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Exploring the diversity of microorganisms and potential pectinase activity isolated from wet fermentation of coffee in northeastern Peru

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Abstract

In this study, the microbiota associated with coffee fermentation from two regions from northern Peru was evaluated. Bacteria and fungi were isolated from coffee farms in the Amazonas and Cajamarca regions and identified using molecular markers 16S rRNA and ITS. The potential pectinase activity was registered by the formation of a transparent halo around colonies. As a result, 29 and 09 species belonging to bacteria and fungi, respectively, were found. The bacterial genera *Lysinibacillus* and *Stenotrophomonas* and the fungal genus *Aspergillus* accounted for the highest number of species isolated from coffee ferments. Forty-one out of 71 isolates showed some type of pectinase enzyme activity, and they included 23 isolates from Cajamarca and 18 from Amazonas. Nevertheless, only three bacterial species registered the formation of transparent halos and showed relevant potential pectinase enzyme activity, namely, *Lysinibacillus xylanilyticus*, *Stenotrophomonas maltophilia*, and *Stenotrophomonas pavanii*, which were all from the Cajamarca region. These species could be further investigated by quantifying enzymes activity and performing other biochemical properties to prototype starter cultures. Accordingly, the study of indigenous microorganisms with biological potential will be essential to increase the coffee value chain and improve the incomes of farmers.

Keywords: coffee; indigenous microorganisms; northern Peru; potential pectinase activity; wet coffee processing.

Practical Application: *Lysinibacillus xylanilyticus*, *Stenotrophomonas maltophilia*, and *S. pavanii* showed potential pectinase activity.

1 Introduction

Coffee is one of the most popular nonalcoholic beverages (Haile & Kang, 2019) and has been considered the most traded and consumed food product in the world for decades (Silva et al., 2013). The coffee fruit consists of an outer layer of skin (exocarp) that covers a layer of pulp followed by mucilage (mesocarp), which is firmly attached to the rigid layer called parchment (endocarp) (Evangelista et al., 2015). Coffee fruits undergo fermentation processes under either wet, dry or semidry conditions (Evangelista et al., 2014; Schwan et al., 2012). During fermentation, the mucilaginous layer is removed and special characteristics are conferred regarding its flavor and aroma because of the production of a wide range of metabolites, such as organic acids, alcohols and esters, which will later add complexity to coffee (De Bruyn et al., 2016; Feng et al., 2016; Vera et al., 2018).

Although the main characteristic flavor of coffee comes from the chemical composition of the bean, some of the metabolites that contribute to the sensory characteristics of the beverage are produced by microbiota associated with the fermentation process (Silva et al., 2013). This microbiota is mainly composed of filamentous fungi, yeasts, archaea, and lactic and acetic acid bacteria (Silva et al., 2005; Huch & Franz, 2015). The main techniques characterizing these microorganisms include culture-independent and culture-dependent approaches (Duong et al., 2020). Culture-independent approaches determine the relative abundance of microorganisms (metabarcoding), potential function of the associated genes (metagenomics), and identification of microorganisms (barcodes), mainly based on DNA techniques (Feng et al., 2016; Oliveira et al., 2013; Santos et al., 2020). Among the main DNA markers used in these approaches are 16S, 18S or 26S/28S rDNA for bacteria (Martins et al., 2020) and ITS or *tef*1 for fungi (Bustamante et al., 2019). The amplification and sequencing of these markers have greatly increased the number of identified microbial species (Duong et al., 2020).

Conversely, the culture-dependent strategy allows the characterization of microorganisms individually on the basis of their morphology and biochemical and functional traits, including their pectinolytic activity (Abdollahzadeh et al., 2020; de Melo-Pereira et al., 2014; Teshome et al., 2017). Pectinase enzymes catalyze the degradation of pectins of the coffee mucilaginous layer by depolymerization and deesterification reactions (Wood & Kellogg, 1988; Xavier-Santos et al., 2004). Pectinase is a general term referring to the mixture of three different enzymatic activities, namely, polygalacturonase (PG), pectin esterase (PE) and pectin lyase (PL), which contribute to the breakdown and modification of pectins and the release of compounds, such as

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galacturonic acid and its oligomers, during the fermentation process (Combo et al., 2012; Patidar et al., 2018; Sunnotel & Nigam, 2002). Currently, the application of pectinolytic enzymes plays an important role in food technology, especially in the fermentation process (Poveda et al., 2018; Souza et al., 2003). These enzymes provide opportunities for future research on the development of a starter culture from microorganisms with pectinolytic activity (Silva et al., 2013). It is important to note that culture-independent and culture-dependent approaches have their own strengths and weaknesses; however, both are of the utmost importance for microorganism analyses, and better results are achieved if they work in a complementary manner (Duong et al., 2020).

Because coffee producers in northern Peru do not employ standardized techniques, the duration of the fermentation process varies among coffee farms (10-48 hours), resulting in a wide range of coffee grades (Puerta & Echeverry, 2015). This scenario might involve a diversity of microorganisms with specific pectinolytic activity during the fermentation process of coffee in northern Peru. Therefore, the aim of this study was to characterize the diversity of microorganisms associated with spontaneous coffee wet fermentation from two regions (Amazonas and Cajamarca) in northeastern Peru through the culture of microorganisms. For this purpose, bacteria and fungi were isolated from coffee farms and identified using Sanger sequencing of molecular markers 16S rRNA for bacteria and ITS for fungi. Additionally, potential pectinase activity was evaluated to list the species that could be subsequently studied and develop a starter inoculum in fermentation to improve coffee quality.

