Chapter

Microbiota of Wild Fruits from the Amazon Region of Ecuador: Linking Diversity and Functional Potential of Lactic Acid Bacteria with Their Origin

Gabriela N. Tenea, Pablo Jarrin-V and Lucia Yepez

Abstract

Subtropical wild fruits are a reservoir of microbial diversity and represent a potential source of beneficial microorganisms. Wild fruits provide essential nutrients, minerals, and antioxidants that contribute to human health. Many of these wild fruits are used by indigenous peoples for medicine and food, but there is yet an unexplored potential in the study of their properties and benefits. Wild fruits from the Amazon region and their associated active substances or bacterial communities can prevent disease, provide appropriate nutrition, contribute to new sources of income, and improve lives. Despite its condition as a megabiodiverse country, Ecuador suffers from limited access to its genetic resources, and particularly for research. A total of 41 isolates were obtained from six wild Amazonian fruit species and were molecularly classified into the genera *Lactiplantibacillus* (31 isolates), *Lactococcus* (3 isolates), *Weissella* (3 isolates), and *Enterococcus* (1 isolate). Three isolates showed large divergence in sequence variability and were not identified by the taxonomic assignment algorithm. Inferred phylogenies on the 16S rRNA gene explained the relationship between lineages and their origin. Carbohydrate metabolism and antimicrobial profiles were evaluated, and the isolates were classified from a functional perspective. Antimicrobial profiles showed a wide-range spectrum against several Gram-positive and Gram-negative bacteria. To our knowledge, this is the first study assessing the diversity of LAB in native tropical fruits from the Amazon region of Ecuador and their promising functional properties. The obtained isolates and their assessed properties are valuable genetic resources to be further investigated for industrial and pharmaceutical applications.

Keywords: microbial diversity, Amazon region, lactic acid bacteria, fruit origin

1. Introduction

Lactic acid bacteria (LAB) are a versatile group of microorganisms and with a long history of use in fermented foods. LAB are distributed in diverse environments and are of considerable economic interest due to their "Food Grade Status" [1].

LAB have been extensively used as starter cultures, as probiotics, and in the production of inhibitory compounds. The latter has been considered as a new generation of antibiotics [2].

The growing number of people with lactose intolerance, allergy to cow's milk protein, or high cholesterol, which cannot consume dairy products, is affected by the lack of access to beneficial probiotic bacteria. Thus, fermented fruits or vegetables, which are an alternative source of probiotic bacteria, have received considerable attention from the consumers. The increasing trend for veganism or vegetarianism has promoted an increase in consumer interest for functional foods that contain beneficial bacteria [3].

When evaluating aspects of safety, taxonomy, and potential to produce pathogenic toxins and resistance to antibiotics, LAB have been recognized by the Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) as "generally recognized as safe" (GRAS) [4]. Although resistance genes in bacteria, including Lactobacillus, are located in transposable elements that can be transferred to other species, the US legislation has no guidelines that contemplate the potential development of resistance for microorganisms used in foods [5]. Alternatively, the European Food Safety Authority (EFSA) established guidelines that define the safety standards for microorganisms used "from farm to fork" [6]. The probiotics belonging to the species included in the category "qualified presumption of safety" (QPS) by EFSA show excellent safety records [4]. Undoubtedly, a full safety assessment of newly identified strains begins with proper identification and an in vitro evaluation of the potential risks [7].

Numerous research studies have attempted to design fermented foods based on fruits or vegetables with probiotic bacteria. LAB can display a remarkable degree of phenotypic and genotypic diversity, allowing them to survive in a variety of habitats and stress conditions. Most species in the LAB group are found colonizing the human or animal intestine but also in several fermented foods, vegetables, and fruits [8, 9, 10].

The capacity to adapt to environmental changes depends on the genetic repertoire and the competency to use micronutrients along with the ability to counteract and overcome externally exerted physical-chemical challenges [11]. Despite an immeasurable arsenal of microbes, each plant or fruit harbors a distinctive microbiota that represents a remarkable niche to several LAB species [12]. However, the microbial composition in these environments is fluctuating and depends on intrinsic (physical and nutritional conditions) and extrinsic (environmental and harvesting conditions) parameters of the plant matrix [13]. Depending on the plant or fruit origin, the LAB species differ; thus, the *Leuconostoc, Lactobacillus, Weissella, Enterococcus,* and *Pediococcus* genera are those most frequently identified as epiphytes within the microbiota [14]. Although several species have been identified and characterized, the selection of new strains with valuable biotechnological properties remains a topic of interest [2].

Ecuador is known as a megabiodiverse country, where access to genetic resources for research is still deficient. Studies on the microbial diversity of unexplored niches and environments are of interest as these might lead to the identification of an endless number of species with unique characteristics and properties. It has been claimed that the microbial population present in raw plant material that has been originated from unexplored niches, differs among samples, as the plant matrix might carry a particular microbiota in a specific geographical region and at a specific time-point [15].

Recently, we proposed to investigate the microbiota associated with wild fruits; especially, to select and characterize strains of beneficial lactic acid bacteria [16]. Tropical forest fruits provide essential nutrients, minerals, and antioxidants that keep a healthy body and provide resilience to disease [17]. The physical and

nutritional properties of fruits, together with the environmental and harvesting conditions of the plant matrix, may influence the final microbial community composition [2]. However, we hypothesize that the wild fruits from the Amazon region differ in their native availability of nutrients and physicochemical conditions, especially when compared to domesticated fruits from commercial crops. These substantial differences might influence the range of potential microbiota.

Wild tropical fruits are used for nutrition and medicine by the local inhabitants; yet, the lack of research makes this natural resource an yet unknown source of benefits to the food industry [18]. The prevention of diseases through appropriate nutrition with such fruits or the active substances extracted from such fruits or the products from the associated bacterial community can save lives and income [19]. The role of LAB isolated from fruits is not clearly understood; nonetheless, as they are naturally present on the surface of fruits, they produce antimicrobial compounds that might be used as a biological agent to control the growth of spoilage bacteria, and without changing the sensory properties of foods [20, 21]. Thus, we expected to find microorganisms with unique characteristics unlike those of the strains found in ordinary fermented materials, such as fermented milk or vegetables. Nonetheless, to use such strains as part of probiotic foods implies the application of numerous functional analyses to determine their susceptibility or resistance to antibiotics, capacity to prevent the growth of harmful bacteria by competitive exclusion, and production of organic compounds.

The resistance of LAB to antibiotics is a relevant biosafety issue. Some strains with intrinsic antibiotic resistance could be useful to restore the antibiotic resistance of the gut microbiota after antibiotic treatments [22]. Specific antibiotic resistance genes are carried by transposable elements and constitute a reservoir of resistance for potential food or gut pathogens, thus representing a major biosafety issue [22, 23]. In this regard, special attention should be given to the presence of antibiotic resistance determinants and their potential mobility. Lactobacilli are usually sensitive to the cell wall-targeting penicillin and β-lactamase, but more resistant to cephalosporins. Also, some species are susceptible to low concentrations of several inhibitors of protein synthesis, such as chloramphenicol and tetracycline [22]. Some LABs are considered the most promising natural food preservatives, as they secrete antimicrobial substances (i.e. bacteriocins) that, when applied as crude-extracts to food, precipitated peptides that protect the products from deterioration by microbial activity [24]. LAB can also be used in the pharmaceutical industry to produce compounds such as esters, through the reduction of fructose, as the most abundant sugar present in fruits [25].

This research aimed to investigate the lactic acid bacteria diversity associated with the microecosystem of wild fruits from the Amazon region of Ecuador. We sought to find unique characteristics, unlike those of the strains found in ordinary fermented materials such as milk or vegetables. As a first step to select the most promising strains with superior functional properties, we present broad insights into this microenvironment. To our knowledge, this is the first study that assessed the diversity and functional properties of LAB in native tropical fruits from the Amazon region of Ecuador.

