes, ketones, organic acids, phenols, pyrazines,

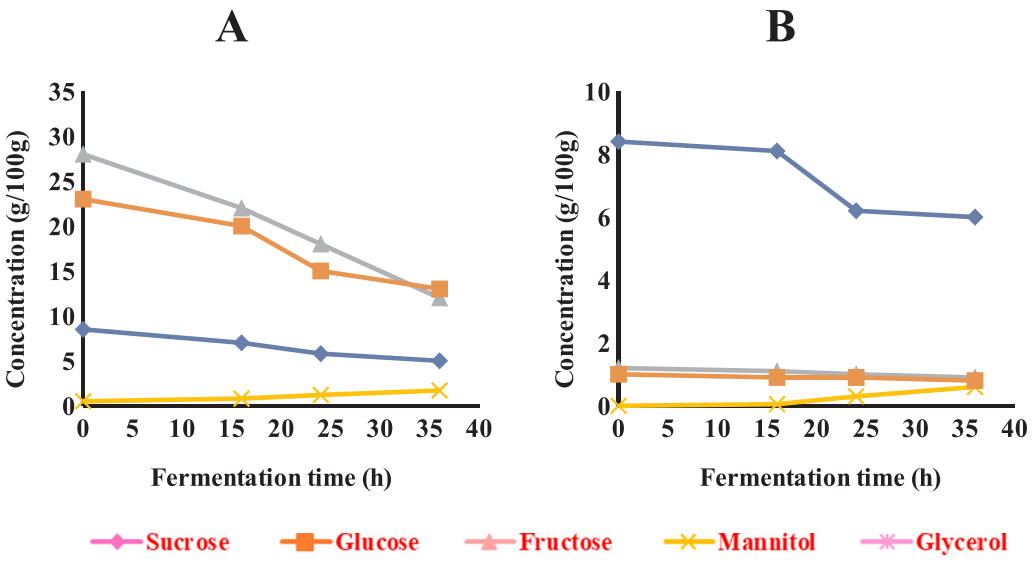

Fig. 5. Changes in the concentration of the sucrose, glucose, fructose, mannitol, and glycerol in the mucilage and endosperm during Natamycin treated fermentation of coffee beans.

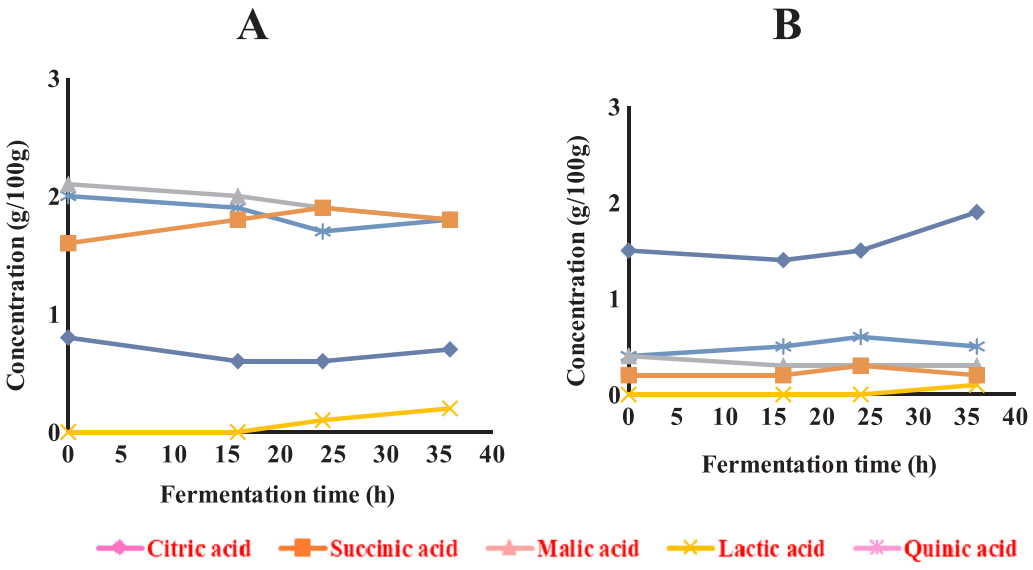

Fig. 6. Changes in the concentration of organic acids in the mucilage (A) and endosperm (B) of coffee beans during Natamycin treated fermentations of coffee beans. The reported data were the means of duplicate analyses with standard deviation ≤ 0.05.

heterocyclic nitrogen, and sulfur-containing compounds.

3.3.3.1. Alcohols. At the beginning of fermentation, there were eight main alcohols detected in the de-pulped green beans; which were ethanol, 2-methyl-3-buten-2-ol, isopentyl alcohol, isoamyl alcohol, 3-methyl-2-buten-1-ol, 1-hexanol, furfural alcohol, 1-nonanol, and phenyl ethyl alcohol, with a total concentration of 1021.9 μg/kg. During the fermentation, the total alcohol concentrations increased by about 3-fold in the absence of Natamycin and 2-fold in the presence of Natamycin ($p < 0.05$). The concentration of ethanol increased > 3-fold, isoamyl alcohol about 37 fold, 1-hexanol and 1-nonanol 3-fold, and 3-methyl-2-buten-1-ol 5-fold, while 2-methyl-3-buten-2-ol, phenyl ethyl alcohol and furfural alcohol remained relatively unchanged in the absence of Natamycin. The levels of ethanol in the green beans in the absence of Natamycin were more than three times higher than that in the presence of Natamycin ($p < 0.5$). Similarly, the levels of isoamyl alcohol and 1-hexnol were > 20 and 4 times higher in the absence of Natamycin, respectively ($p < 0.5$). No significant differences were detected in the remaining alcohols between the two types of beans ($p > 0.05$).

