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Predicting the Microbiome and Metabolome Dynamics of Natural Apple Fermentation Towards the Development of Enhanced Functional Vinegar

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Abstract: Natural vinegar fermentation is a complex process influenced by the interplay between microbial communities and metabolites. This study examined the interplay between the microbiome and the metabolome over a three-month period, with samples collected every ten days. Using Illumina sequencing and chromatographic techniques (HPLC and GC-MS), we mapped microbial shifts and metabolite profiles. Early fermentation showed a diverse microbial presence, including genera such as *Cronobacter*, *Luteibacter*, and *Saccharomyces*. A stable microbial ecosystem established between days 15 and 70, characterized by the dominance of *Leuconostoc*, *Gluconobacter*, and *Saccharomyces*, which facilitated consistent substrate consumption and metabolite production, including various organic acids and ethanol. By day 70, Acetobacter prevalence increased significantly, correlating with a peak acetic acid production of 12.4 g/L. Correlation analyses revealed significant relationships between specific microbes and volatile organic compounds. This study highlights the crucial roles of these microbes in developing sensory profiles suited for industrial applications and proposes an optimal microbial consortium for enhancing vinegar quality. These data suggest that an optimal microbial consortium for vinegar fermentation should include *Saccharomyces* for efficient alcohol production, *Leuconostoc* for ester-mediated flavor complexity, and *Acetobacter* for robust acetic acid production. The presence of *Komagataeibacter* could further improve the sensory and functional qualities due to its role in producing bacterial cellulose.

Keywords: LAB; *Lactobacillus*; acetic acid; natural fermentation

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1. Introduction

Vinegar, with its rich historical background, holds significance as an acidic seasoning utilized in culinary practices and daily life, having served medicinally for centuries in treating wound healing, poison ivy, croup, stomachaches, high fevers, edema, infections, and ulcerations [1]. Vinegar production involves two biochemical processes: alcoholic fermentation followed by acetic acid fermentation. In the first stage, sugars and/or starches are consumed by yeasts in anaerobic conditions, typically from the *Saccharomyces* genus [2]. Subsequently, acetic acid fermentation (AAF) of ethanol by acetic acid bacteria (AAB) occurs in an aerobic environment. The AAF microbial community comprises innumerous genera of AAB, such as *Acetobacter*, *Gluconobacter*, *Komagataeibacter*, and *Gluconacetobacter*. Nevertheless, species within the *Acetobacter* and *Komagataeibacter* genera notably demonstrate a robust capability for acetic acid production [3]. In AF, ethanol is converted to acetic acid through the action of two membrane-bound enzymes located on the outer surface of the cytoplasmic membrane (periplasmic side). Initially, alcohol dehydrogenase (ADH) oxidizes ethanol to acetaldehyde, which is further oxidized to acetic acid by aldehyde dehydrogenase (ALDH).

While all types of vinegar production follow the same sequence of biochemical steps, the composition of microbiota can vary significantly among them. Over the last twenty years, there has been significant progress in the field of studying fermented foods, attributable to advancements in next-generation sequencing, advanced mass analyzers, and other innovative tools [4]. These technologies have substantially increased the capacity and sensitivity of research in the field. Particularly, culture-independent taxonomic methods, predominantly relying on high-throughput sequencing, have been frequently employed and revealed numerous previously undetected non-dominant microbial. Various fermented foods, such as cheese, kefir coffee, cacao, yogurt, and vinegar, have had their microbial genomes sequenced, highlighting the intricate roles and contributions of diverse microorganisms in the fermentation process [5]. In natural vinegar fermentation, NGS has been revealing non-dominant groups such as *Lichtheimia*, *Pediococcus*, *Xanthomonas*, *Kazachstania*, *Gardnerella*, *Streptomyces*, *Pantoea*, *Pseudomonas*, *Serratia*, *Prevotella*, and *Corynebacterium* [6,7]. The unique qualities of various vinegars around the world are shaped by the specific raw materials used, the microbial environment, and the distinct brewing techniques employed [8].

The microbial composition of vinegar directly impacts the product's quality and the formation of volatile compounds during fermentation. Diverse raw materials influence its physical and chemical properties, thus affecting the taste, aroma, and overall quality. Volatile compound profiles of vinegars are closely tied to the raw materials, their composition, and the production methods used [8]. For instance, alcoholic fermentation is a crucial step because the alcohol produced serves as a precursor to many flavor compounds, such as ethyl acetate, isoamyl acetate, isoamyl alcohol, and benzaldehyde, significantly contributing to the final quality of vinegar [9,10]. Conversely, acetic acid fermentation is responsible for oxidizing ethanol into acetic acid and is also essential for the development of flavor compounds including acetaldehyde, benzaldehyde, acetone, succinic acid, and diacetyl. Lactic acid bacteria are present in natural fermentation vinegars, albeit in minor proportions compared to the dominant microbial groups.

The application of controlled fermentations using mixed cultures—comprising lactic acid bacteria and yeast—can enhance the production process by yielding vinegars enriched with nutrients such as vitamin B, flavonoids, and amino acids. This approach not only ensures better microbial regulation but also promotes a targeted increase in the concentration of key volatile compounds, particularly ethyl lactate, ethyl caprate, and ethyl caproate [11]. Although lactic acid bacteria appear in lower proportions compared to the dominant acetic acid bacteria, they play a significant role in shaping the vinegar's sensory profile by contributing to the synthesis of esters and other compounds such as ethyl acetate, acetaldehyde, and diacetyl [12].

Across European nations, a variety of time-honored vinegars, such as Italy's balsamic vinegar and Spain's sherry vinegar, are predominantly produced through liquid-state fermentation, primarily utilizing apple substrates [13]. In Asian countries, such as China, Japan, and Korea, vinegar production began around 1000 BC, and it is a highly appreciated ingredient commonly used to season dishes like seaweed salad, sushi, and boiled and steamed fish [1]. In Brazil, vinegar consumption reaches 170 million liters per year, with 80% being ethanol vinegar. According to the National Association of Vinegar Industries, Brazilian per capita consumption is 0.8 L, while in Europe and the USA, it reaches 1.8 L per capita [2]. Globally, natural vinegar fermentation has been extensively studied; however, there is limited research on the process in Brazil. Brazil's diverse climate, in contrast to Asia and Europe, may lead to the development of new species and biochemical processes unique to the region.