2. Materials and methods

2.1 Sampling, isolation, and purification of lactic acid bacteria

The source wild fruit samples were collected during the rainy season (April–July, 2014-2015) from the tropical forest of the Sucumbíos Province in the Amazon region of Ecuador (**Figure 1**). Several units of fruits from *Chrysophyllum oliviforme,*

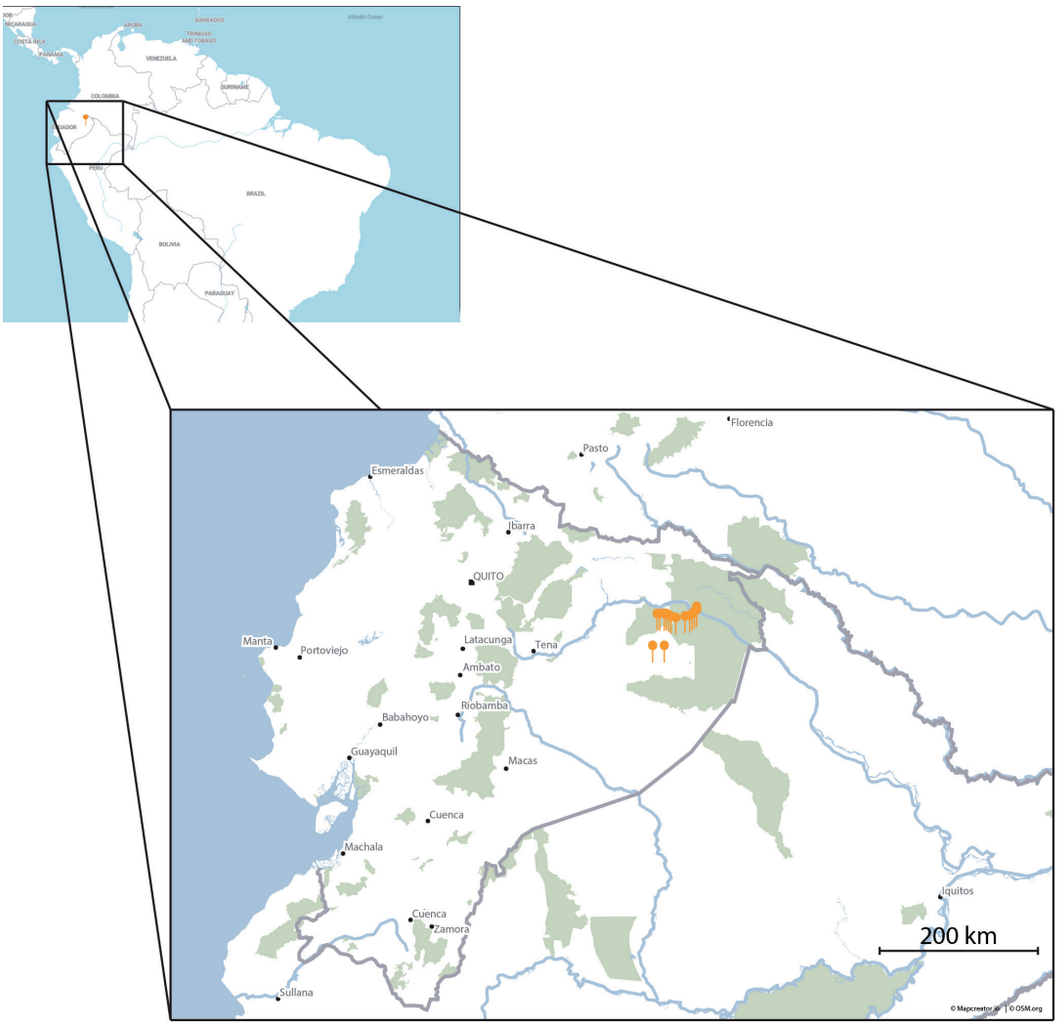

Figure 1.
Location of samples obtained for this study in the Amazon region of Ecuador. Colored areas represent national parks. Most of the samples were obtained in the areas adjacent to the Cuyabeno National Park.

Malus sp., *Costus* sp., *Solanum stramofolium, Cheilocostus specious*, and *Theobroma grandiflorum* were collected aseptically in sterile bags and stored at 4°C for transport to the laboratory. The fruit samples were washed with a 5% bleach solution for 5 min, then twice with distillate water, smashed in small pieces, and transferred into Erlenmeyer flasks (500 ml) that contained peptone water (0.1%, bacteriological peptone, Difco Detroit, MI, USA). Samples were incubated statically for up to 5 days at room temperature. MRS agar [26] plates containing 5 g/l $CaCO_3$ were inoculated and incubated under anaerobic conditions (37°C for 72 hours), and individual colonies were randomly selected and purified. The purified colonies (<75 colonies/each fruit) were Gram-stained, characterized by microscopic morphology, and tested for mobility, indole and catalase production, spore formation, and gas production from glucose [16]. The selected colonies were screened for their capacity to inhibit four bacterial pathogens (Section 2.4), resistance to bile (1%), acidity (pH 2.5-3.0), and temperature range (15-45°C). A total of 41 isolates complying with these criteria were further selected and identified. Each isolate was assigned a collection code (UTN) and was stored at −80°C in MRS medium containing 20% glycerol.

2.2 Classification of isolates using 16S rRNA gene sequence analysis

16S rRNA gene sequencing was used for taxonomical assignment (Macrogen Inc., Korea, custom service). The primers 27F 5′ (AGA GTT TGA TCM TGG CTC

AG) 3′ and 1492R 5′ (TAC GGY TAC CTTGTT ACG ACT T) 3′ were used for the PCR amplification. The PCR reaction was performed with 20 ng of genomic DNA in a 30 μl reaction mixture with EF-Taq (SolGent, Korea) and through the following protocol: activation of Taq polymerase at 95°C for 2 minutes, 35 cycles of 95°C for 1 minute, at 55°C and 72°C for 1 minute, finishing with 10 minutes at 72°C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). The sequencing reaction was performed using a PRISM BigDye Terminator v3.1 cycle sequencing kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95°C for 5 minutes, followed by 5 minutes on ice and then analyzed by an ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

A preliminary fast search of the sequences was conducted using the megablast algorithm on the 16S ribosomal RNA database at NCBI, as implemented in Geneious Prime 2020.2.3 [27]. This first search was used to obtain a maximum of 100 hits and associated search quality parameters that provided an initial reference for taxonomic classification. A final taxonomic assignment was made by the RDP Bayesian classifier algorithm [28] with 100 bootstrap replicates and a K-mer of size 8, as implemented in the function "accurate, high-resolution sample inference from amplicon sequencing data" (assign Taxonomy) of the DADA2 package [29] in R [30]. The Genome Taxonomy Database [31] was used as the reference for the Bayesian classifier algorithm. A threshold for bootstrap values equal or over 80% was used to filter the taxonomic correspondences. The resulting final table of taxonomic assignments was used for downstream statistical analyses.