As expected, roasting caused significant decreases in the concentration of ethanol, 1-hexanol and isoamyl alcohol in both bean types; however, they remained higher in the beans obtained from the spontaneous fermentation than those from the Natamycin treated fermentation ($p < 0.5$). The concentration of furfural alcohol and 1-nonanol increased by about 10 times after roasting, while that of phenyl ethyl alcohol remained relatively unchanged in the absence of Natamycin. Furthermore, 6 additional alcohols were detected after roasting, which were not detected in the green beans, namely 1,4-pentanediol, 2-nonanol, 2,3-butanediol, 1,4-butanediol, 2,3-hexanediol, and 1,4-butanediol, with similar concentrations in both bean types ($p > 0.05$).

3.3.3.2. Aldehydes. Aldehydes were present in green beans at the beginning of fermentation with a total concentration of 933.8 μg/kg, which subsequently decreased during fermentation but increased after roasting. During fermentation, acetaldehyde concentration increased

Table 1a
Analytical parameters for volatiles in coffee beans.

Targeted volatiles	Functional group	*m/z*	LRI*	Sensory description	Identification method
Acetic acid	Acid	43	1458	Vinegar, pungent	L, MS, ST
Butanoic acid	Acid	61	1631	Onion like	L, MS, ST
Heptanoic acid	Acid	60	1974	Acidic	L, MS
Ethanol	Alcohol	45	948	Alcoholic	L, MS, ST
2-Methyl-3-buten-2-ol	Alcohol	71	1054	–	L, MS
Isopentyl alcohol	Alcohol	70	1219	–	L, MS
Isoamyl alcohol	Alcohol	55	1226	Banana and pear	L, MS, ST
3-Methyl-2-buten-1-ol	Alcohol	71	1330	Fruity	L, MS
3-Hexanol	Alcohol	72	1362	Fruity herbal fresh	L, MS, ST
2-Nonanol	Alcohol	45	1565	Herbal	L, MS
2,3-Butanediol	Alcohol	45	1585	Fruity, buttery	L, MS
Furfuryl alcohol	Alcohol	98	1651	Herbal	L, MS, ST
1-Nonanol	Alcohol	70	1652	Floral	L, MS
2,3-Hexanediol	Alcohol	73	1912	Floral	L, MS
Phenylethyl alcohol	Alcohol	91	1933	Floral, fruity	L, MS, ST
1,4-Butanediol	Alcohol	73	1937	Floral	L, MS
Acetaldehyde	Aldehyde	44	701	Fruity, pungent	L, MS, ST
3-Methyl butanal	Aldehyde	44	921	Coffee like	L, MS, ST
Hexanal	Aldehyde	44	1091	Green, fruity	L, MS, ST
3-Methyl-2-butenal	Aldehyde	84	1206	–	L, MS
(*E*)-2-Hexenal	Aldehyde	69	1224	Fatty	L, MS, ST
2,2-Dimethyl hexanal	Aldehyde	57	1456	–	L, MS
Furfural	Aldehyde	96	1469	Sweet, almond	L, MS
Benzaldehyde	Aldehyde	77	1534	Fatty almond	L, MS, ST
5-Methylfurfural,	Aldehyde	110	1581	Caramel, spice, maple	L, MS
Methyl formate	Ester	60	743	Fruity	L, MS
Methyl acetate	Ester	43	826	Fruity	L, MS
Ethyl acetate	Ester	43	893	Fruity	L, MS, ST
Methyl butanoate	Ester	74	992	Fruity	L, MS
Furfuryl formate	Ester	81	1498	Fruity	L, MS
Furfuryl acetate	Ester	81	1540	Fruity	L, MS
Furfural propionate	Ester	80	1602	–	L, MS
2-Methylfuran	Furan	82	867	Fruity, pungent	L, MS
2,5-Dimethylfuran	Furan	95	952	Nutty, caramel	L, MS
2-Vinylfuran	Furan	94	1082	Caramel, fatty	L, MS
2-Butyl furan	Furan	81	1130	–	L, MS
2-Allylfuran	Furan	108	1212	Fatty, nutty	L, MS
2-Pentylfuran	Furan	81	1236	–	L, MS
2-Acetylfuran	Furan	95	1511	Sweet, balsamic	L, MS
2-Propionylfuran	Furan	95	1584	Caramel, nutty	L, MS
2-Furfurylfuran	Furan	91	1612	Fatty, nutty, fruity	L, MS
2(5H)-Furanone	Furan	55	1786	Coffee-like	L, MS
2-(Methoxymethyl)tetrahydrofuran	Furan	71	2048	–	L, MS
2-Butanone	Ketone	43	908	Floral	L, MS
3-Pentanone	Ketone	57	982	–	L, MS, ST
2,3-Butanedione	Ketone	43	983	Oily buttery	L, MS
2,3-Pentanedione	Ketone	43	1070	Oily buttery, caramel	L, MS
2,3-Hexanedione	Ketone	43	1138	Buttery, sweet, creamy	L, MS
2,3-Heptanedione	Ketone	57	1153	Fruity	L, MS
1-Methylpyrrole	N-heterocycle	81	1146	Nutty	L, MS
2-Ethylpyrrole	N-heterocycle		1187	Coffee like	L, MS
Pyridine	N-heterocycle	79	1192	Sour, roasted, burnt	L, MS, ST
2-Methylpyridine	N-heterocycle	93	1229	Coffee-like	L, MS
2-Acetylpyridine	N-heterocycle	79	1611	Coffee-like	L, MS
2-Formyl-1-methylpyrrole	N-heterocycle	109	1625	Nutty, roasted	L, MS
N-acetyl-4(H)-pyridine	N-heterocycle	81	1694	Coffee-like	L, MS
2-Formyl pyrrole	N-heterocycle	81	1842	Nutty, hay-like	L, MS
2-Methoxy phenol (guaiacol)	Phenol	109	1921	Phenolic	L, MS, ST
Phenol (carbolic acid)	Phenol	66	2020	Smoky	L, MS
2-Methyl phenol	Phenol	107	2089	Spicy, sweet	L, MS, ST
2-Methoxy-4-vinylphenol	Phenol	135	2192	Clove	L, MS, ST
Pyrazine	Pyrazine	80	1218	Sweet peanuts	L, MS, ST
2-Methylpyrazine	Pyrazine	94	1270	Nutty	L, MS
2,5-Dimethyprazine	Pyrazine	108	1332	Nutty, roasted	L, MS
2,6-Dimethylpyrazin	Pyrazine	42	1338	Chocolate, nuts	L, MS
2-Ethylpyrazine	Pyrazine	107	1343	Peanut, butter	L, MS
2,3-Dimethylpyrazine	Pyrazine	67	1356	Roasted, nutty	L, MS
2-Ethyl-6-methylpyrazine	Pyrazine	121	1395	Hazelnut, fruity	L, MS
2-Ethyl-5-methylpyrazine	Pyrazine	121	1402	Flowery, hazelnut, fruity	L, MS
2-Ethyl-3-methylpyrazine	Pyrazine	42	1417	Coffee-like	L, MS
3-Ethyl-2,5-dimethylpyrazine	Pyrazine	135	1473	Roasted	L, MS
2-Acetylpyrazine	Pyrazine	43	1620	Roasted	L, MS
2-Acetyl-3-methylpyrazine	Pyrazine	43	1643	Roasted	L, MS
2-Methyl-5-(1-propenyl)pyrazine	Pyrazine	133	1709	Roasted	L, MS