2 Materials and methods

2.1 Sample collection

A total of twenty-four samples of coffee beans under fermentation at different times (from 5 to 32 hours of fermentation) were collected from coffee farmers in the Amazonas and Cajamarca regions. Accordingly, a Drigalski spatula was dipped into the fermented beans and sown directly into Petri dishes with 39 g/L potato dextrose agar medium (PDA, HIMEDIA) (Silva et al., 2013). Petri dishes were sealed with parafilm, labeled and then transported to the Laboratory of Molecular Biology and Genomics of the Universidad Nacional Toribio Rodriguez de Mendoza under sterilized conditions and stored at 28 °C for seven days under 12 h photoperiods (white fluorescent light/darkness) (da Silva et al., 2021).

2.2 Bacterial and fungal isolation

After colony formation, microorganisms were isolated on selective media according to Garofalo et al. (2015). Briefly, ~0.1 g of grown colonies was transferred to De Man, Rogosa and Sharpe (MRS, HIMEDIA) agar media containing 1200 µl/L fluconazole (Elhalis et al., 2020; Oktaviani et al., 2020) for bacterial isolation and yeast extract peptone dextrose (YPD) agar media containing 600 µl/L azithromycin for fungal isolation (de Melo-Pereira et al., 2014; Evangelista et al., 2014). MRS and YPD Petri dishes were incubated at 30 °C with 12 h photoperiods (white fluorescent light/dark) for bacterial (for 48-72 hours) and fungal growth (for 5-7 days) (Elhalis et al., 2020; Roussos et al., 1995).

2.3 DNA sequencing

Isolates were superficially scraped from Petri dishes with a sterilized scalpel and placed in prelabeled 2.0 mL Safelock Eppendorf tubes. Genomic DNA was extracted using a DNA Miniprep Kit (ZymoBIOMICS, California, USA) following the manufacturer's instructions. Two markers were sequenced for bacterial (16S ribosomal RNA, 16S rRNA) and fungal (internal transcribed spacer, ITS) identification. The primer combination for the 16S rDNA gene was 16S_341F: 5'-CCTACGGGAGGCAGCAG-3'; 16S_800R: 5'-CAGGACTACCAGGGTATCTAAT-3' (Lechner et al., 1998) and for ITS1-5.8S, ITS1_F: 5'-TCCGTAGGTGAACCTGCGG-3'; ITS2_R: 5'-TCTCCTCCGCTTATTGATATGC-3' (Masoud et al., 2004). Each marker was amplified using polymerase chain reaction (PCR) with MasterMix (Promega, Madison, Wisconsin, USA) in the following reaction mixture: 10 ng of DNA and 0.25–0.5 pmol of the forward and reverse primers in a total volume of 10 µL. The PCR protocol for 16S rRNA followed a predenaturation step (95 °C for 3 min); 35 cycles of denaturation (94 °C for 1 min), annealing (55 °C for 1 min), and extension (72 °C for 1 min); and a final elongation step (72 °C for 5 min). Additionally, the PCR protocol for ITS followed a predenaturation step (94 °C for 7 min); 40 cycles of denaturation (94 °C for 45 sec), annealing (57 °C for 45 sec), and extension (72 °C for 1 min); and a final elongation step (72 °C for 7 min). Amplicons were purified using a Macherey-Nagel Kit (NucleoSpin Gel and PCR Clean-up, Düren, Germany). Sequences of the forward and reverse strands were commercially determined by Macrogen (Seoul, South Korea) and then edited in Chromas v1.45 software (Technelysium, 1998). A total of 71 sequences were generated and deposited in GenBank (Table S1).

2.4 Phylogenetic analyses

Sequences were initially aligned using MUSCLE algorithms (Thompson et al., 1994) and manually adjusted with MEGA7 (Kumar et al., 2016). In total, eight phylogenetic trees corresponding to the class level were generated for bacteria (Betaproteobacteria, Gammaproteobacteria, Alphaproteobacteria, Firmicutes, and Actinobacteria) and fungal groups (Eurotiomycetes, Sordariomycetes, and Dothideomycetes) on the basis of 16S rRNA and ITS markers, respectively. The nucleotide substitution model that best matched each marker and group was selected using PartitionFinder (Lanfear et al., 2012) (Table S2). The best partitioning strategy and the best sequence evolution model were selected based on the Bayesian information criterion (BIC). Maximum likelihood (ML) analyses were performed with raxmlHPC-AVX (Stamatakis, 2014) implemented in the raxmlGUI 1.3.1 interface (Silvestro & Michalak, 2012) using the best model as appropriate with 1000 bootstrap replicates for both datasets. Bayesian inference (BI) was carried out with MrBayes 3.2.6 (Ronquist et al., 2012) using Metropolis coupled Markov chain Monte Carlo (MCMC) and the best model as appropriate for both datasets. We conducted two runs with four chains each (three hot chains and one cold chain) for 10000000 generations, and trees were sampled every 1000 generations. We plotted the

probability versus generation using Tracer 1.6 6 (Bouckaert et al., 2014) until a probability plateau was reached and set the burn-in value (Bustamante et al., 2021). Intraspecific and interspecific pairwise divergence was estimated using the p-distance model in MEGA7.