This study investigated microbial dynamics and metabolite profiles during vinegar fermentation, addressing a notable research gap in Brazil's vinegar production history. Considering Brazil's distinct climate and traditional methods of vinegar fermentation, elucidating the microbial composition and metabolic pathways involved in this process can offer valuable insights into optimizing raw material selection, fermentation conditions, and processing techniques. These findings are crucial not only for enhancing vinegar

production practices in Brazil but also for advancing the global understanding of vinegar fermentation processes.

2. Materials and Methods

2.1. Fermentation and Sampling

An acetic acid inoculum (also known as "Mother of Vinegar") was collected from a private household that traditionally produces apple vinegar in Curitiba city, Paraná State, Brazil. The inoculum was maintained at ambient temperature (approximately 18.61 ± 2.72 °C) for 4 weeks prior to inoculation. Fermentation process was conducted traditionally in duplicate glass urns of 3 L total volume for 3 months. Fresh organic apples were purchased from a local city market and utilized at a rate of 0.5 kg per liter of must. The must consisted of manually macerated apples and mineral water, while commercial white sugar was incorporated until achieving 20 °Bx. The alcoholic fermentation phase was conducted spontaneously, as traditionally practiced in Brazil. In the sixth week, acetic acid inoculum was added to start acetic fermentation in a proportion of 10%. The urns were kept at ambient temperature (approximately 18.61 ± 2.72 °C). Samples (20 mL) of fermenting vinegar were collected at intervals of 10 days (10 weeks total) to perform microbiological and metabolite target analyses. At each sampling point, the pH was measured using a digital pH meter (LUCA-210 model, Requipal, Curitiba, PR, Brazil).

2.2. DNA Extraction and Metataxonomic Analysis

DNA was extracted from each sample utilizing the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), following the protocol provided by the manufacturer. Following extraction, DNA concentrations were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified from the isolated DNA using primers 341F and 805R. For the amplification of the fungal ITS region, primers ITS 3S and 4R, tagged with Nextera indices, were used in accordance with the manufacturer's instructions (Illumina Inc., San Diego, CA, USA). Paired-end sequencing (2 × 250 bp) was performed on a MiSeq platform using the MiSeq v2 reagent kit (Illumina, San Diego, CA, USA). The resulting raw sequencing reads were analyzed using the QIIME (Quantitative Insights into Microbial Ecology) pipeline, where sequences shorter than 100 bp or containing more than one ambiguous base (N) were filtered out. High-quality sequences were aligned against the SILVA database using the UCLUST algorithm, and taxonomic classification along with the generation of operational taxonomic units (OTUs) was carried out at a 97% sequence identity threshold.

2.3. Bioinformatic Analyses

After completing the sequencing process, chimeric sequences were identified and removed, along with the reduction of noise during pre-clustering and taxonomic assignment, using the default settings of QIIME software version 1.9.0. Employing the UCLUST method (Edgar, 2010), sequences exhibiting greater than 97% similarity were categorized as identical operational taxonomic units (OTUs) according to the SILVA database and QUAST (Quality assessment tool for genome assemblies).

2.4. Co-Occurrence/Co-Exclusion Analysis

The relationships between variables were evaluated using Spearman correlation analysis, performed with R v4.2.3 and the corrplot package. Network diagrams were created and displayed with the open-source software Gephi v0.10.1, employing the Yifan Hu algorithm for node distribution. These maps, showing the Spearman correlation coefficients as edges, illustrate the complex interactions between microbial species and their impact on vinegar flavor profiles during the fermentation process in Brazil. The relationships between variables were evaluated using Spearman correlation analysis, performed with R v4.2.3 and the corrplot package.

2.5. Consumption and Production of Substrates

The determination of sugar consumption and organic acid production was performed through periodic sampling using high-performance liquid chromatography (HPLC) with slight modifications [14]. A 2 mL aliquot was centrifuged at 6000× *g* for 15 min and subsequently filtered through a hydrophilic Polyethersulfone (PES) membrane with a pore size of 0.22 µm (Millipore Corp., Burlington, MA, USA). An aliquot of 100 µL of the filtered samples was injected into the HPLC system, which was equipped with an Aminex HPX 87 H column (300 × 7.8 mm; Bio-Rad, Richmond, CA, USA) and a refractive index (RI) detector (HPG1362A; Hewlett-Packard Company, Palo Alto, CA, USA). The elution of the column was conducted in isocratic mode using a mobile phase of 5 mM H_2SO_4 at 60 °C, with a flow rate set at 0.6 mL/min.

2.6. Secondary Metabolites Formation

The extraction of volatile compounds was performed using a headspace (HS) vial coupled to a SPME fiber (CAR/PDMS df75 µm partially crosslinked; Supelco., Saint Louis, MO, USA). For each determination, 2 mL of sample was stored in a 20 mL HS vial in triplicate. The SPME fiber was exposed for 30 min at 60 °C. The compounds were thermally desorbed into the GC injection system gas phase (GC-MS TQ Series 8040 and 2010 Plus GC-MS; Shimadzu, Tokyo, Japan) at 260 °C. The column oven temperature was maintained at 60 °C for 10 min, followed by two heating ramps of 4 and 10 °C/min until reaching the temperatures of 100 and 200 °C, respectively. The compounds were separated on a 95% PDMS/5% PHENYL column (30 m × 0.25 mm × 0.25 mm film thickness). The GC was equipped with an HP 5972 mass selective detector (Hewlett Packard, Palo Alto, CA, USA). The compounds were identified by comparison to the mass spectra from library databases (Nist'98 and Wiley7n).