(*continued on next page*)

Table 1a (*continued*)

Targeted volatiles	Functional group	*m/z*	LRI*	Sensory description	Identification method
Methanethiol	Sulfur	48	685	Freshness, cooked meat	L, MS
Dimethyl sulfide	Sulfur	62	744	–	L, MS
Dimethyl disulfide	Sulfur	94	1080	Cabbage-like, onion	L, MS
Dimethyl trisulfide	Sulfur	79	1379	Sulfur	L, MS
Furfuryl methyl sulfide	Sulfur	81	1494	Onion, garlic	L, MS
Bis-2-(furfuryl)-disulfide	Sulfur	81	1915	Meaty	L, MS

* LRI = linear retention index; identification method (MS = mass spectrum compared to NIST database, L = literature LRI; ST = standard compound). Literature LRI and sensory descriptors are taken from Czerny and Grosch (2000), Febrianto et al. (2016), Flament et al. (1968), Liu et al. (2019), Mottram (2005), Pereira et al. (2014), and Sunarharum et al. (2014).

about 24-fold, and 3-methyl butanal and benzaldehyde levels almost doubled in the absence of Natamycin. In comparison, the concentration of these volatiles was about 2–8 fold higher compared to unfermented green beans in the presence of Natamycin ($p < 0.5$). The level of hexanal, 3-methyl-2-butenal was relatively unchanged during fermentation in both bean types ($p > 0.5$). In contrast, the level of (*E*)-2-hexenal and 2,2-dimethyl hexanal declined significantly during fermentation, but no statistically significant differences were observed between the two fermentations ($p > 0.05$). After roasting, the total average concentration of aldehydes increased by > 12 times and was higher in roasted beans in the absence of Natamycin compared to the presence of Natamycin ($p < 0.05$). The levels of acetaldehyde, 3-methyl butanal, and benzaldehydes increased in the absence of Natamycin and their concentration more than doubled after roasting in beans fermented with the presence of Natamycin. In contrast, the levels of hexanal, 3-methyl-2-butenal, 2,2-dimethyl hexanal decreased while (*E*)-2-hexenal was not detected after roasting in both bean types with no significant differences ($p > 0.05$) between them. Furthermore, two additional aldehydes were detected in roasted beans, which were not identified in green beans, namely furfural and 5-methyl furfural with relatively high concentrations that were similar in both types of beans without significant differences ($p > 0.05$).

3.3.3.3. Esters. Four ester compounds, namely methyl formate, methyl acetate, ethyl acetate and methyl butanoate, were detected at low concentrations in the green beans at the start of the fermentation. The total concentrations of the esters increased by > 3-fold during fermentation, but then decreased after roasting in both bean types, where they did not show significant differences ($p > 0.05$). The levels of ethyl acetate increased massively from 35.1 to 312.5 μg/kg in the absence of Natamycin during fermentation, which was > 25-fold higher than those in fermented beans when Natamycin was added ($p < 0.05$). The concentration of methyl acetate increased by about 4-fold in both bean types where they did not show significant differences ($p > 0.05$), while the levels of methyl formate and methyl butanoate remained relatively unchanged during fermentation in the presence or absence of Natamycin. After roasting a hug decrease in ethyl acetate occurred, and the level dropped to 23.0 μg/kg in the beans with the absence of Natamycin and non-detectable in the presence of Natamycin. Furthermore, methyl butonate and methyl acetate concentrations dropped by about 4-fold, while methyl formate increased by about 7-fold after roasting in both bean types where they did not show significant differences ($p > 0.05$). In addition, three extra ester compounds, which were not observed in green beans, were detected after roasting, namely furfuryl formate, furfuryl propionate and furfuryl acetate, and the levels were significantly different between beans with or without the presence of Natamycin ($p > 0.05$).

3.3.3.4. Acids and phenols. The unfermented green beans contained three main organic acids, butanoic (65.3 μg/kg), acetic (56.2 μg/kg), and heptanoic (6.9 μg/kg) acids. The total concentration of organic acids increased by about 3-fold and 6-fold during fermentation in the absence and presence of Natamycin, respectively. The concentrations of acetic and butanoic acids were about 2–3 times higher in the presence of Natamycin than where it was absent ($p < 0.05$), while the heptanoic acid level remained relatively after fermentation in both samples ($p > 0.05$). As expected, after roasting the levels of these organic acids were decreased in both bean types; however, the concentration of acetic acid and butanoic acid remained higher in the presence of Natamycin than where it was absent ($p < 0.05$). Three main phenolic compounds, guaiacol (3.3, 1.6 μg/kg), 2-methyl phenol (57, 50 μg/kg) and 2- methoxy-4-vinylphenol (20, 21 μg/kg), were identified after roasting, which not detected in green beans; the values in parenthesis corresponded to the amount of compounds in roasted beans in the absence and presence of Natamycin, respectively, with no significant differences observed between the two types of beans ($p > 0.05$).