We used reference samples to root the inferred phylogenetic hypotheses and provide a comparison to the experimental samples used in this study. This reference samples were: KJ660075.1: *Brachyspira_hampsonii*; AJ276460.1: *Enterococcus_faecalis;* NR_114312.1: *Weissella oryzae*; NR_104573.1: *Lactobacillus*; LC063164.1: *Weissella confusa;* NR_029133.1: *Lactobacillus.*

LAB represent a small fraction of the autochthonous microbiota in the fruit and plant phyllosphere. However, due to the inherent limitations of in vitro cultures, the most frequent isolates often belong to the genera *Lactobacillus, Leuconostoc, Weissella, Enterococcus*, and *Pediococcus* [14]. More recently, based on conserved pairwise average amino acid identity, core genome phylogeny, physiological criteria, clade-specific signature genes, and ecology, *Lactobacillaceae*, and *Leuconostocaceae* were merged into *Lactobacillaceae* [32]. The genus *Lactobacillus* was split into 25 genera including *Lactiplantibacillus, Fructiplantibacillus, Lactobacillus*, among others. Thus, we allocate the study samples according to this new classification.

2.3 Metabolic profile of the selected isolates

The BBL Crystal anaerobe identification system (cat # 245010, BD Company, US) is a miniaturized identification method for tests of fermentation, oxidation, degradation, and hydrolysis of diverse substrates. It also includes chromogen and fluorogenic linked substrates to detect enzymes that microbes metabolize. The results obtained after incubation of strips for 24 hours at 37°C were analyzed using the BBL Crystal analysis software (according to the manufacturer instructions). The results were qualitatively defined as negative (−) or positive (+) according to the detected color. As a reference for the metabolic profile test, we used *Lactobacillus plantarum* ATCC8014 and *Lactobacillus fermentum* CNCM 1-2998 (Lacc), which were recovered from the commercial probiotic Lacteol Fort (*Lactobacillus* LB, Axcan Pharma, France).

2.4 Antibiotic susceptibility testing

Susceptibility to several antibiotics was determined by commercial discs of Amoxicillin (AMX: 25 μg), Ampicillin (AM: 10 μg) Gentamicin (CN: 10 μg), Kanamycin (K: 30 μg), Amoxicillin/Clavulanic Acid (AMC: 20/10 μg), Tetracycline (TE: 30 μg), and Cefuroxime (CXM: 30 μg). For the disk diffusion assay, we used concentrations recommended by the Scientific Committee on Animal Nutrition (discs provided by Merck) [12]. We used *Escherichia coli* ATCC 25922 as quality control. The microbiological breakpoints reported by the FEEDAP standards were used to categorize lactobacilli as susceptible or resistant [4]. *Lactobacillus plantarum* ATCC8014 and *L. fermentum* CNCM 1-2998 (Lacc) were used as a reference to the test. Results were qualified as R (resistance), I (intermediate), and S (susceptible).

2.5 Antimicrobial spectrum of the selected isolates

The antimicrobial spectrum of the selected isolates was determined against Gram-positive and Gram-negative bacteria, as previously described by Garzon et al. [33]. The indicator bacteria were: *Salmonella enterica subsp. enterica* (Kauffmann and Edwards) Le Minor and Popoff ATCC 51741, *Salmonella enterica subsp. enterica* serovar Abaetetuba ATCC35640, *Escherichia coli* ATCC25922, *E. coli* ATCC10536, *Shigella sonnei* ATCC25931, *Streptococcus thermophilus* ATCC19258, *Enterobacter aerogenes* UTNEag1 (laboratory collection), *Salmonella* UTNSm2 (laboratory collection), *Shigella* UTNShg1 (laboratory collection), and *E. coli* UTNEc1 (laboratory collection). LAB was grown in MRS broth at 34°C for 24–27 hours and the supernatants were collected by centrifugation at 13,000 x g for 20 minutes at 4°C. The crude extract (CE) was recovered and filtered with a 0.22 μm porosity syringe filter. The indicator strains (100 μl) were grown in broth medium (7 log CFU/ml) and mixed with 3.5 ml of soft MRS agar (0.75%). It was then overlaid on nutrient agar plates and incubated at 37°C for 2 hours. The CE of each strain (100 μl) was transferred onto the reaction wells (6 mm) on overlaid agar, incubated at 37°C and subsequently examined for the presence of an inhibition zone at 48 hours. To rule out the possible inhibitory activity of organic acids, the CE was heated at 80°C for 10 minutes, the pH adjusted at 6.0 and the activity was determined. *Lactobacillus plantarum* ATCC8014 and *L. fermentum* CNCM 1-2998 (Lacc) were used as reference strains. MRS broth was used as a negative control. The experiments were run in triplicate and the mean value of the inhibition zone was determined. A numeric scale from zero to ten was included in the statistical analysis and the results were also qualitatively defined as narrow (inhibit less than 5 indicator strains) or broad activity (inhibit more than 5 indicator strains).

2.6 Statistical and phylogenetic analyses

The interpretation of the antibiogram results was assisted by the package AMR [34], which provided corresponding frequencies on the qualitative responses. The distances (Bray-Curtis) among samples were then projected in canonical space through a non-metric multidimensional scaling. Either putative genera of bacteria, assigned through the RDP Bayesian classifier algorithm [28], or the host plants were included as the grouping variable.

The metabolic profiling resulted in a matrix that could be interpreted in binary form, and from which it was possible to determine a set of distances (binary Bray-Curtis) for classifying samples through a cluster analysis (unweighted pair group method with arithmetic averages, UPGMA). Ordination methods were carried out through vegan [35] and ggdendro [36] in R [30].

Sequences were aligned trough Clustal Omega, as implemented in Geneious Prime 2020.2.3 [27]. A proper substitution model was obtained through jModeltest v. 2.1.10 [37] and selected by a Bayesian information criterion. A phylogenic hypothesis was inferred by Bayesian inference with Mr. Bayes 3.2.6 [38]. The selected HKY85 model included a proportion of invariable sites and varying rates across sites with a discretized gamma distribution (HKY85 + I + G). The Bayesian analysis included 1.1 million generations and four chains per run. Hypotheses were sampled every 200 generations and the first 10% of these samples were discarded. The remaining 90% of the trees and parameters were respectively summarized in a 50% majority-rule consensus tree.

3. Results and discussion

3.1 Wild fruits: A microenvironment of diverse lactic acid bacteria

Out of 41 isolates, the most frequently observed genus was *Lactiplantibacillus* (31 isolates), followed by *Lactococcus* (3 isolates), *Weissella* (3 isolates), and *Enterococcus* (1 isolate). Three isolates showed large divergence and were not identified by the taxonomic assignment algorithm (i.e. UTN39, UTN41, and UTN88) (**Figure 2a**). The former three isolates may represent unreported lineages or species. The presence of *Lactococcus* in plants or fruit is rare: thus, we only found few isolates belonging to such genus.

Isolates showed a remarkable distance to the outgroup reference samples, and most were included within a clade formed by *Lactiplantibacillus,* where they showed relatively small distances (i.e. small branch lengths). However, within this inclusive group of *Lactiplantibacillus* there was an ingroup with strong support (Bayesian posterior probabilities = 1) and formed by *Weissella, Enterococcus,* and *Lactococcus*. This paraphyletic ingroup, within a more inclusive group formed mostly by *Lactiplantibacillus* should be eventually resolved by the inclusion of additional molecular markers (**Figure 2a**). The paraphyletic ingroup contains samples that belong to the plant species *Costus* sp., which occurs exclusively to this clade (**Figure 2b**).

3.2 The metabolic profile reveals the divergent properties of selected LAB

LAB strains may present specific metabolic traits as a result of their microenvironmental origin (i.e. different species of fruits) and possess a unique portfolio of enzymes that allow them to metabolize various compounds found in the host plant or fruit matrices. We present a metabolic profile together with other properties that were analyzed in the obtained isolates (**Figure 3**). Within *Lactiplantibacillus*, the isolates showed a variable capacity to ferment sugars and hydrolyze esculin. These features were strain-dependent. Among the available isolates, UTN39 and UTN76 were the only two samples that metabolized ARG, while UTN37 and UTN39 hydrolyzed urea. The latter is a relevant characteristic for selecting probiotic strains [2]. Of particular relevance were the locations of UTN76 and UTN39 on the previously presented phylogenetic hypotheses (**Figure 2**); these two isolates showed large genetic distances relative to the other samples, or unique positions in clades that diagnose them as different or remarkable lineages. The observed patterns in the metabolic profile are rather complex. UTN37, UTN39, and UTN76 show noteworthy properties; they, however, differ in their response to other substrates (**Figure 3**); thus, the corresponding dendrogram, which results from clustering the observed metabolic profile, hints to four main groups of isolates (**Figure 4**).