3. Results and Discussion

3.1. Microbial Dynamics Profile During Spontaneous Vinegar Fermentation

A total of 2,044,774 paired-end reads were obtained from Illumina sequencing for prokaryotes and 2,148,806 for eukaryotes, grouped into 237 and 32 OTUs, respectively, at 97% sequence similarity. Figure 1 displays the rarefaction curves for the prokaryotic (A) and eukaryotic (B) rRNA gene sequences, showing an increase in alpha diversity with deeper sequencing for both datasets. In both cases, the curves reach a plateau at higher depths, indicating that the sequencing coverage was sufficient. The prokaryotic dataset exhibited greater diversity compared to the eukaryotic dataset, suggesting a higher species richness or evenness in the bacterial community over time. The sequences were classified at the genus level, the lowest taxonomic rank, using QIIME and the SILVA database, with the results presented in Figure 2.

The observed microbial succession underscores the dynamic nature of spontaneous fermentation, where the initial microbial diversity gradually transitions to communities dominated by key functional microorganisms. In Figure 2A, the initial bacterial community composition was characterized by a high genera diversity, with predominant populations including *Cronobacter*, *Luteibacter*, *Erwinia*, *Agrobacterium*, *Pseudomonas*, and *Methylobacterium*. The early presence of diverse bacteria likely contributed to the breakdown of complex substrates, setting the stage for the establishment of AAB, LAB, and yeast. These microbial groups are associated with environmental sources, substrates, and human intervention in traditional vinegar production. For example, *Cronobacter* is found in various food matrices, *Luteibacter*, *Erwinia*, and *Agrobacterium* are plant-associated, and *Pseudomonas*, *Methylobacterium*, and *Sphingomonas* are abundant in apple ecosystems [15–19].

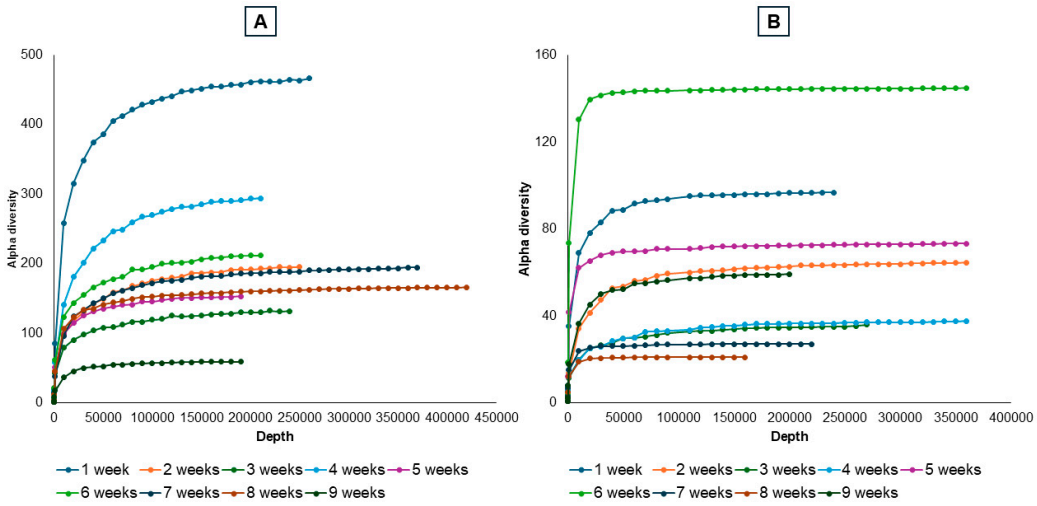

Figure 1. Rarefaction curves for 16S rRNA (**A**) and 18S rRNA (**B**) gene sequences obtained from NGS analysis of microbial samples collected over a three-month vinegar fermentation period.

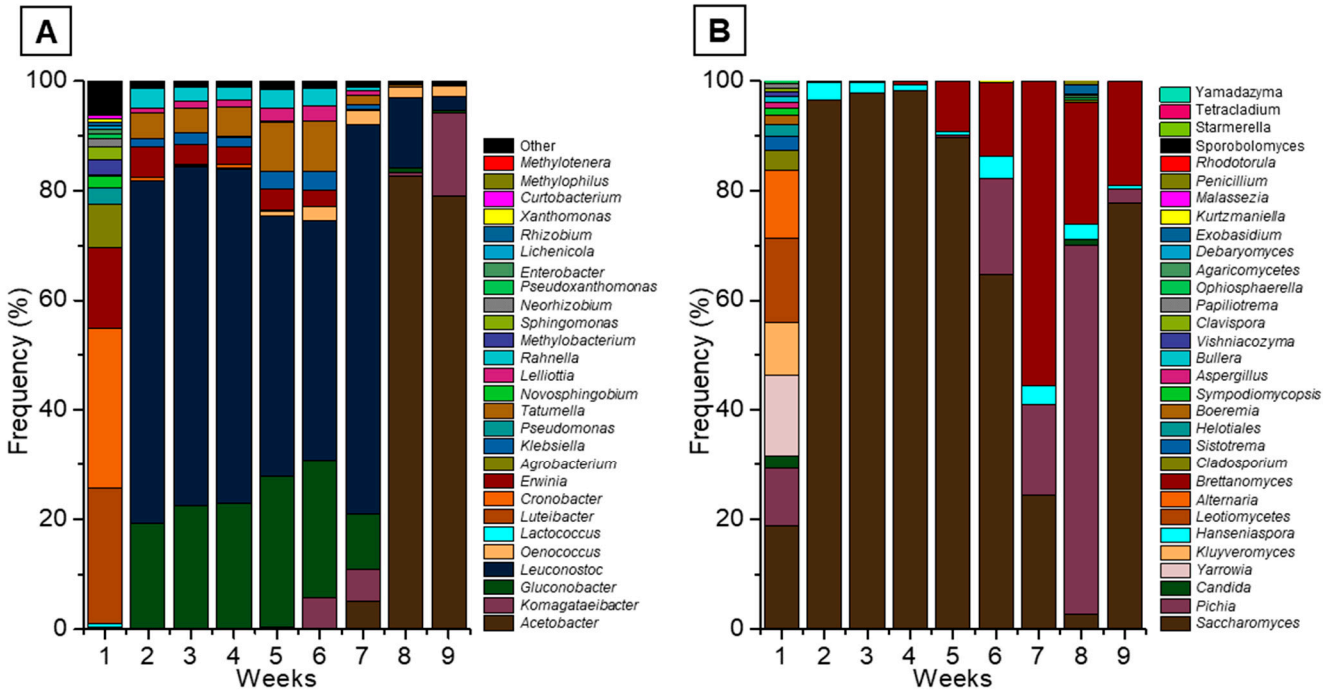

Figure 2. Microbial dynamics during vinegar fermentation analyzed using Illumina amplicon sequencing for bacteria (**A**) and fungi (**B**). The bar plots represent the relative abundance of microbial taxa across nine fermentation weeks, illustrating the succession and dominance of key groups involved in the process. The complete list of microbial groups is reported in the Supplementary Materials section (Table S1).