3.3.3.5. Ketones. Two ketones were present at the beginning of fermentation, which were 2-butanone (148.5 μg/kg) and 3-pentanone (207.5 μg/kg). After fermentation, the concentration of 2-butanone declined by about 3 fold, while 3-pentanone remained relatively unchanged in the absence of Natamycin. No significant differences in the concentration of these volatiles were detected in the presence of Natamycin ($p > 0.05$). After roasting, the concentration of 2-butanone increased 10 fold, while 3-pentanone slightly dropped in both bean types with no significant differences detected between them. Furthermore, 5 additional volatile ketones which were not identified in green beans were detected after roasting, 2,3-butanedione (356.0, 204.0 μg/kg), 2,3-pentanedione (454.0, 292.0 μg/kg), 2,3-hexanedione (6.1, 5.0 μg/kg) and 2,3-heptanedione (1.6, 1.3 μg/kg), with the values in parenthesis corresponding to the amount of volatiles in roasted beans in the absence and presence of Natamycin, respectively, with no statistical significance differences between the two types of beans ($p > 0.05$).

3.3.3.6. Pyrazines, N-heterocycles and furans. Pyrazines, pyridine, pyrroles, and furans were not detected in green beans during fermentation. They were mainly formed during roasting. A total of 27 compounds belonging to these groups were detected with 2,5-dimethylfuran, 2-methylpyrazine, 2-ethyl-6-methylpyrazine, pyridine, and 2-formyl-1-methylpyrrole being the most abundant. The concentrations of most of these volatiles were similar in beans fermented with or without the presence of Natamycin with no significant differences between them ($p > 0.05$).

3.3.3.7. Sulfides. One main sulfide compound was detected at the start of fermentation, which was dimethyl sulfide, and its concentration dropped during the fermentation in the absence of Natamycin. After roasting, dimethyl sulfide increased by > 3-fold in the absence of Natamycin. Furthermore, three additional compounds were detected, which were not present in the green beans, with dimethyl trisulfide being the most abundant, followed by methanethiol, and bis-2-(furfuryl)-disulfide. No significant differences in the concentration of these volatiles were detected between beans fermented in the absence or presence of Natamycin ($p > 0.05$).

Table 1b
Changes in the volatile concentrations in green and roasted fermented coffee beans.

Volatiles	Green beans			Roasted beans	
	Initial	Control	N. treated*	Control	N. treated
Acid					
Butanoic acid	65.3^{a}	13.2^{cA}	38.8^{bA}	2.9^{bB}	9.8^{aB}
Acetic acid	56.2^{c}	383.0^{bA}	690.3^{aA}	250.1^{aB}	388.6^{aB}
Heptanoic acid	6.9^{a}	17.7^{aA}	22.8^{aA}	5.4^{aB}	2.3^{aB}
Total acids	128.4^{cA}	413.9^{bA}	751.9^{aA}	258.4^{bB}	400.7^{aB}
Alcohols					
Ethanol	147.0^{b}	482.3^{aA}	129.5^{bA}	5.1^{aB}	2.0^{bB}
2-Methyl-3-buten-2-ol	16.2^{a}	17.6^{aA}	16.5^{aA}	74.9^{aB}	64.8^{Ab}
Isopentyl alcohol	466.1^{a}	446.8^{aA}	564.7^{aA}	63.9^{aB}	56.2^{aB}
Isoamyl alcohol	15.9^{b}	586.6^{aA}	27.1^{bA}	4.7^{B}	ND
3-Methyl-2-buten-1-ol,	240.3^{b}	1277.6^{aA}	1293.3^{aA}	123.1^{aB}	153.2^{aB}
1-Hexanol	93.0^{c}	259.6^{aA}	60.1^{bA}	13.0^{aB}	5.2^{bB}
2-Nonanol	ND	4.1^{aA}	2.9^{aA}	3.7^{aA}	2.0^{aA}
2,3 Butanediol	ND	ND	ND	39.5^{a}	37.3^{a}
Furfuryl alcohol	3.2^{aA}	1.6^{aA}	1.4^{aA}	150.1^{aB}	147.0^{aB}
1-Nonanol	12.1^{b}	39.0^{aA}	30.1^{aA}	303.8^{aB}	391.2^{aB}
2,3-Hexanediol	ND	ND	ND	58.9^{a}	46.9^{a}
Phenylethyl alcohol	28.0^{a}	29.4^{aA}	32.2^{aA}	19.7^{aB}	23.4^{aB}
1,4-Butanediol	ND	ND	ND	5.0^{a}	1.0^{b}
Total alcohols	1021.9^{cA}	3144.7^{aA}	2154.9^{bA}	865.4^{aB}	930.2^{aB}
Aldehyde					
Acetaldehyde	2.4^{c}	59.0^{aA}	7.5^{bA}	204.5^{aB}	78.6^{bB}
3-Methyl butanal	17.4^{c}	40.4^{aA}	20.2^{bA}	65.0^{aB}	19.2^{bA}
Hexanal	24.4^{a}	34.6^{aA}	25.1^{aA}	15.3^{aB}	12.3^{aB}
3-Methyl-2-butenal	66.3^{a}	57.1^{aA}	83.8^{aA}	29.3^{aB}	17.1^{aB}
2-Hexenal	461.5^{a}	30.0^{b}	28.9^{b}	ND	ND
2,2-Dimethyl hexanal	226.0^{a}	92.6^{bA}	75.1^{bA}	25.3^{aB}	26.2^{aB}
Furfural	ND	ND	ND	4933.2^{a}	4275.0^{a}
Benzaldehyde	135.8^{b}	221.90^{aA}	110.8^{bA}	320.7^{aB}	176.2^{bB}
5-Methyl furfural	ND	ND	ND	1294.2^{a}	1080.6^{a}
Total aldehydes	933.8^{aA}	535.6^{bA}	351.4^{cA}	6887.5^{aB}	5685.2^{bB}
Ester					
Methyl formate	13.4^{a}	14.1^{aA}	13.4^{aA}	87.7^{aB}	101.1^{aB}
Methyl acetate	704.8^{b}	2492.9^{aA}	3043.9^{aA}	487.4^{aB}	760.0^{aB}
Ethyl acetate	35.1^{b}	312.5^{aA}	12.3^{cA}	23.0^{B}	ND
Methyl butanoate	152.6^{a}	106.4^{aA}	110.2^{aA}	28.1^{aB}	14.9^{aB}
Furfuryl formate	ND	ND	ND	10.0^{a}	8.0^{a}
Furfuryl propionate	ND	ND	ND	144.0^{a}	104.3^{a}
Furfuryl acetate	ND	ND	ND	246.1^{a}	339.4^{a}
Furfural propionate	ND	ND	ND	89.1^{a}	68.9^{a}
Total esters	905.9^{b}	2925.9^{aA}	3179.8^{aA}	1115.4^{aB}	1396.6^{aB}
Furan					
2-Methyl furan	ND	ND	ND	119.2^{a}	178.7^{a}
2,5-Dimethyl furan	ND	ND	ND	4327.3^{a}	4095.7^{a}
2-Vinylfuran	ND	ND	ND	28.1^{a}	20.8^{a}
2-Butyl furan	ND	ND	ND	26.6^{a}	37.9^{a}
2(5H)-Furanone	ND	ND	ND	7.1^{a}	5.4^{a}
2-Allylfuran	ND	ND	ND	5.6^{a}	8.0^{a}
2-Pentylfuran	ND	ND	ND	325.5^{a}	312.0^{a}
2-Acetylfuran	ND	ND	ND	266.9^{a}	375.8^{a}
2-Propionylfuran	ND	ND	ND	199.6^{a}	116.3^{a}
2-Furfurylfuran	ND	ND	ND	8.6^{a}	13.1^{a}
Tetrahydro-2-(methoxymethyl)-furan	ND	ND	ND	11.8^{a}	7.3^{a}
Total furans	ND	ND	ND	5326.3^{a}	5171.0^{a}
Ketones					
2-Butanone	148.5^{a}	44.9^{bA}	38.9^{bA}	442.3^{aB}	400.8^{aB}
3-Pentanone	207.5^{a}	163.9^{aA}	169.8^{aA}	112.9^{aB}	114.6^{aB}
2,3-Butanedione	ND	ND	ND	356.0^{a}	204.0^{a}
2,3-Pentanedione	ND	ND	ND	454.0^{a}	292.0^{a}
2,3-Hexanedione	ND	ND	ND	6.1^{a}	5.0^{a}
2,3-Heptanedione	ND	ND	ND	1.6^{a}	1.3^{a}
Total ketones	356^{a}	208.8^{bA}	208.7^{bA}	1372.9^{aB}	1017.7^{aB}
N-heterocycle					
1-Methyl-pyrrole	ND	ND	ND	3.1^{a}	1.0^{a}
2 Ethyl-pyrrole	ND	ND	ND	14.0^{a}	12.2^{a}
Pyridine	ND	ND	ND	459.6^{a}	330.9^{a}
Pyridine, 2-methyl	ND	ND	ND	4.0^{a}	3.4^{a}
2-Acetylpyridine	ND	ND	ND	6.2^{a}	6.5^{a}
2-Formyl-1-methylpyrrole	ND	ND	ND	325.0^{a}	369.9^{a}