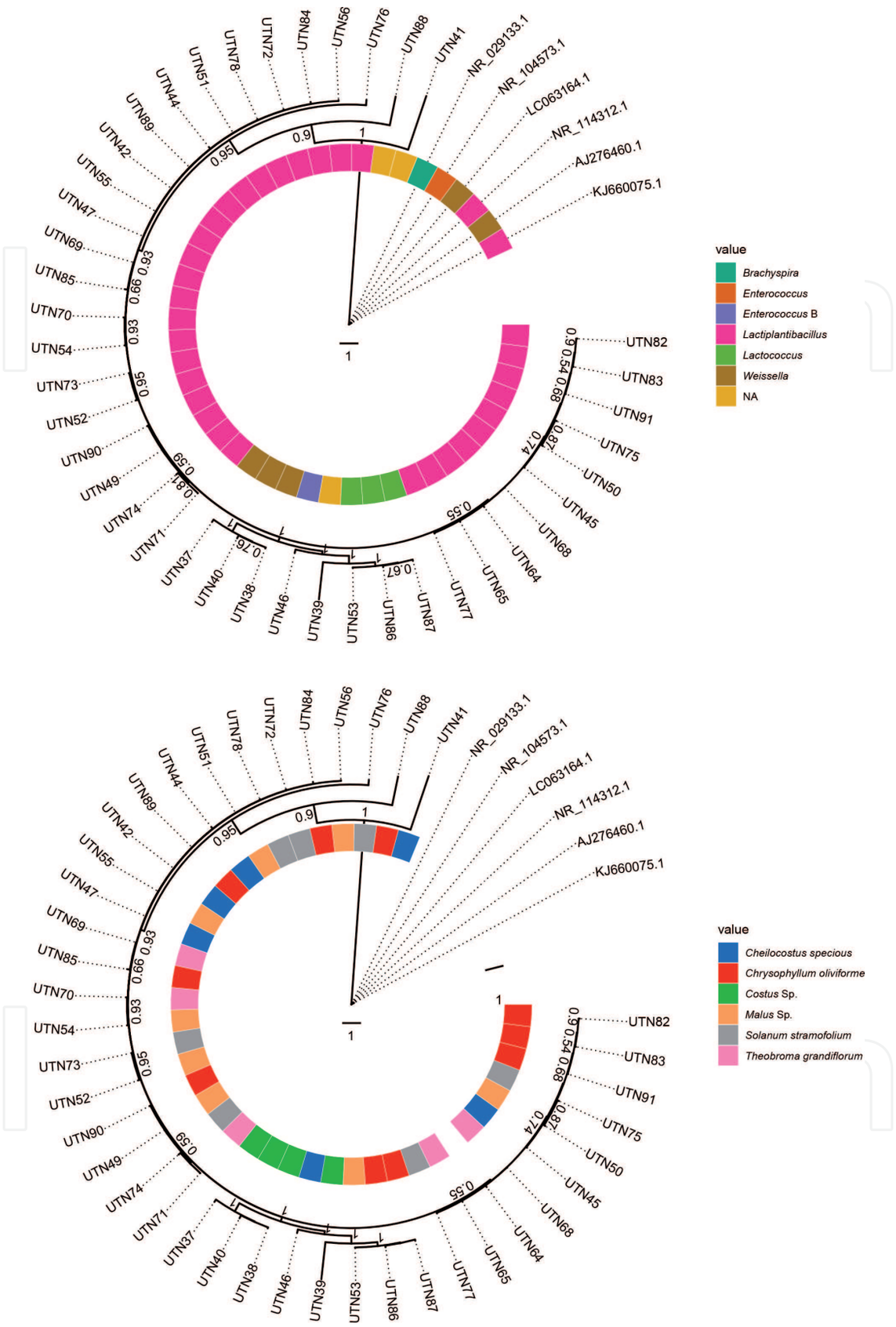

Figure 2.
(a) Phylogenetic hypothesis of the isolated samples and corresponding genera of bacteria. (b) The hypothesis with the corresponding host genera of plants. Branch support values are posterior probabilities from the applied Bayesian inference.

The ability to utilize the α-galacto-oligosaccharides-family (αGOS), d-melibiose [α-Gal-(1 → 6)-Glu], as well as the raffinose-family oligosaccharide (RFO) d-raffinose, seems to be a common feature among all the tested isolates. On the

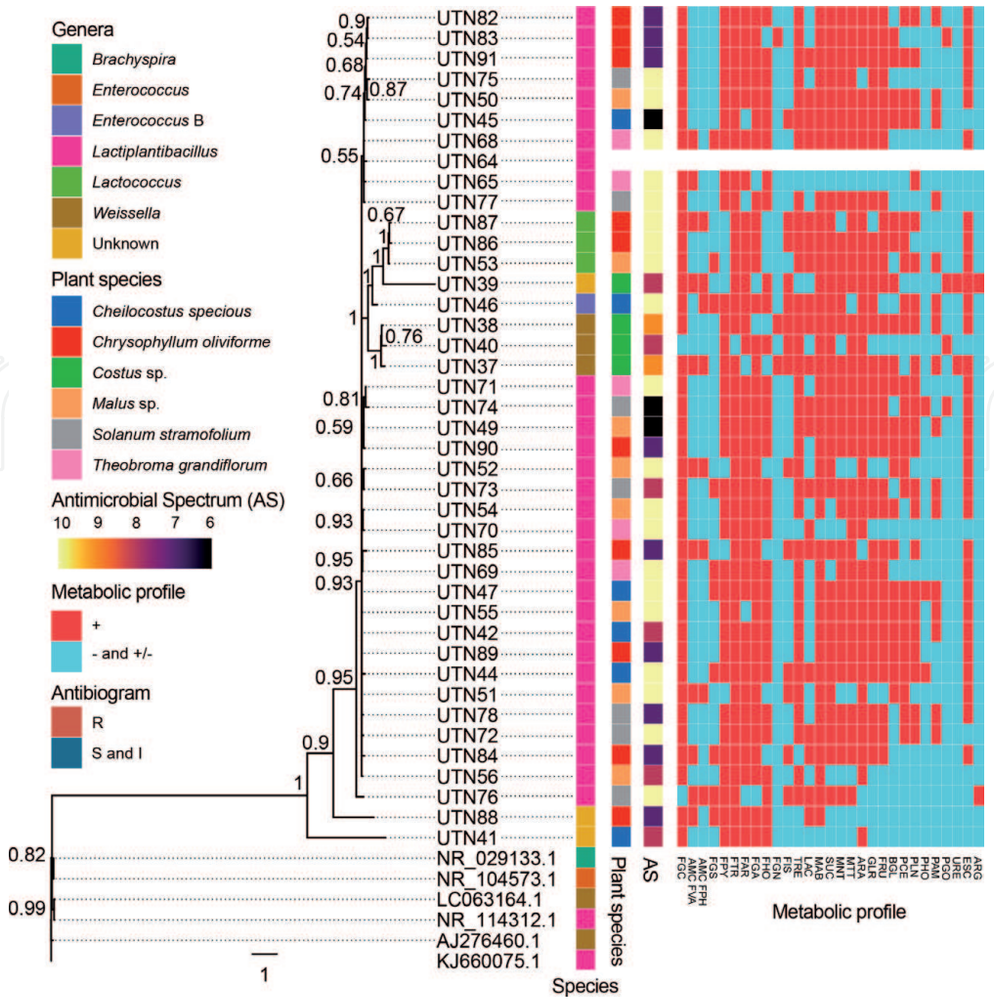

Figure 3.
Annotated phylogeny and heatmap showing the metabolic profiles of the LAB isolates. The phylogeny is an alternative depiction of the one shown in ***Figure 2*** *and serves as a reference to the profiles. The antimicrobial spectrum (AS) is also presented for each isolate.*