As fermentation progressed, the bacterial community underwent significant changes. From weeks two to seven, *Leuconostoc* dominated the bacterial community, indicating its crucial role in the mid-stages of fermentation. This genus plays a key part in shaping the fermentation environment by producing lactic acid, which lowers the pH and creates conditions favorable for subsequent microbial activities [20]. Although *Leuconostoc* is a common genus in vinegar fermentation [21], it is typically less prevalent than other LAB such as *Lactobacillus* (recently reclassified as *Lacticaseibacillus*) [22]. However, this is the first documented case where *Leuconostoc* has been found in higher concentrations than *Lactobacillus* during the traditional fermentation of apple vinegar. This discovery suggests a

potentially significant shift in microbial dynamics, highlighting the importance of *Leuconostoc* in the development of flavor and quality characteristics that may have been previously underestimated in apple vinegar fermentation. Furthermore, *Leuconostoc* species secrete bacteriocins and other antimicrobial compounds—such as acetic acid, phenyllactic acid, and hydroxyphenyllactic acid—which inhibit spoilage microorganisms and pathogens, contributing to microbial stability [23]. This finding highlights the potential role of *Leuconostoc* in shaping the microbial dynamics and contributing to unique flavor profiles in apple vinegar, suggesting that it may play a more influential role than previously recognized.

Gluconobacter was prevalent from weeks two to seven, contributing to the oxidative conversion of sugars into organic acids, lowering the pH, and shaping the fermentation profile [24]. Commonly found on harvested apples, pomace, and juice [25], it plays a key role in vinegar production by oxidizing sugars to acids without complete breakdown, enhancing the acidity and flavor [26]. This genus also produces gluconic acid, keto gluconates, and bioactive compounds like riboflavin (B2), improving both product quality and microbial stability [3,27]. *Gluconobacter* species, including *G. japonicus*, are found in the early fermentation stages of various vinegars such as Persian date vinegar [28]. Its versatile contributions underscore its importance in enhancing the vinegar quality and health benefits.

In the final stages (weeks eight to nine), *Acetobacter* became dominant, representing over 80% of the reads. Known for its essential role in vinegar production, Acetobacter oxidizes ethanol into acetic acid, the primary component of vinegar. Its efficient metabolism enables it to thrive in acidic environments with high ethanol levels, ensuring the complete conversion of alcohol into acetic acid and finalizing the fermentation process. Additionally, *Komagataeibacter* (15.23%) appeared and shared dominance in the final week. This genus is recognized for its efficient acetic acid production and robust biofilm formation, which contribute to the stability and quality of the vinegar product. *Komagataeibacter* species are known for their ability to produce cellulose, creating a thick biofilm that protects the bacteria and helps maintain optimal fermentation conditions. This biofilm formation is crucial for the continuous and consistent production of acetic acid, as it helps in maintaining the bacterial population in a stable state, ensuring a high yield and quality of vinegar. Furthermore, *Komagataeibacter* can tolerate high acetic acid concentrations, making it highly effective in the latter stages of fermentation when acetic acid levels are at their peak [29,30]. European studies on vinegar fermentation reveal a trend of *Komagataeibacter* dominating in red wine vinegar, while in apple vinegar, both *Komagataeibacter* and *Acetobacter* are typically balanced throughout the process [31,32]. However, in the present study, *Acetobacter* were unexpectedly far more prevalent than *Komagataeibacter*. This shift in microbial dominance suggests that environmental factors, fermentation conditions, or substrate composition may favor *Acetobacter* over *Komagataeibacter*, potentially altering the fermentation dynamics and influencing the final product's acidity and flavor profile.

Figure 2B presents the fungal community succession over a nine-week fermentation period. Initially, a diverse array of fungi genera was observed, including *Saccharomyces* (18.77%), *Leotiomycetes* (15.52%), *Yarrowia* (14.68%), *Alternaria* (12.22%), *Pichia* (10.54%), *Kluyveromyces* (9.52%), *Cladosporium* (3.76%), *Sistotrema* (2.41%), *Helotiales* (2.27%), *Boeremia* (1.76%), *Candida* (2.41%), *Sympodiomycopsis* (1.16%), and *Aspergillus* (1.11%). As fermentation progressed, *Saccharomyces* became increasingly dominant, maintaining a frequency of over 70% from the second week of fermentation.

Saccharomyces play a crucial role in the alcoholic fermentation phase, converting sugars into ethanol, which serves as a precursor for acetic acid production by AAB [33]. This yeast exhibits superior competitiveness in the fermentative environment due to its tolerance to high ethanol concentrations, efficient sugar metabolism, and ability to thrive under low oxygen conditions [34]. Additionally, *Saccharomyces* contributes to the flavor profile by generating esters and alcohols during fermentation, which enhance the sensory qualities of the final product [28]. Thus, the careful selection of yeast strains for mixed cultures is critical to vinegar fermentation, as it directly influences the balance between alcoholic

and acetic fermentations, ultimately shaping the beverage's quality. Yeast strains can also modulate the interplay between acetic acid fermentation and lactic acid production, further affecting the complexity and stability of the final product [35].