2.5 Potential pectinase activity

Potential pectinolytic activity was determined using the methods described by Hankin & Lacy (1984) and Schwan et al. (1997). Isolated yeasts and bacteria were cultured at 30 °C for four days with 12 h photoperiods on plates with mineral medium containing 5 g/L polygalacturonic acid (MP5) at pH 5.5 for detecting polygalacturonase (PG) activity and mineral medium containing 5 g/L pectin (MP7) at pH 7.2 for detecting pectin lyase (PL) activity (Hankin & Lacy, 1984).The mineral medium contained 5 g/L glucose, 6 g/L KH_2PO_4, 1 g/L yeast extract, 2 g/L $(NH_4)2SO_4$, 15 g/L agar and 0.1 ml/L solutions of 0.1 g/L $FeSO_4$, 20 g/L $MgSO_4$, 0.1 g/L $CaCl_2$, 0.2 g/L H_3BO_3, 0.2 g/L $MnSO_4$, 1.4 g/L $ZnSO_4.7H_2O$, 1 g/L $CuSO_4.5H_2O$, and 0.2 g/L MoO_3 (Carrim et al., 2006; Silva et al., 2008a). Potential enzymatic activity was registered by the formation of a transparent halo around colonies due to the cell lysis and precipitation of polysaccharides during enzymatic reactions with pectin after the addition of 1% cetyl trimethylammonium bromide (CTAB) (Wang & Stegemann, 2010; Zhao et al., 2015). Briefly, 5 mL of CTAB solution was added to each plate and incubated at room temperature for three hours in dark conditions (García et al., 2007; García & Chafla, 2015). Afterward, the excess CTAB solution was removed. Six measurements for the area, perimeter, length, and diameter of halos were recorded from each culture using ImageJ software (Huang et al., 2007).

3 Results

3.1 Phylogenetic analyses

A total of 71 microorganisms were isolated from coffee fermentation processes in northeastern Peru. Fifty-eight (81.69%) isolates were bacterial, and thirteen (18.31%) isolates were fungal. Twenty-four bacterial and 5 fungal isolates were identified from Amazonas, whereas 34 bacterial and 8 fungal isolates were identified from Cajamarca (Figure S1). The genus *Lysinibacillus* was the most abundant bacteria (28%), and the genus *Aspergillus* (46%) was the dominant fungi. Additionally, 29 species of bacteria from Amazonas (15 species) and Cajamarca (19 species) were identified and distributed in 14 genera. The most frequent genera in Amazonas were *Lysinibacillus* (46%) and *Bacillus* (13%), whereas such genera in Cajamarca were *Stenotrophomonas* (29%) and *Lysinibacillus* (14%). Nine fungal species were identified and distributed in seven genera. The most frequent genera in Amazonas were *Aspergillus* (60%), *Fusarium* (20%), and *Metarhizium* (20%), whereas those in Cajamarca were *Aspergillus* (38%), *Epicoccum* (25%), and *Penicillium* (13%). Eight phylogenetic trees were constructed using 16S rRNA for bacteria (258 sequences) and ITS for fungi (125 sequences). Species identity was assigned based on grouping with sequences of type species and low values of genetic divergence. In either case, we followed a conservative approach where not every lineage was recognized as a species.

3.2 Bacterial groups

Class Actinobacteria

The phylogeny of the class Actinobacteria included species of the genus *Streptomyces* (29 sequences, 1451 bp) (Figure S2). *Kitasatospora kazusensis* Li was used as an outgroup. In this class, *Streptomyces* sp. (MET119) was recognized in samples from Cajamarca, and it was sisters to *S. coelescens* Krassilnikov and *S. violaceoruber* Waksman & Curtis. The low genetic divergences among these species confirm no variations between sequences of type sequences (i.e., *S. violaceoruber- S. coelescens*; *S. niveoruber- S. griseoviridis*).

Class Alphaproteobacteria

The phylogeny of the class Alphaproteobacteria included members of the genera *Pseudochrobactrum* and *Ochrobactrum* (29 sequences, 1429 bp) (Figure S3). *Alcaligenes faecalis* Castellani & Chalmer was used as the outgroup. Three species were recognized from Amazonas and three from Cajamarca. *P. asaccharolyticum* Kämpfer (MET110), *Pseudochrobactrum* sp.1 (MET10), and *Pseudochrobactrum* sp.2 (MET116) were identified. The last two species differed by 0.3% in genetic variation. Other *Pseudochrobactrum* species ranged from 0 to 4.2%. Additionally, *Ochrobactrum* showed a paraphyletic relationship and low divergence between its species. For instance, type specimens of *O. tritici* Lebuhn and *O. cystisi* Zurdo-Piñeiro varied by 0.1%. *Ochrobactrum pseudogrignonense* Kämpfer (MET88), *Ochrobactrum* sp.1 (MET71 and MET115), and *Ochrobactrum* sp.2 (MET19) were identified. The genetic divergence between *Ochrobactrum* sp. 1 and *Ochrobactrum* sp. 2 was 1.5%.

Class Betaproteobacteria

The phylogeny of the class Betaproteobacteria included members of the genera *Alcaligenes*, *Achromobacter*, and *Delftia* (42 sequences, 1491 bp) that were grouped into three different clades (Figure S4). *Paenibacillus amylolyticus* Nakamura was used as an outgroup. One species was recognized from Amazonas and five from Cajamarca. Clade A contained three specimens of *Alcaligenes* sp. with identical sequences (MET72, MET85, and MET106). The genetic divergence between the type sequences of *A. aquatilis* van Trappen and *A. faecalis* Castellani & Chalmers was 0.5%. In clade B, *Achromobacter spiritinus* Vandamme (MET81 and MET117) and *Achromobacter* sp. (MET20) were found and differed by 1.3%. Genetic divergences between type sequences of *Ach. animicus* Vandamme - *Ach. mucicolens* Vandamme and *Ach. ruhlandii* Packer and Vishniac - *Ach. denitrificans* Rüger & Tan were 0.4% and 0.5%, respectively. Genetic variation in *Ach. spiritinus* Gomila lineage ranged from 0.1-0.3%. In clade C, *Delftia lacustris* Jørgensen (MET12, MET80) and *Delftia* sp. (MET23) were found. Genetic divergences between our *Delftia* specimens were 2.4%, whereas the type sequences of *D. tsuruhatensis* Shigematsu - *D. litopenaei* Chen and *D. acidovorans* den Dooren de Jong - *D. litopenaei* Chen differed by 2.0% and 2-2.6%, respectively.