other hand, the metabolisms of the trisaccharide d-melezitose (α-Glu-(1 → 3)-β-Fru-(2 → 1)-α-Glu) and the disaccharides d-trehalose [α-Glu-(1 → 1)-α-Glu] and d-turanose (α-Glu-(1 → 3)-α-Fru) were strain-specific and restricted mainly to the *Lactoplantibacilaceae.* Although not classified, in a distinct lineage near the base of the tree, and with relatively large genetic distances, the isolates UTN41 and UTN88 did not metabolize most of the oligosaccharide substrates. The dissimilarity of individual isolates was supported by the different use of glycerol, trehalose, and sucrose. UTN40 was in the same lineage group as UTN37 and UTN38; however, it showed a distinct metabolic and antibiotic pattern, being the only one with resistance to AM10 and CN10. The isolate UTN76 showed a similar metabolic pattern to UTN88, both were the only isolates that did not metabolize p-n-p-β-D-cellobioside, p-n-p-phosphate, and proline. The *Lactococcus* species (UTN53, UTN86, and UTN87) were grouped in the same clade but were different in both the metabolic and antibiotic profiles.

The results revealed the production of β-galactosidase, α-glucosidase, β-glucosidase, and p-n-p-α-β-galactoside in some isolates. Although all isolates were originated from plants, there were differences in the utilization of mannitol and fructose. Previous studies have indicated that strains of *L. plantarum*, which were isolated from plant environments, often were able to metabolize these carbohydrates, and which has been considered as a fructophilic property [39].

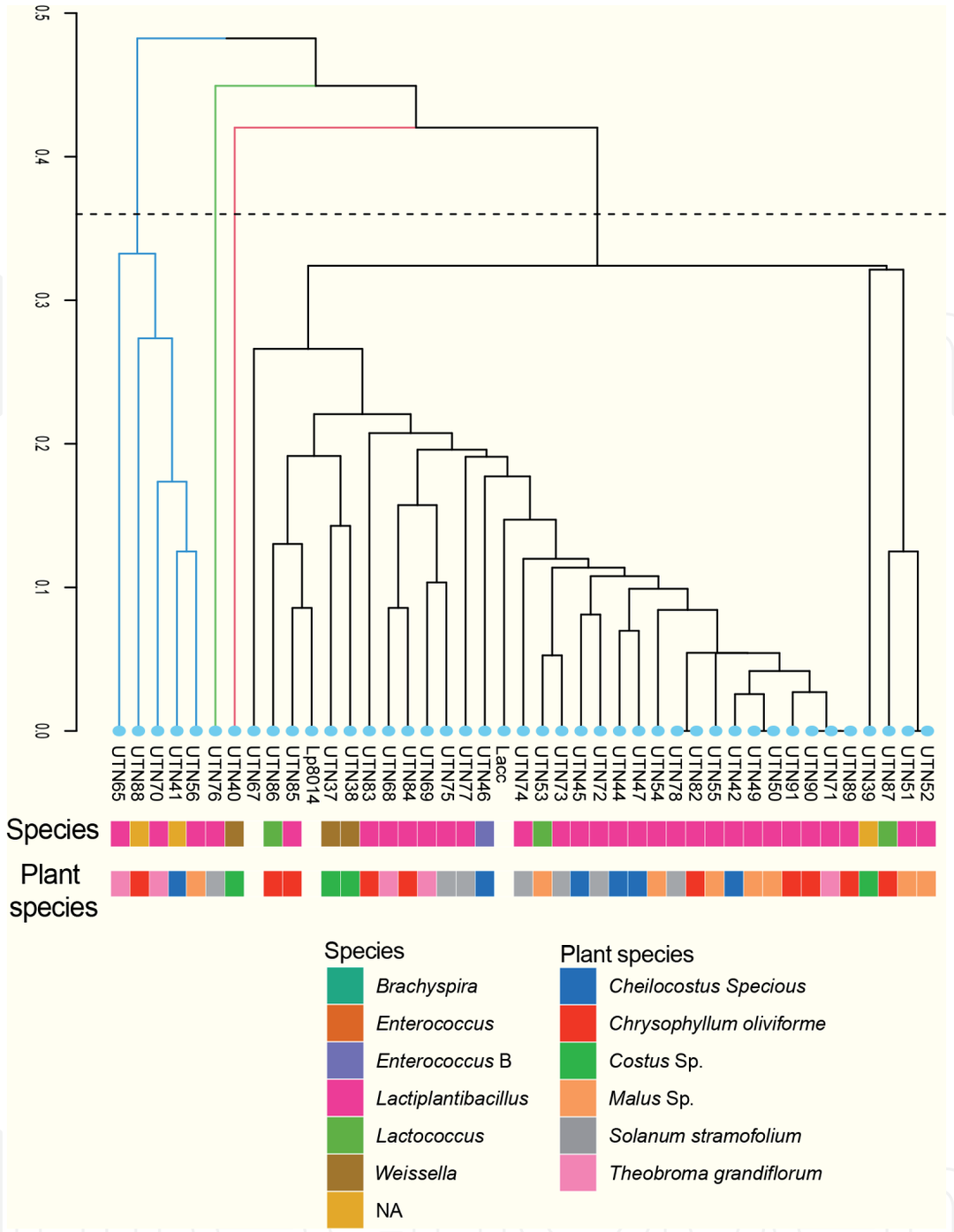

Figure 4.
Dendrogram from a cluster analysis on the metabolic profile for the obtained isolates. Four groups are possible to define at the established distance (dotted vertical line). The assigned species and the host plant species are represented as color codes.

3.3 The antimicrobial profile reveals that inhibitory activity is related to microenvironmental origin

AMX25 was the only antibiotic for which all isolates showed susceptibility, while all isolates showed innate resistance to MET5. Previous studies have indicated that Lactobacilli have a high natural resistance to metronidazole, as well as antibiotics that inhibit the synthesis of proteins such as chloramphenicol, erythromycin, clindamycin, and tetracyclines [40]. UTN41 was susceptible to almost all antibiotics. Among the *Lactiplantibacillus* genus, the isolates UTN76 and UTN88 were resistant to AMC30, and UTN84 and UTN89 were resistant to TE30. However, this relevant pattern did not show consistency with the fruit host, as the plant species

Chrysophyllum oliviforme, which was the origin of the resistant samples, was also the origin of other isolates in *Lactiplantibacillus*, but which did not coincide in their resistance pattern (**Figure 5**). Another relevant aspect was that of UTN68 (*Lactiplantibacillus*), which originated from *Theobroma grandifolium* and UTN40 (*Weissella*) from *Costus* sp., which were the only isolates showing resistance to AM10 (**Figure 5**).

Although resistance to gentamicin and kanamycin is considered a health concern, the isolates that showed resistance to both antibiotics were control reference strains that were isolated from dairy and human intestine. In general, patterns in the antibiogram were broad and yet inconclusive, as the position of isolates and plant hosts in canonical space show that further evidence is necessary to establish definitive patterns of association (**Figure 6**). However, this is also evidence of large variability across samples, with no conclusive patterns in isolated species or their host plants.