(*continued on next page*)

Table 1b (*continued*)

Volatiles	Green beans			Roasted beans	
	Initial	Control	N. treated*	Control	N. treated
4(H)-Pyridine,*N*-acetyl-	ND	ND	ND	56.9^{a}	65.7^{a}
2-Formyl pyrrole	ND	ND	ND	136.8^{a}	161.2^{a}
Total N-heterocycles	ND	ND	ND	1005.6^{a}	950.8^{a}
Phenol					
2 Methoxy phenol (guaiacol)	ND	ND	ND	3.3^{a}	1.6^{a}
Phenol	91.4^{a}	47.3^{aA}	53.4^{aA}	75.2^{aA}	58.6^{aA}
2-Methyl phenol	ND	ND	ND	57.2^{a}	50.3^{a}
2-Methoxy-4-vinylphenol	ND	ND	ND	19.5^{a}	20.9^{a}
Total phenols	91.4^{a}	47.3^{aA}	53.4^{aA}	155.2^{aB}	131.4^{aB}
Pyrazine					
Pyrazine	ND	ND	ND	267.3^{a}	183.4^{a}
2-Methylpyrazine	ND	ND	ND	2910.5^{a}	2531.4^{a}
2,5-Dimethypyrazine	ND	ND	ND	1184.1^{a}	1242.1^{a}
2,6-Dimethylpyrazine	ND	ND	ND	380.1^{a}	197.9^{a}
2-Ethylpyrazine	ND	ND	ND	62.5^{a}	74.8^{a}
2,3-Dimethylpyrazine	ND	ND	ND	375.1^{a}	445.1^{a}
2-Ethyl-6-methylpyrazine	ND	ND	ND	752.9^{a}	714.9^{a}
2-Ethyl-5-methylpyrazine	ND	ND	ND	403.0^{a}	382.6^{a}
2-Ethyl-3-methylpyrazine	ND	ND	ND	272.2^{a}	253.2^{a}
3-Ethyl-2,5-dimethylpyrazine	ND	ND	ND	96.9^{a}	116.1^{a}
2-Acetylpyrazine	ND	ND	ND	2.7^{a}	4.2^{a}
2-Acetyl-3-methylpyrazine	ND	ND	ND	18.9^{a}	19.6^{a}
2-Methyl-5-(1 propenyl)pyrazine	ND	ND	ND	6.8^{a}	5.7^{a}
Total pyrazines	ND	ND	ND	6733.0^{a}	6171.0^{a}
Sulfide					
Methanethiol	ND	ND	ND	13.7^{a}	15.8^{a}
Dimethyl sulfide	11.9^{a}	5.8^{bA}	4.2^{bA}	39.9^{aB}	31.3^{aB}
Dimethyl disulfide	ND	ND	ND	16.7^{a}	32.6^{a}
Dimethyl trisulfide	ND	ND	ND	27.1^{a}	29.0^{a}
Furfuryl methyl sulfide	ND	ND	ND	19.7^{a}	30.9^{a}
Bis-2-(furfuryl)-disulfide	ND	ND	ND	5.1^{a}	8.5^{a}
Total sulfides	11.9^{a}	5.8^{bA}	4.2^{bA}	122.2^{aB}	148.1^{aB}