Class Firmicutes

The phylogeny of the class Firmicutes included members of the genera *Alkalihalobacillus*, *Bacillus*, *Clostridium*, *Lysinibacillus*, and *Paenibacillus* (109 sequences, 1529 bp) (Figure S5). *Proteus*

hauseri O'Hara was used as the outgroup. Six species were recognized from Amazonas and eight from Cajamarca. In clade A, *Clostridium algidixylanolyticum* Broda (MET11) was identified. This species was in sistership to *C. aerotolerans* van Gylswyk & van der Toorn. In clade B, *Paenibacillus campinasensis* Yoon (MET87) and *Paenibacillus* sp. (MET22) were identified. The latter was closely related to *P. mobiliz* Yang. In clade C, the following species were identified: *Bacillus cereus* Frankland & Frankland (MET112 and MET113), *B. graminis* Bibi (MET109), *B. subtilis* Ehrenberg (MET68), *Lysinibacillus fusiformis* Priest (MET78, MET83, MET86, MET92, MET93, MET97, MET98, MET107, MET111, MET120, and MET118), *L. xylanilyticus* Lee (MET9), *Lysinibacillus* sp.1 (MET8 and MET17), and *Lysinibacillus* sp.2 (MET84 and MET114). The genetic divergence between *Lysinibacillus* sp. 1 and *Lysinibacillus* sp. 2 ranged from 0.6-0.8%. In this clade, *A. clausii* Nielsen (MET13) and *Alkalihalobacillus* sp. (MET64 and MET6) were also identified, and they differed by 4.6-5.3%.

Class Gammaproteobacteria

The phylogeny of the class Gammaproteobacteria included members of the genera *Morganella*, *Proteus*, and *Stenotrophomonas* (49 sequences, 1436 bp) (Figure S6). *Clostridium algidixylanolyticum* Broda was used as an outgroup. Five species were recognized from Amazonas and two from Cajamarca. In clade A, *Morganella* sp. (MET91), *P. columbae* Dai (MET89), and *Proteus* sp. (MET90) were identified. *Morganella* sp. differed from *M. morganii* Winslow by 0.3-0.7%. In clade B, *Stenotrophomonas maltophilia* Hugh (MET5, MET65, MET66, MET69, and MET70) and *S. pavanii* Ramos (MET1, MET2, MET4, MET7, MET18, MET67, and MET77) were identified. The genetic variation between *S. maltophila* and *S. pavanii* was 1.2%.

Fungi

Class Dothideomycetes

The phylogeny of the class Dothideomycetes included members of the genera *Cladosporium* and *Epicoccum* (37 sequences, 539 bp) (Figure S7). *Aspergillus creber* Jurjević, S.W. Peterson & B.W. Horn was used as the outgroup. Two species were recognized from Cajamarca. *Cladosporium* sp. (MET131) was identified in clade A, while *Epicoccum ovisporum* Valenz-Lopez, Stchigel, Crous, Guarro & J.F. Cano (MET105 and MET132) was placed in clade B.

Class Eurotiomycetes

The phylogeny of the class Eurotiomycetes included members of the genera *Aspergillus, Penicillium*, and *Talaromyces* (51 sequences, 732 bp) (Figure S8). *Fusarium keratoplasticum* Geiser, O'Donnell, D.P.G. Short & Ning Zhang was used as the outgroup. Two species were recognized from Amazonas and four from Cajamarca. Clade A contained *Talaromyces cnidii* T.S.H. Yu, T.J. An & H.K. Sang (MET104), while clade B contained *Penicillium citrinum* Thom (MET126). Clade C comprised *Aspergillus creber* Jurjević, S.W. Peterson & B.W. Horn (MET122 and MET123) and two unidentified species, *Aspergillus* sp.1 (MET127 and MET128) and *Aspergillus* sp.2 (MET121 and MET129). *Aspergillus* sp.1 differed by 0.5% from *Aspergillus* sp.2.

Class Sordariomycetes

The phylogeny of the class Sordariomycetes included members of the genera *Fusarium* and *Metarhizium* (37 sequences, 564 bp) (Figure S9). *Beauveria bassiana* (Bals.-Criv.) Vuill. was used as the outgroup. Two species were recognized from Amazonas. Clade A contained *Fusarium* sp. (MET103), whereas clade B contained *Metarhizium guizhouense* Chen & Guo (MET130). Genetic variation within *M. guizhouense* ranged from 0.2-1.5%.

3.2 Potential pectinase activity

The 71 isolated microorganisms were cultured in the media types MP5 (with pectin) and MP7 (with polygalaturonic acid) to perform an initial screening of the pectinase enzyme activity. After adding 1% CTAB to the plates and incubating for three hours, the formation of halos around the growing colonies was evaluated. In total, 41 isolates showed the formation of different types of halos around the colony in MP5 (35 isolates) and MP7 (24 isolates) media (Table 1, Figure 1, Figure S10). Eighteen isolates from Amazonas and 23 from Cajamarca showed halos. However, only three species (i.e., *Lysinibacillus xylanilyticus*, *Stenotrophomonas maltophilia*, and *S. pavanii*) formed transparent halos of 4.16-7.55 cm (Table 2, Figure 2).