Although antibiotic resistance is a criterium to be considered from a biosafety perspective, it has been shown that the probiotic strains of starter culture strains

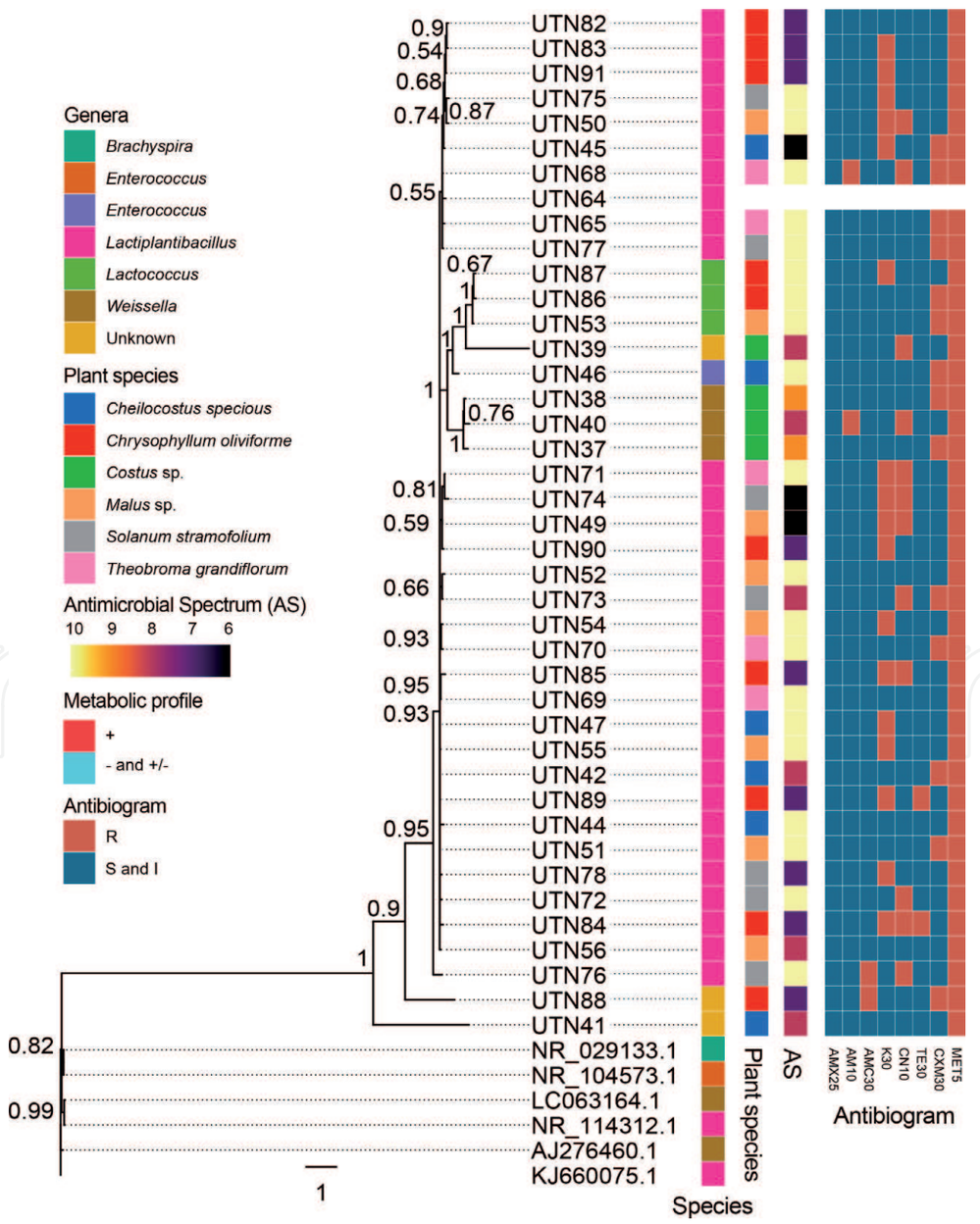

Figure 5.
Annotated phylogeny and heatmap showing antimicrobial profiles of the LAB isolates.

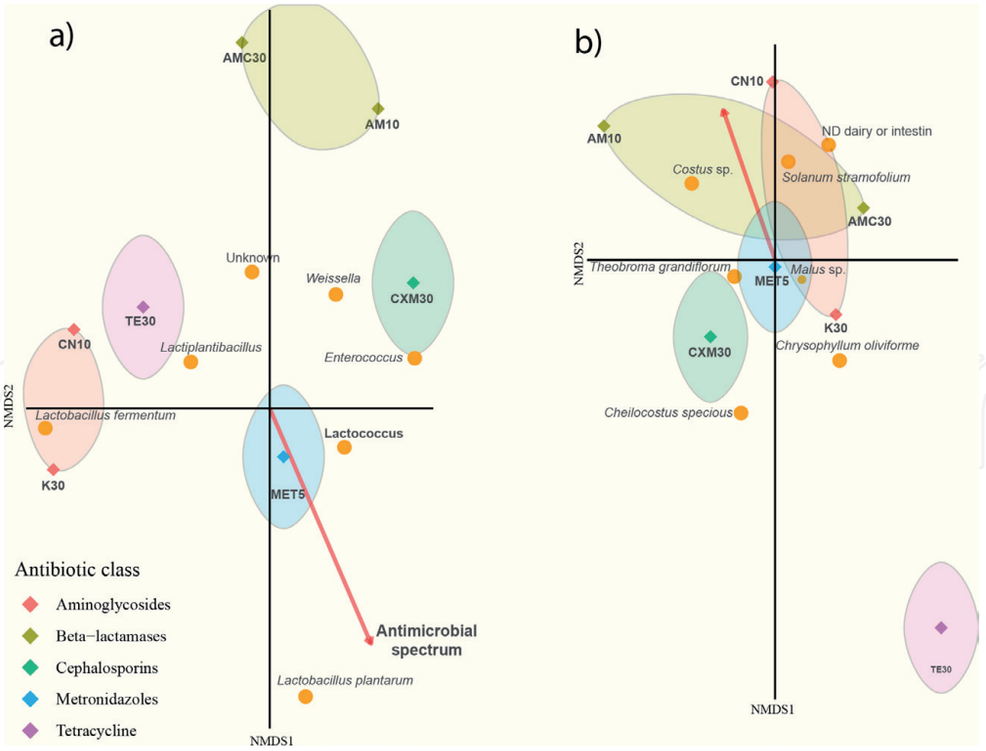

Figure 6.
Non-metric multidimensional scaling (NMDS) that represents, in two dimensions, the distances among isolates and host plants according to the observed pattern of antibiotic resistance. a) Tile for the spatial relationships in NMDS space between antibiotics and the studied bacterial isolates. b) Tile for spatial relationships in NMDS space between antibiotics and the host plants of the studied bacterial isolates. Short distances among these objects in the canonical space represent strong associations of either resistance or susceptibility. The antimicrobial spectrum is shown here as a cofactor showing a directional trend. AMX25 was removed from this analysis as it was not informative (i.e. susceptibility as a zero-response constant). The colored areas facilitate the interpretation of the antibiotics position. L. fermentum *and* L. plantarum *were used as a reference in the tests.*

are resistant to ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracyclines, and chloramphenicol [4, 7]. Another study has reported that Lactobacilli isolated from fermented olives were resistant to cephalosporins, streptomycin, and kanamycin [41]. Overall, the observed pattern is for high variability or diversity in the response to antibiotics as there is considerable dispersion in the response within and among bacterial species or plant hosts (**Figure 6**). However, there were also isolates with unique resistance patterns, which will require a further inquiry into their molecular and physiological properties. Undoubtedly, a full safety assessment with a robust identification of the strains and an in vitro evaluation of the potential risks is needed; particularly if these are intended to be used as additives in food products.