Brettanomyces and *Pichia* emerged in the final stages of the fermentation process (Figure 2). *Brettanomyces* are known for producing a range of volatile compounds, including phenolic compounds such as 4-ethylphenol and 4-ethylguaiacol, which can impart complex flavors like smoky, spicy, or barnyard notes [36]. While these compounds add depth and character in controlled amounts, an excess of *Brettanomyces* activity can result in off-flavors, making its presence a double-edged sword, valuable in moderation but potentially detrimental if overexpressed. *Pichia*, on the other hand, have been identified in organic apple cider vinegars, showing resistance to acetic acid concentrations up to 12 g/L [33,34,36,37]. Their acid tolerance and persistence during fermentation promote high ester production, contributing to the creation of a flavorful vinegar.

3.2. Substrates and Metabolites

The observed dynamics of the HPLC analysis of the compounds revealed distinct metabolic patterns within the vinegar fermentation process (Figure 3). Glucose and fructose from supplemented sugar, along with apple-derived fructose, gradually decreased across sampling points, reflecting their utilization by yeasts and LAB for energy and biosynthesis. By the end of fermentation, their concentrations were 44.28 and 5.77 g/L, respectively, indicating that fructose was the preferred substrate for consumption. A declining trend suggests the conversion of glucose and fructose in two different moments. Glucose was sharply consumed until week three, after which its consumption stabilized until the end of fermentation. This is likely due to most microorganisms being glucophilic and preferentially utilizing glucose to convert into final metabolites. However, fructose was drastically consumed from week five onwards, resulting in low residual levels by the end of the vinegar fermentation. This is consistent with the fact that the genus *Acetobacter*, which utilizes fructose for cellulose production, begins to appear in the Illumina analysis in weeks six to seven, reaching nearly 80% dominance by the end of fermentation [38].

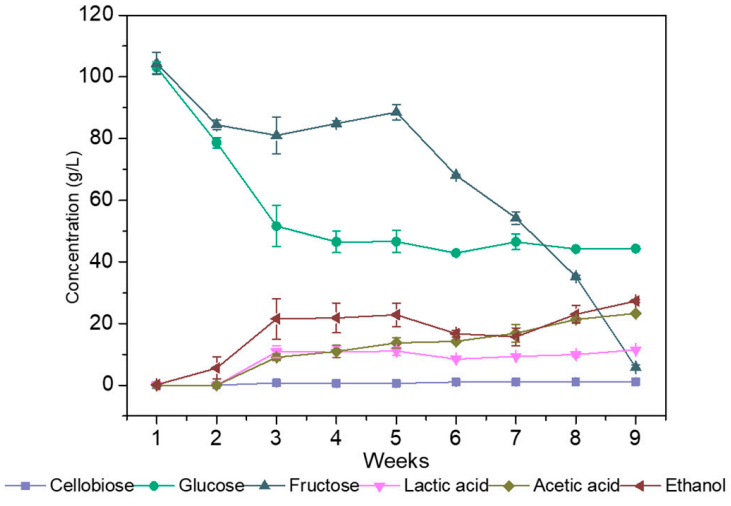

Figure 3. Sugar consumption and organic acids production (g L^{-1}) during a three-month vinegar fermentation.

Lactic acid production increased with the rise of the LAB populations, peaking at 11.48 g/L by the end of 10 weeks, followed by a plateau. This plateau may indicate that the LAB populations stabilized, continuing to metabolize available glucose and fructose while other microbial groups began contributing to fermentation dynamics. The early and continuous presence of LAB reflects their pivotal role in establishing favorable conditions, such as pH reduction, that shape the microbial community throughout the process.

Ethanol levels steadily increased from the start of fermentation, peaking at week eight, reflecting continuous microbial activity and effective sugar conversion despite fluctuations in substrate availability. The high ethanol concentration at this stage is essential, as it serves as a precursor for acetic acid production by the AAB during the subsequent acetous fermentation. The presence of ethanol throughout the process also highlights the persistence of fermentative yeast, which play a critical role in sustaining the microbial community dynamics.

By week nine, ethanol levels declined as the AAB oxidized it into acetic acid, which accumulated progressively throughout the acetous phase, peaking at 23.30 g/L. This gradual increase in acetic acid confirms the efficient conversion of ethanol, ensuring the completion of vinegar fermentation. Notably, the acetic acid concentration remained moderate compared to other vinegar fermentations, such as persimmon vinegar, where acetic acid levels surpass 40 g/L [39], a concentration lethal to sensitive species like *Acetobacter* and *Komagataeibacter*.

The moderate acidity in the present fermentation supported the persistence of both *Acetobacter* and *Komagataeibacter*, with *Acetobacter* showing a particular advantage under these conditions. This lower acetic acid concentration fostered microbial diversity, ensuring that sensitive species could thrive and contribute to the fermentation process, leading to a more complex microbial ecosystem.

The dynamic interplay between glucose and fructose utilization, ethanol production, and acetic acid accumulation reflects the intricate balance of metabolic pathways. The effective management of substrate availability and fermentation conditions is essential to maintain microbial diversity, optimize product quality, and ensure stability throughout the process. These interactions highlight the importance of precision in fermentation to achieve a desirable balance between microbial activity and product attributes.

3.3. Volatile Compound Formation

The HS-SPME/GC analysis identified 56 volatile compounds, including 17 esters, 14 alcohols, 13 carboxylic acids, 6 aldehydes, 3 ethers, 2 alkanes, and 1 ketone (Figure 4). The sample underwent two distinct fermentation phases. In the initial alcoholic fermentation, glucose was converted into ethanol by yeasts present in the microbiota, as expected [40]. This was followed by acetic fermentation, during which acetic acid bacteria (AAB) transformed the ethanol into acetic acid. This sequential fermentation not only produced a variety of volatile compounds but also resulted in the consumption or bioconversion of other substances, contributing to the complexity and depth of the final product.