Values are of three replicates. Mean values in each row with different lower-case letters (a–c) indicate significant differences among the green beans or the roasted beans. Mean values in the same row with different upper-case letters (A, B) indicate significant differences between the green beans and roasted beans. Control, spontaneous fermentation without treatments. ND, not detected.

* N. treated, Natamycin treated.

3.3.4. Sensory evaluation of coffee beverages

No significant difference ($p > 0.05$) was detected by the Q-graded coffee certified panel between coffee brewed from fermented beans in the presence and absence of Natamycin for sweetness, balance, clean up and uniformity. However, coffee from beans fermented in the absence of Natamycin was rated with significantly higher scores for fragrance, flavor, acidity, body and overall score compared with beans fermented in the presence of Natamycin ($p < 0.05$). In contrast, a higher score for aftertaste was awarded to the latter (in the presence of Natamycin) than the former (Fig. 7). The panel reported yellow grape and apple fruity aroma, vanilla, peanut, chocolate notes and smooth body for coffee from beans from the spontaneous fermentation, while mild fruity and trace cocoa flavor notes characterized coffee from beans fermented in the presence of Natamycin.

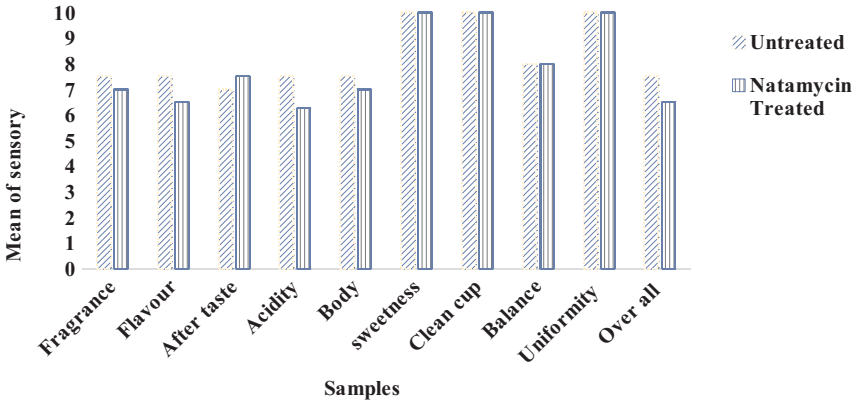

Fig. 7. Mean scores of cup test by three Q-graded coffee certified panelists for coffee brewed from beans produced by spontaneous and Natamycin treated fermentation (mean ± standard deviation).

4. Discussion

4.1. Microbial ecology

Analysis by both culture-dependent and independent methods showed that yeasts, LAB, AAB and *Enterobacteriaceae* grew in the laboratory scale wet spontaneous fermentation of coffee beans, similar to results reported in previous studies (Agate and Bhat, 1966; Masoud et al., 2004; Pereira et al., 2014; Silva, 2014). Furthermore, results by both methods proved that adding Natamycin to the fermentation mass selectively suppressed yeast growth and their metabolic activities without significantly altering bacterial growth and diversity. Inhibition of yeast growth during coffee fermentation without significantly impacting on bacterial growth could help evaluate the role of yeast in the fermentation and their contribution to coffee quality. Natamycin is a well-known food-grade antifungal agent used to control fungal spoilage in many foods at concentrations up to 2000 ppm (Delves-Broughton et al., 2005). Preliminary experiments using different concentrations of Natamycin were first tried and 300 ppm was chosen, as this was the lowest concentration where yeast growth was suppressed under the experimental conditions and no significant changes occurred to bacterial growth. Similar findings have been reported elsewhere (Ho et al., 2014).

As reported in our previous study, in the spontaneous fermentation, the population of yeasts grew by > 10 times; the level of LAB declined slightly, while AAB increased initially and then declined (Elhalis et al., 2020). In the fermentation with added Natamycin, the growth of yeasts declined from the start and dropped to non-detectable levels by 24 h. The species and growth patterns of LAB and AAB in the Natamycin treated fermentation were similar to those in the spontaneous fermentation, but the populations were higher in the former. Similar findings on LAB and AAB growth pattern in spontaneous coffee fermentation have been reported elsewhere (Avallone et al., 2001; De Bruyn et al., 2017; Evangelista et al., 2015; Ribeiro et al., 2017; Zhang et al., 2019). Bacteria (especially LAB) and yeasts are known to co-exist and co-operate in several fermented foods and beverages such as kefir, cheese, sourdough and wine (Alexandre et al., 2004; Cheirsilp et al., 2003; Corsetti et al., 2001; De Vuyst et al., 2010). Our results showed that with the absence of yeast growth and their metabolic activities, LABs and AABs grew to higher populations than that with the presence of yeasts. The likely cause of this higher bacterial population is the lesser competition from yeasts for nutrients and possible suppression of bacterial growth (especially LAB) by toxic yeast metabolites such as alcohol.