It is known that some LAB strains produce a wide variety of anti-pathogenic compounds, like bacteriocins, ethanol, organic acids, diacetyl, acetaldehydes, hydrogen peroxide (H_2O_2), and peptides [42, 43]. When we analyzed the antimicrobial spectrum against ten Gram-negative and Gram-positive bacteria, including closely related species and pathogens such as *Salmonella enterica, Shigella sonnei, Escherichia coli, Enterobacter, Staphylococcus aureus,* we observed that the isolates showed high inhibitory potential, as none had values below six and were defined as broad-spectrum (**Figures 3** and **4**). The inhibitory effect of LAB strains may result from a combination of competition for metabolic substrates, growth suppression by organic acids, and bacteriocin secretion. Recently, we showed that some of the Lactobacilli strains inhibited *Salmonella enterica subsp. enterica* ATCC51741 and *E. coli* ATCC25922 at both the early and logarithmic stages of bacterial growth *in vitro* and *ex vitro* [44, 45, 46]. Also, we showed that one selected LAB strain from the

Lactococcus genera harbored interesting functional properties to be used in starter culture formulations for dairy-based fermented food products [46].

4. Conclusions

The Amazon rainforest is a sizeable reservoir of plants, animals, and bacterial diversity. For Ecuador, the Amazon region could be a significant source of new bioproducts, based on the transformation of biodiversity [47]. Subtropical wild fruits have a relevant ethnobotanical significance, as they are mostly consumed by indigenous people as food or natural medicine; however, the bacterial microbiota of those fruits has not been assessed. In this research, we investigated the lactic acid bacteria diversity associated with several wild fruits collected from the Amazon region of Ecuador. Their remarkable inhibitory potential towards Gram-negative bacteria might be related to their capacity to produce various antimicrobial substances, that when applied to food products might prevent the growth of undesirable microorganisms. A better understanding of the metabolic capacity of these microorganisms will further complement our knowledge about the development of a novel starter or preservative culture for fruit- and vegetable-based foods. The prospective comparative exploration of the genomes of LAB strains from various plant or fruit origins would be of particular interest to provide information on their adaptations to different food-matrices and to further explore biotechnological applications.

Genotype-functional correlation studies contribute to the discovery of new biotechnological properties for several species. The results from the present study supported our hypothesis that LAB strains from wild fruits of the Amazon Region of Ecuador carry noteworthy characteristics that could be inherent to their ecological niches or environmental origin and that could be developed for biotechnological applications. Several strains were found capable of producing antimicrobials with high inhibitory potential against commensal and spoilage bacteria and are promissory natural food preservatives.

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Conflict of interest

The authors report no conflicts of interest. The authors are responsible for the content and writing of this article.

Author details

Gabriela N. Tenea[1]*, Pablo Jarrin-V[2] and Lucia Yepez[1]

1 Biofood and Nutraceutics Research and Development Group, Technical University of the North, Ibarra, Ecuador, Av. 17 de Julio s-21 y José María Córdova, Barrio El Olivo, 100150, Ibarra, Ecuador

2 Population and Environment Research Group, Universidad Regional Amazónica Ikiam, Tena, Ecuador

*Address all correspondence to: gntenea@utn.edu.ec; gtenea@hotmail.com

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References

[1] Garcia EF, Luciano WA, Xavier DE, da Costa WC, de Sousa Oliveira K, Franco OL. et al. Identification of lactic acid bacteria in fruit pulp processing byproducts and potential probiotic properties of selected *Lactobacillus* strains. Frontiers in Microbiology. 2016;7: 1371. DOI: 10.3389/fmicb.2016.01371

[2] Ruiz Rodríguez, LG, Aller K, Bru E, De Vuyst L, Hebert EM, Mozzi F. Enhanced mannitol biosynthesis by the fruit origin strain *Fructobacillus tropaeoli* CRL 2034. Applied Microbiology and Biotechnology. 2017;101:6165-6177. DOI: 10.1007/s00253-017-8395-1

[3] Szutowska J. Functional properties of lactic acid bacteria in fermented fruit and vegetable juices: a systematic literature review. European Food Research and Technology. 2020;246:357-372. DOI: 10.1007/s00217-019-03425-7

[4] European Food Safety authority (EFSA). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J. 2012: 10:2740-9.16. 732:1-15. DOI: 10.2903/j.efsa.2008.732

[5] Mattia A, Merker R. Regulation of probiotic substances as ingredients in foods: Premarket approval or "generally recognized as safe" notification. Clinical Infectious Diseases. 2008; 46(supplement_2):S115-S118. DOI: 10.1086/523329

[6] Laulund S, Wind A, Derkx PM, Zuliani V. Regulatory and safety requirements for food cultures. Microorganisms. 2017;5:E28. DOI: 10.3390/microorganisms5020028

[7] Fraqueza MJ. Antibiotic resistance of lactic acid bacteria isolated from dry-fermented sausages. International Journal of Food Microbiology. 2015, 212, 76-88. DOI: 10.1016/j. ijfoodmicro.2015.04.035

[8] Chao SH, Huang HY, Kang YH, Watanabe K, Tsai YC. The diversity of lactic acid bacteria in a traditional Taiwanese millet alcoholic beverage during fermentation. LWT - Food Sci and Technology. 2013;51:135-142. DOI: 10.1016/j.lwt. 2012.09.015

[9] Emerenini E, Afolabi O, Okolie P, Akintokun A. Isolation and molecular characterization of lactic acid bacteria isolated from fresh fruits and vegetables using nested PCR analysis. Brazilian Microbiology Research Journal. 2013;3:368-377. DOI: 10.9734/ BMRJ/2013/2520

[10] Mustopa AZ, Fatimah F. Diversity of Lactic acid bacteria Isolated from Indonesian traditional fermented foods. Microbiology Indonesia. 2014;8(2):48-57. DOI: 10.5454/mi.8.2.2

[11] Barraclough T, Balbi K, Ellis R. Evolving concepts of bacterial species. Evolutionary Biology. 2012;39:148-157. DOI: 10.1007/s11692-012-9181-8

[12] Naeem M, Ilyas M, Haider S, Baig S, Saleem, M. Isolation characterization and identification of lactic acid bacteria from fruit juices and their efficacy against antibiotics. Pakistan Journal of Botany. 2012;44:323-328

[13] Di Cagno R, Filannino P, Gobbetti M. Vegetable and fruit fermentation by lactic acid bacteria. In: Mozzi F, Raya RR, Vignolo GM, editors. Biotechnology of Lactic Acid Bacteria: Novel Applications. Chichester:John Wiley & Sons; 2015. p. 216-230. DOI: 10.1002/9781118868386.ch14

[14] Filannino P, Di Cagno R, and Gobbetti M. Metabolic and functional paths of lactic acid bacteria in plant foods: get out of the labyrinth. Current Opinion in Biotechnology. 2018;49: 64-72. DOI: 10.1016/j.copbio.2017.07.016

[15] Samuni-Blank M, Izhaki I, Laviad S, Bar-Massada A, Gerchman Y, Halpern M. The role of abiotic environmental conditions and herbivory in shaping bacterial community composition in floral nectar. PLoS ONE. 2014;9:e99107. DOI: 10.1371/journal.pone.0099107

[16] Benavidez A, Ulcuango M, Yépez L, Tenea, GN. Assessment of the in vitro bioactive properties of lactic acid bacteria isolated from native ecological niches of Ecuador. Revista Argentina de Microbiologia. 2016;48:236-244. DOI: 10.1016/j.ram.2016.05.003