4 Discussion

In this study, the diversity of the microbial community in coffee wet fermentation was evaluated under culture approaches using Sanger sequencing of molecular markers for identification (i.e., 16S rRNA and ITS) while an initial screening of the pectinase activity was performed (i.e., MP5 and MP7 media). The higher number of identified species and isolates with PG and PL activities were found in Cajamarca region, which typically uses a longer fermentation time and produces higher coffee quality than the Amazonas region (Agencia Peruana de Noticias, 2019). Several bacteria and fungi identified in this study were previously reported in other coffee fermentation processes. For instance, bacterial species such as *B. cereus*, *B. subtilis*, *L. fusiformis*, and *Paenibacillus* sp. from Minas Gerais, Brazil (Evangelista et al., 2015; Silva et al., 2008a,b) and *Proteus* sp. from Mysore, India (Agate & Bhat, 1966) were isolated from either wet or semidry fermentation processes. On the fungal side, members of *Aspergillus*, *Fusarium*, and *Penicillium* were isolated from Brazil and Colombia (Cruz-O'Byrne et al., 2021; Silva et al., 2000) from wet coffee fermentation. The identification of these bacteria and fungi in our study suggests that they are commonly isolated in specific Petri dishes (Hamdouche et al., 2016; Vilela et al., 2010). Conversely, this is the first time that the following microorganisms were isolated from coffee ferment in either bacterial or fungal media, *Alkalihalobacillus*, *Pseudochrobactrum*, *Stenotrophomonas*, and *Talaromyces*, confirming the importance of this approach to characterize the diversity of microorganisms that has not been recorded previously in fermentation processes (Carvalho et al., 2017; Elhalis et al., 2020).

Table 1. Isolates from Amazonas (A) or Cajamarca (C) Regions that showed halo formation around the colony in MP5 and MP7 media.

Species Bacteria	Code	Region	Time*	Media	Features of halo
Bacillus cereus	MET112	A	5	MP5	White color, non-uniform intensity, no definite shape
Lysinibacillus fusiformis	MET78	A	5	MP5	Whitish color, uniform intensity, continuous rounded shape
Lysinibacillus fusiformis	MET97	A	5	MP5, MP7	Both: beige in color, uniform intensity, continuous rounded shape
Lysinibacillus fusiformis	MET98	A	5	MP5	White color, uniform intensity, discontinuous rounded shape
Lysinibacillus fusiformis	MET111	A	5	MP5	Yellowish white color, uniform intensity, continuous rounded shape
Lysinibacillus sp.2	MET84	A	5	MP5	Yellowish white color, uniform intensity, continuous rounded shape
Pseudochrobactrum asaccharolyticum	MET110	A	5	MP5	White color, uniform intensity, no definite shape
Pseudochrobactrum sp.2	MET116	A	5	MP7	Beige color, uniform intensity, continuous rounded shape
Alcaligenes sp.	MET106	C	6	MP7	Beige color, uniform intensity, continuous rounded shape
Clostridium algidixylanolyticum	MET11	C	6	MP5	Yellowish white color, non-uniform intensity, discontinuous rounded shape
Delftia lacustris	MET12	C	6	MP5, MP7	MP5: yellowish white color, MP7: beige color, both: uniform intensity, continuous rounded shape
Delftia lacustris	MET80	C	6	MP5	Yellowish white color, uniform intensity, continuous rounded shape
Paenibacillus sp.	MET22	C	6	MP5	Beige color, uniform intensity, continuous rounded shape
Stenotrophomonas maltophilia	MET69	A	9	MP5	Beige color, uniform intensity, continuous rounded shape
Stenotrophomonas pavanii	MET18	A	9	MP7	Yellowish white color, non-uniform intensity, discontinuous rounded shape
Lysinibacillus fusiformis	MET86	A	10	MP5, MP7	MP5: yellowish white color, MP7: beige color with yellowish border, both: non-uniform intensity, discontinuous rounded shape
Lysinibacillus fusiformis	MET83	C	12	MP5, MP7	In MP5: yellowish white color, in MP7: yellowish beige color, Both: uniform intensity, continuous rounded shape
Ochrobactrum sp.2	MET19	C	12	MP5	White color, non-uniform intensity, discontinuous rounded shape
Achromobacter spiritinus	MET81	C	18	MP5, MP7	MP5: Transparent white color, MP7: Beige color, both: uniform intensity, continuous rounded shape
Delftia sp.	MET23	C	18	MP5, MP7	Both: beige-yellowish color, uniform intensity, continuous rounded shape
Lysinibacillus fusiformis	MET107	C	22	MP5	Yellowish white color, uniform intensity, continuous rounded shape
Stenotrophomonas maltophilia	MET65	C	22	MP5, MP7	MP5: yellowish white color, in MP7: transparent color with beige gradient edges, Both: uniform intensity, continuous rounded shape.
Stenotrophomonas pavanii	MET1	C	22	MP5, MP7	MP5: yellowish-white color, MP7: transparent, brownish-white gradient edges, Both: uniform intensity, continuous rounded shape
Stenotrophomonas pavanii	MET2	C	22	MP5	Brownish beige color, uniform intensity, non-continuous rounded shape
Bacillus graminis	MET109	C	27	MP7	Beige color, uniform intensity, continuous rounded shape
Lysinibacillus sp.1	MET8	C	27	MP5	Yellowish white color, uniform intensity, continuous rounded shape
Morganella sp.	MET91	A	27	MP5, MP7	MP5: white color, MP7: beige color, Both: uniform intensity, continuous rounded shape
Proteus sp.	MET90	A	27	MP5, MP7	MP5: beige color, MP7: white color, Both: uniform intensity, continuous rounded shape.
Stenotrophomonas maltophilia	MET70	C	27	MP5, MP7	MP5: yellowish white color, MP7: pinkish color with beige gradient edges, Both: uniform intensity, continuous rounded shape.

* Fermentation time (5h, 6h, 9h, 10h, 12h, 18h, 22h, 27h, 32h) at the moment of collection.

Table 1. Continuação...