The predominant yeasts in the fermentation were *H. uvarum* and *Pichia kudriavzevii*, followed by *Candida*, *Wickerhamomyces* and *P. fermentans*. *H. uvarum* was detected during wet coffee fermentations in Tanzania and was the second most abundant yeast with an initial population of 6.2 log CFU/g that increased to 7.1 log CFU/g in 48 h (Masoud et al., 2004), but *P. kudriavzevii* was isolated in coffee wet fermentation for the first time in our previous report (Elhalis et al., 2020) and confirmed by this study. Other *Pichia* members such as *P. fermentans*, *P. guilliermondii*, *P. caribbica*, *P. kluyveri*, and *P. anomala* were reported in previous studies (Masoud et al., 2004; Pereira et al., 2014), but not in our study. Surprisingly, *Saccharomyces* spp. were not detected in this study at any time during the fermentation, while they are common isolates with high counts in similar studies conducted in other regions (Agate and Bhat, 1966; Pereira et al., 2014). These findings suggest that yeasts isolated from wet fermentation of coffee beans in different regions have both common and region-specific species (Elhalis et al., 2020).

Results of the culture-independent analysis were broadly consistent with the data of cultural methods but revealed a wider microbial diversity. Over 40 bacterial reads belonging to five families, and 232 fungal reads belonging to 15 families were found in the fermenting beans. *H. uverium* was the most abundant OUTs yeast reads followed by *Candida* and *Wickerhamomyces*, which were consistent with the culture-dependent analysis (Elhalis et al., 2020). Surprisingly, no *Pichia* was detected by culture-independent methods at any stage of the fermentation, but this genus was found by the cultural method. One possible reason for this might be the database limitations used in the culture-independent analysis for yeasts, which were not as complete as those for bacteria. Most of the identified yeasts are well known for their high pectinolytic activities, and are believed to contribute to the mucilage degradation during wet coffee fermentation (Agate and Bhat, 1966; Pereira et al., 2014; Silva, 2014). Furthermore, yeasts such as *Pichia*, *Candida* and *Hanseniaspora* have also been detected in cocoa bean fermentations and believed to be involved in the degradation of pectin of the cocoa pulp (Crafack et al., 2013; Ho et al., 2014; Samagaci et al., 2016). However, such findings were not supported in our study, because in fermentations either with the absence or presence of Natamycin, complete degradation of mucilage was observed, suggesting that yeasts made little or no contribution to the degradation of mucilage. Avallone et al. (2001) reported weak enzymatic activities in the endogenous yeast isolates of coffee fermentation and believed that the main cause of mucilage degradation was the acidification process, rather than pectinolytic activities from yeasts or other microorganisms. Interestingly, early observations on mucilage degradation during coffee fermentation appeared to support our findings as it was found that the degradation was completed before microbial activities became predominate in the fermentation (Lilienfeld-Toal, 1931).

A relatively high abundance of filamentous fungi was detected at the initial stage, which progressively declined during fermentation and mostly disappeared at the end. Possible reasons of such reduction might be the production of organic acids, alcohols and heat as well as the consumption of available oxygen during fermentation as such changes were not favourable to the growth and survival of filamentous fungi (Satyanarayana and Kunze, 2009). Furthermore, endogenous isolates, such as yeasts, were reported to produce antifungal metabolites that suppress the growth of filamentous fungi (Masoud et al., 2004; Payne and Bruce, 2001). The suppression of filamentous fungi would likely favour product safety.

4.2. Microbial metabolism

As expected, the suppression of yeast growth by the addition of Natamycin significantly slowed the consumption rate of mucilage sugars during fermentation. In contrast, suppression of yeast growth did not show a significant impact on the sugars in the endosperm. Endosperm sucrose declined moderately during fermentation which is likely due to the endogenous invertase activities converting sucrose to glucose and fructose (Hansen et al., 1998). However, the levels of fructose and glucose did not show a corresponding increase but remained relatively stable during fermentation. This phenomenon has been observed in our previous report and we believe that it could be due to 1) the sugars leaching out from the endosperm to the outside environment during the fermentation process (Wootton, 1974), and 2) switching of the metabolism of the bean from aerobic to anaerobic pathways (Elhalis et al., 2020). The concentration of the reducing sugars inside the beans has a crucial impact on the coffee bean color, aroma and flavor during roasting, where sugars are key participants of Maillard reaction and caramelization (Fischer et al., 2001; Rohan and Stewart, 1967). These findings are noteworthy because they suggest that the higher levels of residual mucilage in the yeast suppressed fermentation did not diffuse, as predicted, into the endosperm raising the sugar level, which would otherwise have an impact on the coffee flavor and aroma through Maillard reactions during roasting.

Suppression of yeasts by Natamycin also had a significant impact on the production of key microbial metabolites during coffee fermentation. Glycerol was detected in the absence of Natamycin but not detected when Natamycin was added to the fermentation. This finding suggests that yeasts were the microbial group responsible for the production of

glycerol during coffee bean fermentation. Glycerol was reported to be produced by the sugar metabolism of yeasts and has a sweet taste and smooth mouthfeel (Swiegers et al., 2005). In contrast, mannitol concentration was higher when Natamycin was added than when it was absent. Mannitol is mainly produced by the fructose metabolism of heterofermentative LABs such as *Leuconostoc mesenteroides* (De Vuyst et al., 2010; Saha and Racine, 2011), which agrees with the result of higher populations of LAB in the presence of Natamycin. Mannitol has a favourable cool taste and can contribute positively to the sensory quality of coffee.

4.3. Volatile compounds

The growth and activity of yeasts during coffee bean fermentation had a huge impact on the concentration of alcohols in both the green and roasted beans. Greens beans from spontaneous fermentation had a much higher levels of total alcohols than those where the growth of yeasts was suppressed by Natamycin. In particular, the levels of alcohols, isoamyl alcohol and 1-hexnol were 3–20 times higher in the former than in the latter. Ethanol has an essential role in the beverage viscosity and serves as a solvent for volatiles, while isoamyl alcohol is known for its desirable banana and pear flavor (Tamang and Fleet, 2009). Although large proportions of them were lost during roasting, their concentrations remained much higher in the beans from spontaneous fermentation than those where yeasts were suppressed, which is likely to impact on the flavor and aroma of brewed coffee. Both volatiles were reported as yeast metabolites in other fermented products, which were consistent with our results (Pereira et al., 2015; Tamang and Fleet, 2009). 2-Buten-1-ol-3-methyl, which has a fruity aroma, was another major alcohol found in the coffee beans. It was also reported in several fermented products, and believed to be produced by both yeasts and LAB during the metabolism of amino acids such as leucine (Carballo, 2012). However, its levels were not significant different between the two types of beans, suggesting that the yeasts might not be a main producer of this alcohol.