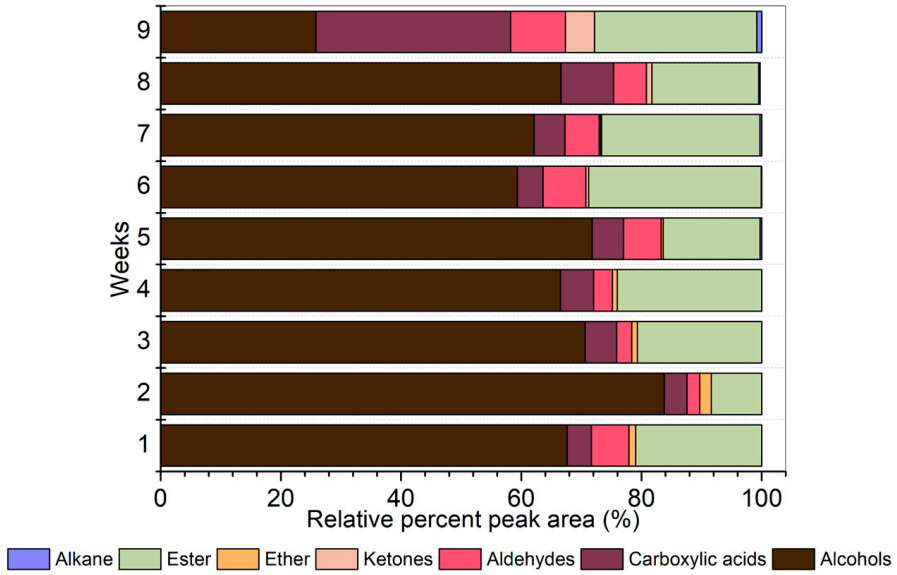

Figure 4. Volatile compounds (area $\times 10^5$) identified in apple vinegar fermentation by GC-MS analysis.

At the beginning of fermentation (Table 1), aldehydes, alcohols, and esters were detected. Aldehydes and alcohols were particularly prevalent in the early stages due to alcoholic fermentation. Alcohols are synthesized through the primary and secondary metabolism of yeasts, such as the Shikimate Pathway, which is responsible for the biosynthesis and conversion of aromatic amino acids into various compounds [41]. Phenethyl alcohol, a higher alcohol with a rose-like aroma, is produced by the Shikimate Pathway and exhibited the highest concentration (703) among the alcohols at the onset of fermentation. This can be correlated with the dominance of *S. cerevisiae*, which is known for producing this compound. The majority of alcohols act as precursors for other compounds and are subsequently consumed over the course of the fermentation process.

Table 1. GC-MS profile aroma of apple natural vinegar expressed by concentration of volatile aroma compounds (area $\times 10^5$).

Compound	Odor	Taste	Beginning (Week 1)	Middle (Week 5)	End (Week 9)
Carboxylic acids					
Octanoic acid	Faint/Fruity-acid	Slightly sour	26,177	24,157	41,033
4-Terpineol	Pine	Herbal pepper	18,017	13,141	0
Nonanoic acid	Fatty	Coconut	0	3586	13,084
Butyric acid	Rancid	Butter-fat	0	0	66,785
Isovaleric acid	Rancid-cheesy	Acid	0	0	57,223
Caproic acid	Characteristic goat-like	ND	0	0	14,583
Aldehydes					
2,4-dimethyl Benzaldehyde	Bitter almond	ND	115,062	129,278	53,130
Decanal	Floral-fatty/citrus	Sharp orange	5552	6717	4844
Nonanal	Orange-rose	ND	4406	7.5	5698
Benzaldehyde	Almond oil	Burning aromatic/Bitter almond	0	0	27,199
Ketones					
Acetoin	Buttery	Fatty creamy	0	10,032	48,329
Alcohols					
Phenylethyl alcohol	Rose-like	Initially bitter then sweet/Reminiscent of peach	703,008	652,024	76,180
Isoamyl alcohol	Disagreeable	Pungent/Repulsive	361,700	338,652	3310
1-Butanol	Harsh fusel with banana	Banana/Fusel	51,492	39,933	0
1-Hexanol	Sweet alcohol	Fatty/Fruity	34,249	32,602	0
Isoamyl acetate	Pear-like	Bittersweet reminiscent of pear/Slight apple	24,254	24,714	0
Benzyl alcohol	Faint aromatic	Sharp burning	23,079	19,076	0
2,3-Butanediol	Odorless	Sweet	136,944	0	0
2-Ethyl-1-hexanol	Mild/Oily/Sweet/Floral/ Reminiscent of rose	Sweet/Fatty-floral/Fruital note	4458	5116	40,784

Table 1. *Cont.*

Compound	Odor	Taste	Beginning (Week 1)	Middle (Week 5)	End (Week 9)
Ether					
Estragole	Reminiscent of anise	Sweet	13,933	0	0
Benzene	Aromatic	ND	8537	0	0
Ester					
Phenethyl acetate	Fruity	Flower/Honey/Rose	165,065	171,126	52,570
Ethyl palmitate	Waxy	ND	115,960	116,372	37,692
Ethyl decanoate	Oily brandy-like	Brandy/Grape/Pear	70,352	109,201	0
Ethyl octanoate	Wine/Brandy/Fruity/Floral	Apricot/Brandy/Fat/Floral/ Pineapple	32,700	74,141	0
Ethyl hexanoate	Wine-like	Apple Peel/Brandy/Fruit Gum/Overripe Fruit/Pineapple	7287	12.9	0
Ethyl dodecanoate	Fruity/Floral	Floral/Fruit/Leaf	0	50,400	0
Ethyl tetradecanoate	Waxy/Reminiscent of orris	Wax	0	17,668	8526
Ethyl butyrate	Banana/Pineapple	Sweet/Pineapple	0	0	16,697

ND: not detected.

Aldehydes are formed from the oxidation of the alcohols and fatty acids present in apples. 2,4-dimethyl benzaldehyde, an aldehyde with a bitter almond aroma, exhibited its highest concentration (115) at the beginning of fermentation and its lowest concentration (53) at the end. This compound is not typically associated with microorganisms or fruits. Conversely, benzaldehyde was observed only at the end of fermentation. This almond-like aldehyde is produced by certain yeasts, such as *Saccharomyces cerevisiae* and *Candida* spp., as the byproduct of alcoholic fermentation, especially under stress conditions. Long-term fermentation can elevate stress within microorganisms due to several factors, such as metabolite accumulation and nutrient limitation, which can explain the increase in benzaldehyde levels only during the final stages of fermentation [42].