Species Bacteria	Code	Region	Time*	Media	Features of halo
Stenotrophomonas pavanii	MET7	C	27	MP7	Yellowish-white color, non-uniform intensity, continuous rounded shape
Stenotrophomonas pavanii	MET77	C	27	MP5, MP7	MP5: yellowish white color, MP7: pinkish color with beige gradient edges, Both: uniform intensity, continuous rounded shape
Alcaligenes sp.	MET72	C	32	MP5, MP7	MP5: yellowish white color, MP7: beige color with yellowish edges, Both: uniform intensity, continuous rounded shape
Lysinibacillus fusiformis	MET92	A	32	MP5, MP7	In MP5: White color, in MP7: Beige color, both with non-uniform intensity and discontinuous rounded shape.
Lysinibacillus fusiformis	MET93	A	32	MP5	Yellowish white color, non-uniform intensity, continuous rounded shape
Lysinibacillus xylanilyticus	MET9	C	32	MP5, MP7	MP5: yellowish white color, MP7: transparent color with beige edges, Both: uniform intensity, continuous rounded shape
Lysinibacillus sp.1	MET17	A	32	MP5, MP7	MP5: beige color, non-uniform intensity, MP7: yellowish white color, uniform intensity, Both: continuous rounded shape
Ochrobactrum sp.1	MET71	C	32	MP5	MP5: yellowish color, MP7: yellowish white color, Both: uniform intensity, continuous rounded shape
Pseudochrobactrum sp.1	MET10	C	32	MP7	Yellowish-white color, non-uniform intensity, no definite shape
Fungi					
Penicillium citrinum	MET126	C	12	MP5, MP7	Both: beige in color, uniform intensity, continuous rounded shape
Aspergillus sp.1	MET127	C	22	MP5, MP7	MP5: beige color with yellowish edges, MP7: pink color with beige edges, Both: uniform intensity, continuous rounded shape
Talaromyces cnidii	MET104	C	27	MP5	Beige color, uniform intensity, continuous rounded shape

* Fermentation time (5h, 6h, 9h, 10h, 12h, 18h, 22h, 27h, 32h) at the moment of collection.

Table 2. Measurements of halos and colonies of selected microorganisms with pectinolytic activity.

Code	Genus	Halo Diameter (HD)	Colony Diameter (CD)	HD/CD Ratio
MET65	*Stenotrophomona maltophilia*	5.71	3.63	1.57
MET1	*Stenotrophomonas pavanii*	7.55	5.04	1.50
MET9	*Lysinibacillus xylanilyticus*	4.16	2.42	1.72

The most common microorganisms reported in coffee fermentation are lactic acid bacteria (LAB), such as *Bacillus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Weissella* (Melo-Pereira et al., 2017; Pothakos et al., 2020), whereas the most commonly reported fungal species belonged to *Candida*, *Hanseniaspora*, *Pichia*, and *Saccharomyces* (Oliveira-Junqueira et al., 2019; Zhang et al., 2019). However, the predominant species in this study corresponded to the bacteria *Lysinibacillus* and the fungi *Aspergillus*. This highlights the importance of region-specific evaluations that allow the characterization of indigenous microbiota in coffee fermentation processes (Carvalho et al., 2017, Elhalis et al., 2020). This might be a consequence of specific environmental factors (e.g., temperature and altitude) (Evangelista et al., 2015; Körner, 2007).

Furthermore, some of the identified species have been associated with good coffee quality, such as *Cladosporium cladosporioides* (Chalfoun et al., 2007; Pereira et al., 2005), while others were linked to low quality, such as *Fusarium* and *Penicillium* species (Masoud & Kaltoft, 2006; Souza et al., 2017) and some *Aspergillus* species, which represent potential ochratoxin A producers (Joosten et al., 2001; Mantle & Chow, 2000). However, once fermentation is finished, their abundances are not significant, ensuring that the final coffee is safe for human consumption consume (Masoud & Kaltoft, 2006; Souza et al., 2017).

Out of 71 isolated microorganisms, 41 showed potential pectinolytic enzyme activity (pectin degradation) on the basis of their PG (85.4%) and PL (58.5%) and combined PG and PL (43.9%) activities. PG and PL enzymes have been extensively reported in species of the genera *Aspergillus*, *Bacillus* and *Penicillium* (Chaudhri & Suneetha, 2012; Kashyap et al., 2000; Murad & Azzaz, 2011). This study confirms potential PG and PL activities in these three and 12 other genera (Table 1). Pectinase enzyme activity is associated with the presence of transparent halos due to the formation of alcohols and acids (e.g., butyric acetic, lactic and other long-chain carboxylic acids) (Barragán et al., 2014; Beg et al., 2000; Carrim et al., 2006; García & Chafla, 2015).