Yeasts also had a huge impact on the level of esters in the coffee beans, especially for ethyl acetate, the concentration of which in the spontaneously fermented beans was > 25 times higher than in those where yeast growth was suppressed. Although a large amount of ethyl acetate was lost during roasting, it remained to be present after roasting in the spontaneously fermented beans, while it was not detected in the beans fermented with the addition of Natamycin. The presence of ester compounds such as ethyl acetate in beverages can significantly affect coffee flavor and aroma as it has a fruity note with a low threshold concentration (Cristiani, 2001). Both yeasts and AAB were reported to produce these volatiles in several fermented products (Peddie, 1990; Saerens and Swiegers, 2016). However, higher populations of AAB in fermentation with the presence of Natamycin, did not correspond with higher levels of esters in the beans, suggesting that the main producer of such compounds was the yeasts rather than AAB. Similarly, aldehydes, especially 3-methyl butanal, benzaldehyde and hexanal, were present in high levels in the spontaneously fermented green beans, which persisted after roasting. These aldehydes were reported as secondary metabolites of LAB and yeasts as intermediates of ethanol production, and have fruity, apple, and almond aroma (Mukisa et al., 2017; Rosca et al., 2016). In the presence of Natamycin, the LAB population was higher than in the spontaneous fermentation; however, concentrations of these aldehydes were lower compared with the latter, suggesting that yeasts were more likely the main producer of such volatiles during the fermentation. Previous studies reported a high level of aldehydes in fermented foods inoculated with yeasts (Pereira et al., 2015; Rosca et al., 2016), which broadly agrees our results. The presence of these volatiles might contribute to the high flavor score and the fruity aroma of the coffee beverage made from the beans fermented in the absence of Natamycin.

High concentrations of acetic acid were detected in beans from both fermentations; however, its level was almost 2 times higher when yeast growth was suppressed. Acetic acid is mainly synthesized either by direct sugar metabolism or ethanol oxidization by AAB, LAB, and yeasts (Awad et al., 2012; De Vuyst et al., 2010; Swiegers et al., 2005). The higher level of acetic acid in the presence of Natamycin was likely related to the higher population of AAB and LAB in the Natamycin treated fermentation. Acetic acid adds to coffee a pleasant clean and sweet taste at low concentrations but an undesirable pungent and vinegary flavor at high concentrations (> 1 mg/ml) (Bertrand et al., 2012). Similarly, butanoic acid concentration was 2-fold higher in the presence of Natamycin. Butanoic acid confers an onion taste and is not preferred in high-quality coffee (Amorim and Amorim, 1977). These findings indicate that the growth of yeasts during coffee bean fermentation reduced the production of these acids, which might be achieved by either the ability of yeasts to suppress the population of the microbial groups that produce acetic and butanoic acids or by taking up and catabolize these metabolites (Casal et al., 1999; Giannattasio et al., 2013; Hohmann, 2009; Mollapour et al., 2009).

Suppression of yeast growth did not significantly impact on the concentration of ketones, pyrazines, pyrroles, pyridines, furans, phenols and sulfur-containing volatiles in the green beans. Previous studies reported the capability of yeasts to produce ketones during cocoa beans fermentation (Ho et al., 2014). Pyrazines were also created by *Bacillus* species during soybeans fermentation (Besson et al., 1997). Furthermore, several yeasts and LAB were reported to produce sulfur-containing volatiles (Carballo, 2012). These findings are not supported in the current study. Most of these volatiles were only detected after roasting and were either not detected or did not show an accumulation during the fermentation process, suggesting that microorganisms in the fermentation were responsible for their synthesis. It is known that bean components such as sugars and amino acids are the main precursors for these volatiles, which are mainly generated by thermal reactions such as Maillard reaction and sugar and protein degradations (Baggenstoss et al., 2008; Daglia et al., 2007; Yaylayan and Keyhani, 1999). Thus, it is possible that microbial activities might contribute to the formation of these volatiles during roasting indirectly through their impact on the bean components during fermentation. However, it was previously reported that fermentation had no significant impact on the coffee bean protein and peptide contents (Elhalis et al., 2020; Ludwig et al., 2000). Such observations might explain the similar concentrations of these compounds in both bean types after roasting. Overall, these volatiles confer a wide range of coffee sensory characteristics such as sweetness, sour, roast, caramel, buttery, woody and earthy (Mayer et al., 2000), and their similar levels in both bean types might explain the similar sensory scores given to the sweetness, balance, clean up and uniformity of coffees brewed from the two types of bean.

5. Conclusion

Results of the current study demonstrated that the growth and activities of yeasts during coffee bean fermentation had a significant impact on coffee flavor and aroma. Specifically, yeasts significantly increased the concentration of alcohols, esters, aldehydes, glycerol, and organic acids in the fermented green beans as well as roasted beans compared with beans from fermentation where yeast growth was suppressed by Natamycin. Furthermore, the growth and activities of yeasts reduced the growth of undesirable microorganisms such as filamentous fungi and the production of undesirable metabolites such as butanoic and acetic acids. Sensory analysis showed that coffee brewed from beans produced by yeast suppressed fermentations had lower flavor and aroma scores compared with those by spontaneous fermentation. Moreover, yeasts did not appear to have a significant impact on the mucilage degradation during coffee bean fermentation. Overall, this study demonstrates that yeasts play a crucial role for wet coffee fermentation in producing coffee with superior flavor, aroma and overall sensory quality.

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.

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Ethical approval

This experiment was conducted at the University of New South Wales Sydney with human ethics approval by the HREAP Executives (HC number: HC190689).

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