Esters that enhance the aromatic profile of vinegar can be formed during fermentation or occur naturally in apples. These compounds are completely or partially consumed during the fermentation process, serving as precursors for other volatile compounds. Phenethyl acetate (165), ethyl palmitate (116), and ethyl decanoate (70) were the main esters at the beginning of fermentation. Phenethyl acetate, which has a pleasant fruity and floral aroma, is produced by the conversion of phenyl pyruvic acid in the Shikimate Pathway, primarily through the activity of *S. cerevisiae* [43]. Phenethyl acetate and isoamyl acetate have been detected during apple vinegar fermentation across multiple regions, including China, Japan, and Spain [44–46]. These esters contribute significantly to the aromatic profile of the final product, imparting the fruity and floral notes essential for high-quality vinegar.

Ethyl palmitate and ethyl decanoate are esters formed through the reaction between palmitic acid and decanoic acid (present in apple pulp) and ethanol, and are produced by yeasts during fermentation. These reactions are catalyzed by microbial lipases, facilitating esterification despite the esters not being directly synthesized by the microorganisms themselves. Towards the end of fermentation, an increase in ethyl butyrate—an ester with distinct banana and pineapple aromas—was observed. This compound is linked to both yeasts and acetic acid bacteria from the *Acetobacter* genus, which exhibit significant activity in the later fermentation stages. The delayed accumulation of this ester could be due to the late production of butyric acid, a precursor with a rancid-like aroma that plays a critical role in its formation [47]. This interplay between microbial metabolism and ester formation highlights the complexity of fermentation, where timing and substrate availability greatly influence the development of the aromatic compounds in the final product.

At the end of fermentation, most alcohols, ethers, and esters were consumed, while carboxylic acids, ketones, and some aldehydes were formed. The carboxylic acids are primarily produced by the oxidation of ethanol by microorganisms [48]. The main organic acids produced were acetic, butyric, and isovaleric acids. Acetic acid, with a concentration of 12 g/L, is produced by the *Acetobacter* genus through the oxidation of ethanol into acetaldehyde, followed by the oxidation of acetaldehyde into acetic acid. The concentration of acetic acid increases by the end of the fermentation period as *Acetobacter* dominate and suppress other bacteria.

On the other hand, during acetous fermentation, the AAB *Acetobacter* was the genus exhibiting the highest number of strong positive correlations with the compounds in question (Figure 5). The robust positive correlations observed between this genus and the diverse array of compounds suggest a significant metabolic involvement in either the generation or consumption of these substances. Isopentyl alcohol/acetate, recognized for their fruity aromas that are often synthesized during fermentation processes, potentially undergo synthesis or breakdown facilitated by *Acetobacter*, potentially influencing the system's flavor profile (pleasant fruity aroma). Additionally, *Acetobacter*'s correlation with fatty acids, the crucial constituents of cellular membranes and energy sources, hints at its role in lipid metabolism, potentially impacting cell membrane synthesis and energy metabolism within the microbial community. It presented a high correlation to nonanoic acid, providing a cheese and butter flavor. Furthermore, the correlation with 3-cyclohexen-1-ol, a cyclic alcohol with fragrance applications, suggests *Acetobacter*'s potential involvement in its metabolism, potentially influencing the system's aroma profile. Lastly, the strong positive correlation between *Acetobacter* and compounds such as butanoic acid, ethanol, phenol, caproic acid, and benzaldehyde, known for various industrial applications and as intermediates in microbial metabolism, underscores *Acetobacter*'s potential involvement in their production, utilization, or transformation within the microbial community. The AAB *Komagateibacter* has similar metabolite correlations to *Acetobacter*; however, it differs for its stronger correlation to benzaldehyde 2,4-dimethyl, which has a pleasant almond-like aroma.

Figure 5. Correlation analysis between microbiota, organic acids, and volatile compounds (**A**) and concentration of volatile aroma compounds (area $\times 10^5$) (**B**) during vinegar fermentation.

3.4. Correlation Analysis

Radar plots illustrating the correlation analysis between microbiota, organic acids, and volatile compounds are presented in Figure 6. During the initial stage of fermentation, bacteria such as *Leuconoctoc* and *Gluconobacter*, alongside the yeast *Saccharomyces*, showed a strong correlation. It can also be observed that *Saccharomyces* exhibited a negative correlation with lactic acid, which explains its decrease in prevalence throughout the fermentation process. This trend was similarly observed in Chinese traditional Shanxi aged vinegar [39]. *Leuconostoc* showed moderate correlations (0–0.5) with various compounds, suggesting potential interactions in the vinegar production. Lactic acid, octanoic acid, isovaleric acid, and butanoic acid contribute to flavor and aroma. Benzyl alcohol and 2-ethyl-1 hexanol are known to contribute to the aroma of beverages like wine. Ethyl 9-decenoate and tetradecanoic acid ethyl ester provide fruity aromas, while propanoic acid acts as a preservative compound. Hexanoic acid ethyl ester enhances the fruity aroma of beverages. In addition, it presented a positive correlation to acetoin, similar to other works [49].

Gluconobacter, unlike *Leuconostoc*, exhibited moderate correlations (0–0.5) with other kinds of compounds, including dodecanoic acid, thymol, 2-methyltetracosane, malonic acid, benzeneacetic acid, butanoic acid ethyl ester, heneicosane, and hexadecanoic acid ethyl ester. Dodecanoic acid may influence the cellular membrane structure and lipid metabolism [50]. Thymol, known for its antimicrobial and aromatic properties, is used to enhance flavor and food safety [51]. Benzeneacetic acid contributes to complex and unique aromas in fermented foods [52]. Butanoic acid ethyl ester provides fruity aromas to fermented beverages. Finally, hexadecanoic acid ethyl ester influences aroma and may be involved in the formation of pleasant ester aromas in fermented foods. Additionally, *Gluconobacter* present a similar correlation as *Leuconostoc* to hexanoic acid, octanoic acid,

linalool, isovaleric acid, butanoic acid, ethyl 9-decenoate, tetradecanoic acid ethyl ester, propanoic acid, hexanoic acid, and methyleugenol benzene.