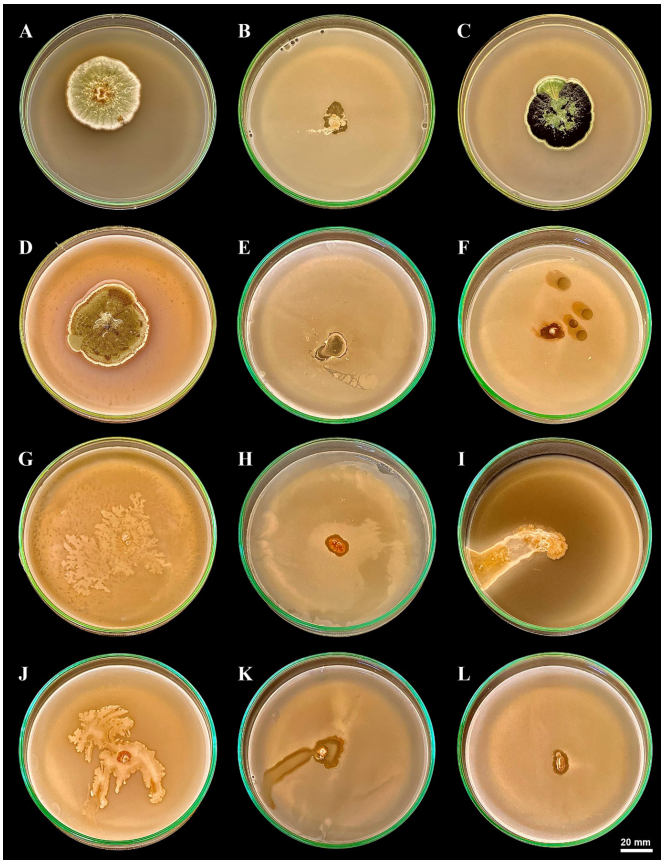

Figure 1. Formation of halos in MP5 and/or MP7. Microorganisms isolated from the Cajamarca Region: *Penicillium citrinum* (A, MP5), *Delftia lacustris* (B, MP5), *Aspergillus* sp.1 (C, MP5; D, MP7), *Ochrobactrum pseudogrignonense* (E, MP5), *Stenotrophomonas pavanii* (F, MP5), *Alcaligenes* sp. (G, MP7), *Clostridium algidixylanolyticum* (H, MP5). Microorganisms isolated from Amazonas: *Lysinibacillus fusiformis* (I, MP7; J, MP7; K, MP5; L, MP5).

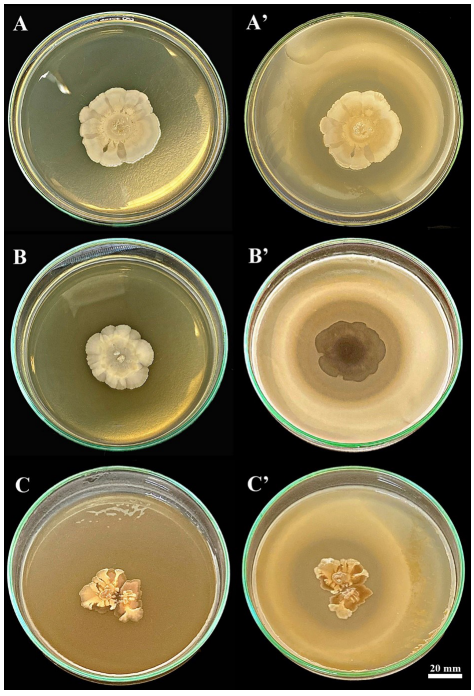

Figure 2. Microorganism colonies before and after precipitating 1% CTAB on MP7 media and forming transparent halos. A: *Stenotrophomonas maltophilia* without CETAB, A': *Stenotrophomonas maltophilia* with CETAB, B: *Stenotrophomonas pavanii* without CETAB, B': *Stenotrophomonas pavanii* with CETAB, C: *Lysinibacillus xylanilyticus* without CETAB, C': *Lysinibacillus xylanilyticus* with CETAB.

This scenario was reported exclusively in MP5 medium for the following three bacterial species *Lysinibacillus xylanilyticus* (MET9), *Stenotrophomonas maltophilia* (MET65), and *S. pavanii* (MET1). These species showed higher halo diameters (5.8 ± 1.7 cm) than those reported in other species (2.33 ± 0.4 cm and 1.6 ± 0.82 cm), suggesting their potential as pectin degraders (García & Chafla, 2015; Hurtado & Otálvaro, 2020; Oumer & Abate, 2018).

A higher number of microorganisms with potential pectinase activity (43.9%) was reported from the Cajamarca region, including the three species *L. xylanilyticus*, *S. maltophilia*, and *S. pavanii*, which might explain the high-quality coffee produced in this region according to the International Specialty Coffee Fair awards obtained in 2018 and 2019 (Agencia Peruana de Noticias, 2019; Cafelab.pe, 2018). These findings provide a starting point for further exploration and quantification of the enzyme activity of indigenous microorganisms from northern Peru and subsequent analyses should be conducted to develop starter cultures of coffee fermentation

The increasing market demand for coffee consumption has led to the study of indigenous microbiota associated with coffee cultivars to understand the role of these microorganisms in the organoleptic characteristics of the final beverage (Haile & Kang, 2019; Silva et al., 2008a). Indigenous microorganisms with relevant enzyme activity might have the potential to be used as starter cultures, thus allowing controlled fermentation and enhancing the quality of coffee (Carvalho et al., 2017). This scenario might increase the global value chain of coffee and improve the economic incomes of coffee farmers (Melo-Pereira et al., 2017). Therefore, the isolation, identification, and molecular and physicochemical characterization of indigenous microorganisms are essential to accomplish these goals.

5 Conclusions

In this study, the microbiota inhabiting wet coffee fermentation from two regions in northern Peru were evaluated. Based on Sanger sequencing and initial screening of pectinolytic activity, we observed that the species composition of fungi and bacteria varied from one coffee farm to another (Amazonas vs. Cajamarca regions). Fungal and bacterial populations were more diverse and abundant in Cajamarca than in the Amazon region. This probably resulted from the longer fermentation time of fruits in Cajamarca that were subjected to a longer degradation and the prevalence of anaerobic microorganisms. The ability of naturally occurring microbiota to degrade mucilage is still unexplored in northern Peru, thereby limiting the development of starter cultures to reduce fermentation times, improve process control, and enhance cup quality. Here, we identify three bacterial species with potential pectinolytic activity (*L. xylanilyticus, S. maltophilia* and *S. pavanii*) isolated from the Cajamarca region. This finding should be further investigated by quantifying enzymes activity, performing other biochemical properties of these species (i.e., carbohydrate assimilation, ethanol resistance), and prototyping starter cultures (as a single species or in a consortium) according to coffee variety, local geographic conditions, and fermentation time.

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Supplementary material

Supplementary material accompanies this paper.