[17] Xing J, Wang G, Zhang Q, Liu X, Gu Z, Zhang H, Chen YQ, Chen W. Determining antioxidant activities of lactobacilli cell-free supernatants by cellular antioxidant assay: a comparison with traditional methods. PLoS ONE. 2015;10(3):e0119058. DOI: 10.1371/journal.pone.0119058

[18] Khoo HE, Azlan A, Kong KW, Ismail A. Phytochemicals and medicinal properties of indigenous tropical fruits with potential for commercial development. Evidence-Based Complementary and Alternative Medicine. 2016;ID 7591951. DOI: 10.1155/2016/7591951

[19] Gul K, Singh AK, Jabeen R. Nutraceuticals and functional foods: The foods for the future world. Critical Reviews in Food Science and Nutrition. 2016;56(16):2617-2627. DOI: 10.1080/10408398.2014.903384

[20] Tenea GN, Hurtado P, Ortega C. Inhibitory effect of substances produced by native *Lactococcus lactis* strains of tropical fruits towards food pathogens. Preventive Nutrition and Food Science. 2018;23(3):260-268. DOI: 10.3746/pnf.2018.23.3.260

[21] Tenea GN, Pozo Delgado T. Antimicrobial peptides from *Lactobacillus plantarum* UTNGt2 prevent harmful bacteria growth on fresh tomatoes. Journal of Microbiology and Biotechnology. 2019;29:1553-1560, DOI: 10.4014/jmb.1904.04063

[22] Gueimonde M, Sánchez B, de Los Reyes-Gavilán GC, Margolles A. Antibiotic resistance in probiotic bacteria. Frontiers in Microbiology. 2013;4:202. DOI: 10.3389/fmicb.2013.00202

[23] Ceapa C, Lambertb J, van Limptb K, Welse M, Smokvinaa T, Knol J, Kleerebezemd M. Phenotype profiling links genotype to carbohydrate utilization signatures in the species *Lactobacillus rhamnosus*. Applied Environmental Microbiology. 2015; DOI: 10.1128/AEM.00851-15

[24] Bali V, Panesar PS, Bera MB, Kennedy JF. Bacteriocins: Recent trends and potential applications. Critical Review in Food Science and Nutrition. 2016;56(5):817-34. DOI: 10.1080/10408398.2012.729231

[25] Saha BC. A low-cost medium for mannitol production by *Lactobacillus intermedius* NRRL B-3693. Applied Microbiology and Biotechnology. 2006;72:676-680. DOI: 10.1007/s00253-006-0364-z

[26] De Man JC, Rogosa M, Sharpe E. A medium for the cultivation of lactobacilli. Journal of Applied Microbiology. 1960;23:130-155. DOI: 10.1111/j.1365-2672.1960.tb00188.x

[27] Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz A, Duran D, Thierer, T, Ashton B, Meintjes P, Drummond A. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28(12):1647-1649. DOI: 10.1093/bioinformatics/bts199

[28] Wang Q, Garrity G, Tiedje J, Cole J. Naïve bayesian classifier for rapid assignment of rRNA Sequences into the new bacterial taxonomy. Applied and Environmental Microbiology. 2007;73(16):5261-5267. DOI: 10.1128/aem.00062-07

[29] Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods. 2020; 13:581-583. DOI: 10.1038/nmeth.3869

[30] R Core Team R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. 2020. URL: https://www.R-project.org/

[31] Alishum A. DADA2 formatted 16S rRNA gene sequences for both bacteria & archaea (Version 3). Zenodo. 2020; DOI: 10.5281/zenodo.3951383

[32] Zheng J, Wittouck S, Salvetti E, Franz C, Harris H, Mattarelli P, O'Toole PW, Pot B, Vandamme P, Walter J., Watanabe K, Wuyts S, Felis GE, Gänzle MG, Lebeer S. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. International journal of systematic and evolutionary microbiology. 2020;70(4):2782-2858. DOI: 10.1099/ijsem.0.004107

[33] Garzón K, Ortega C, Tenea GN. Characterization of bacteriocin-producing lactic acid bacteria isolated from native fruits of Ecuadorian Amazon. Polish Journal of Microbiology. 2017; 66(4): 473-481. DOI: 10.5604/01.3001.0010.7037

[34] Berends MS, Luz CF et al. AMR - An R Package for working with antimicrobial resistance data. bioRxiv. 2019. DOI: 10.1101/810622

[35] Oksanen JF, Blanchet G, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens HH, Szoecs E, Wagner H. Vegan: community ecology package. R package version 2.5-6. 2019. https://CRAN.R-project.org/package=vegan

[36] de Vries A, Ripley BD. ggdendro: Create dendrograms and tree diagrams using 'ggplot2'. R package version 0.1.21. 2020. https://CRAN.R-project.org/package=ggdendro

[37] Darriba D, Taboada GL, Doallo R Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nature Methods. 2012;9(8):772

[38] Ronquist F, Huelsenbeck JP. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003;19:1572-1574

[39] Gustaw, K., Michalak, M., Polak-Berecka, M. et al. Isolation and characterization of a new fructophilic Lactobacillus plantarum FPL strain from honeydew. Annals of Microbiology. 2018;68:459-470. DOI: 10.1007/s13213-018-1350-2

[40] Abriouel H, Casado Muñoz MD, Lerma LL, Perez Montoro B, Bockelman W, Pichner R, et al. New insights in antibiotic resistance of *Lactobacillus* species from fermented foods. Food Research International. 2015;78:465-481. DOI: 10.1016/j.foodres.2015.09.016

[41] Casado Muñoz MC, Benomar N, Lerma LL, Gálvez A, Abriouel H. Antibiotic resistance of *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* isolated from naturally-fermented Aloreña table olives throughout fermentation process. International Journal of Food Microbiology. 2014;172:110-118. DOI: 10.1016/j.ijfoodmicro.2013.11.025

[42] Venegas-Ortega MG, Flores-Gallegos AC, Martínez-Hernández JL, Aguilar CN, Nevárez-Moorillón GV. Production of bioactive peptides from lactic acid bacteria: a sustainable approach for healthier foods. Comprehensive Review in Food Sciences and Food Safety. 2019;18:1039-1051. DOI: 10.1111/1541-4337.12455

[43] Simons A, Alanout K, Duval RE. Bacteriocins, antimicrobial peptides from bacterial origin: overview of their biology and their impact against multidrug-resistant bacteria. Microorganisms. 2020;8:639. DOI: 10.3390/microorganisms8050639

[44] Tenea GN, Lara MI. Antimicrobial compounds produced by *Weissella confusa* Cys2-2 strain inhibit Gram-negative bacteria growth. CyTA – Journal of Food. 2019;17(1):105-111. DOI: 10.1080/19476337.2018.1561520

[45] Tenea GN, Olmedo D, Ortega C. Peptide-based formulation from lactic acid bacteria Impairs the pathogen growth in *Ananas comosus* (Pineapple). Coatings. 2020;10: 457. DOI: 10.3390/coatings10050457

[46] Tenea GN, Suárez J. Probiotic Potential and Technological Properties of Bacteriocinogenic *Lactococcus lactis* subsp. *lactis* UTNGt28 from a native Amazonian fruit as a yogurt starter culture. Microorganisms. 2020;8:733. DOI: 10.3390/microorganisms8050733

[47] Jarrín Valladares PS, Carrillo LT, Zamora G. The internal colony as a current issue: transformation of the human territory in the Amazonian region of Ecuador. Letras Verdes. 2016;20:22-43. DOI: 10.17141/letrasverdes.20.2016.2063