Figure 6. Radar plots of Spearman correlation coefficients showing correlation analysis between microbiota, organic acids, and volatile compounds.

In addition, during acetous fermentation, *Oenococcus* showed a strong correlation to succinic acid diethyl ester, isopentyl alcohol acetate, and benzaldehyde 2,4-dimethyl, representing the LAB to contribute the most flavor to the final vinegar product aroma. *Oenococcus*, a LAB frequently associated with food fermentation, particularly wine production, exhibit a notable capacity for producing compounds such as succinic acid, diethyl ester, isopentyl alcohol acetate, and benzaldehyde 2,4-dimethyl [53]. These compounds play multifaceted roles in food fermentation processes. Firstly, isopentyl alcohol acetate (isoamyl acetate), renowned for its fruity aroma akin to banana, and benzaldehyde 2,4-dimethyl, characterized by its distinctive almond-like scent, contribute significantly to the aromatic profile of the fermented food, enriching it with fruity nuances and sensory intricacies. Secondly, succinic acid and diethyl ester impart specific flavors to the fermented food, enhancing its overall taste and palatability. Additionally, the existence of these aromatic compounds introduces strata of sensory intricacy into the fermented item, thereby augmenting its allure and fascination to consumers. In summary, *Saccharomyces* contribute to alcohol formation, enhancing the fruity aroma. *Leuconostoc* are linked to ester production, enhancing flavor complexity, while *Acetobacter* are associated with acetic acid and VOCs, influencing the overall aroma profile.

The correlation analysis revealed that the predominant microbial groups—*Saccharomyces*, *Gluconobacter*, *Leuconostoc*, *Acetobacter*, *Komagataeibacter*, and *Pichia*—exhibited antagonistic relationships with several undesirable microorganisms, including *Erwinia*, *Aspergillus*, *Candida*, *Pseudomonas*, and *Cronobacter*. These antagonistic interactions suggest that the dominant

beneficial microbes help suppress the growth of spoilage organisms, contributing to the microbial stability of the vinegar. Furthermore, the undesirable groups showed a negative correlation with acetic acid and lactic acid, reinforcing the idea that higher levels of these organic acids—produced by the key fermentation microorganisms—enhance product safety by creating an unfavorable environment for contaminants. A similar trend was observed in Shanxi aged vinegar [40], where a beneficial microbial consortia dominated the fermentation environment, restricting the presence of potential spoilage organisms. The microbial dynamics observed in Shanxi vinegar, which involves a complex multi-stage fermentation, highlight the importance of organic acid production in maintaining the balance between desirable and undesirable microbes. This also underscores the critical role of fermentation management in achieving both microbial safety and enhanced sensory quality in traditional vinegars.

Saccharomyces had a strong correlation with *Leuconostoc*, *Gluconobacter*, and *Hanseniaspora*, and a weak correlation with *Acetobacter* and *Komagataeibacter*. This is because *Saccharomyces* can utilize carbon sources similarly to *Leuconostoc* and *Gluconobacter* [54]. On the other hand, the weak interaction with *Acetobacter* and *Komagataeibacter* is related to the nature of these microorganisms, which use the ethanol produced by *Saccharomyces* when its growth is reduced [29]. *Acetobacter* presented a strong connection with *Komagataeibacter* and *Pichia* due to the high presence of these microorganisms. This interaction is interesting because while *Acetobacter* produce ethanol, the metabolism of *Komagataeibacter* and *Pichia* can contribute to the vinegar volatile profile [3]. It is noteworthy that *Pichia* species can thrive in low pH environments.

Additionally, an intriguing interaction occurs between *Saccharomyces* and *Hanseniaspora* during the mid-stages of fermentation. In wine, for example, the cooperative interaction between both genders in mixed fermentations is characterized by *Hanseniaspora* enhancing the aroma through unique ester production in the initial fermentation phases, followed by *Saccharomyces*'s suppression of *Hanseniaspora* via cell-to-cell contact and competition for essential nutrients, ultimately shaping the final drink profile [55]. Thus, the data show that the ideal consortium for complex vinegar fermentation includes the initial co-inoculation of *Leuconostoc*, *Gluconobacter*, and *Saccharomyces* for the alcoholic phase, followed by the addition of *Acetobacter* and *Komagataeibacter* for the acetic acid phase. Additionally, the addition of *Hanseniaspora* at the initial stage of fermentation and *Pichia* at the final stage can be tested to produce vinegars with more complex and distinctive flavors. These interactions highlight the multifaceted roles of different microbiota in shaping the sensory qualities of traditional fermented vinegar in Brazil.

4. Conclusions

This study elucidated the microbial dynamics and metabolite profiles during traditional Brazilian apple vinegar fermentation, highlighting the key microbial species and their roles in the fermentation process. Dominant species such as *Saccharomyces*, *Leuconostoc*, *Gluconobacter*, and *Acetobacter* were identified as crucial players in the different stages of fermentation. *Saccharomyces* were significant in early alcohol formation, *Leuconostoc* contributed to fruity and floral notes through ester production, and *Acetobacter* were essential in the acetic fermentation stage, enhancing acetic acid production and volatile organic compound formation.

The data suggest that an optimal microbial consortium for vinegar fermentation should include *Saccharomyces* for efficient alcohol production, *Leuconostoc* for ester-mediated flavor complexity, and *Acetobacter* for robust acetic acid production. The presence of *Komagataeibacter* could further improve the sensory and functional qualities due to their role in producing bacterial cellulose.

Understanding these microbial interactions and their metabolic pathways is critical for the vinegar industry. This knowledge enables the optimization of fermentation conditions, improves vinegar quality and consistency, and supports the development of novel vinegar varieties with tailored flavor profiles to meet consumer preferences and market

demands. The findings from this study contribute significantly to the advancement of vinegar production practices.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fermentation10110552/s1: Table S1: Relative abundance (%) of bacteria and fungi identified during the fermentation of Brazilian apple cider vinegar.

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