Table S1. List of taxa used in the molecular analyses along with strain and, if known, country. (T) represents sequence obtained from type strains. GenBank accession numbers under each marker; if marker not sequenced indicated by "–". Sequences generated in present study are in bold.

Table S2. Best models for maximum likelihood and Bayesian inferences by group and taxonomical class.

Figure S1. Number of species per taxonomical class for bacteria and fungi isolated from coffee ferments in Amazonas and Cajamarca Regions.

Figure S2. Phylogenetic tree of the Actinobacteria class based on maximum likelihood inferences of 16S rRNA gene sequences. Bootstrap values of maximum likelihood (BS; ≥ 50%)/Bayesian posterior probabilities (BPP; ≥ 0.9) are indicated above the branches. Values below 50% (BS) or 0.90 (BPP) are indicated with dashes (-). The scale bar indicates the number of nucleotide substitutions per site. Sequence accession numbers are shown in parentheses, with T representing sequences obtained from type strains. Sequences for taxa in bold were generated in this study.

Figure S3. Phylogenetic tree of class Alphaproteobacteria based on maximum likelihood inference of 16S rRNA gene sequences. Bootstrap values of maximum likelihood (BS; ≥ 50%)/Bayesian posterior probabilities (BPP; ≥ 0.9) are indicated above the branches. Values below 50% (BS) or 0.90 (BPP) are indicated with dashes (-). The scale bar indicates the number of nucleotide substitutions per site. Sequence accession numbers are shown in parentheses, with T representing sequences obtained from type strains. Sequences for taxa in bold were generated in this study.

Figure S4. Phylogenetic tree of class Betaproteobacteria based on maximum likelihood inference of 16S rRNA gene sequences. Bootstrap values of maximum likelihood (BS; ≥ 50%)/Bayesian posterior probabilities (BPP; ≥ 0.9) are indicated above the branches. Values below 50% (BS) or 0.90 (BPP) are indicated with dashes (-). The scale bar indicates the number of nucleotide substitutions per site. Sequence accession numbers are shown in parentheses, with T representing sequences obtained from type strains. Sequences for taxa in bold were generated in this study.

Figure S5. Phylogenetic tree of the class Firmicutes based on maximum likelihood inference of 16S rRNA gene sequences. Bootstrap values of maximum likelihood (BS; ≥ 50%)/Bayesian posterior probabilities (BPP; ≥ 0.9) are indicated above the branches. Values below 50% (BS) or 0.90 (BPP) are indicated with dashes (-). The scale bar indicates the number of nucleotide substitutions per site. Sequence accession numbers are shown in parentheses, with T representing sequences obtained from type strains. Sequences for taxa in bold were generated in this study.

Figure S6. Phylogenetic tree of class Gammaproteobacteria based on maximum likelihood inference of 16S rRNA gene sequences. Bootstrap values of maximum likelihood (BS; ≥ 50%)/Bayesian posterior probabilities (BPP; ≥ 0.9) are indicated above the branches. Values below 50% (BS) or 0.90 (BPP) are indicated with dashes (-). The scale bar indicates the number of nucleotide substitutions per site. Sequence accession numbers are shown in parentheses, with T representing sequences obtained from type strains. Sequences for taxa in bold were generated in this study.

Figure S7. Phylogenetic tree of class Dothideomycetes based on maximum likelihood inference of ITS sequences. Bootstrap values of maximum likelihood (BS; ≥ 50%)/Bayesian posterior probabilities (BPP; ≥ 0.9) are indicated above the branches. Values below 50% (BS) or 0.90 (BPP) are indicated with dashes (-). The scale bar indicates the number of nucleotide substitutions per site. Sequence accession numbers are shown in parentheses, with T representing sequences obtained from type strains. Sequences for taxa in bold were generated in this study.

Figure S8. Phylogenetic tree of class Eurotiomycetes based on maximum likelihood inference of ITS sequences. Bootstrap values of maximum likelihood (BS; ≥ 50%)/Bayesian posterior probabilities (BPP; ≥ 0.9) are indicated above the branches. Values below 50% (BS) or 0.90 (BPP) are indicated with dashes (-). The scale bar indicates the number of nucleotide substitutions per site. Sequence accession numbers are shown in parentheses, with T representing sequences obtained from type strains. Sequences for taxa in bold were generated in this study.

Figure S9. Phylogenetic tree of class Sordariomycetes based on maximum likelihood inference of ITS sequences. Bootstrap values of maximum likelihood (BS; ≥ 50%)/Bayesian posterior probabilities (BPP; ≥ 0.9) are indicated above the branches. Values below 50% (BS) or 0.90 (BPP) are indicated with dashes (-). The scale bar indicates the number of nucleotide substitutions per site. Sequence accession numbers are shown in parentheses, with T representing sequences obtained from type strains. Sequences for taxa in bold were generated in this study.

Figure S10. Microorganisms before and after 1% CTAB precipitation in MP5 and/or MP7 media and the formation of different types of halos. Microorganisms from the Cajamarca Region: Penicillium citrinum without CETAB (A, MP5), Penicillium citrinum with CETAB (A', MP5), Aspergillus sp.1 without CETAB (B, MP7; C, MP7), Aspergillus sp.1 with CETAB (B', MP7; C', MP7), Alcaligenes sp. without CETAB (D, MP7), Alcaligenes sp. with CETAB (D', MP7), Clostridium algidixylanolyticum without CETAB (E, MP5), Clostridium algidixylanolyticum with CETAB (E', MP5). Microorganisms from the Amazonas region: Lysinibacillus fusiformis without CETAB (F, MP7; G, MP7; H, MP5) and Lysinibacillus sp. 4 with CETAB (F', MP7; G', MP7; H', MP5).

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