

Article

Effects of Sequential Fermentation with *Metschnikowia* sp. Mp58 Belonging to the *M. pulcherrima* Clade and *Lactiplantibacillus plantarum* Cro3-2 on the Quality Characteristics of a Fermented Apple Beverage

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Abstract

This study developed an ABTS-targeted sequential fermentation strategy for fermented apple beverages using *Metschnikowia* sp. Mp58 belonging to the *M. pulcherrima* clade (Mp) and *Lactiplantibacillus plantarum* Cro3-2 (Lp). Thirteen *Metschnikowia* isolates and ten *Lactiplantibacillus* isolates were initially screened according to their effects on ABTS radical-scavenging activity, after which Mp58 and Cro3-2 were selected for further investigation. Different fermentation strategies were compared, and sequential inoculation of Mp followed by Lp produced the highest ABTS radical-scavenging activity. Single-factor experiments, an orthogonal design, and response surface methodology were subsequently used to optimize the inoculation interval, fermentation temperature, and total fermentation time, with ABTS radical-scavenging activity as the sole response variable. The selected conditions consisted of Mp inoculation at 0 h, Lp inoculation after 25 h, and fermentation for a total of 46 h at 21.50 °C. The experimentally obtained ABTS radical-scavenging activity was $78.17 \pm 0.49\%$, which was close to the model-predicted value. Under the selected conditions, Mp–Lp sequential fermentation significantly altered the physicochemical, bioactive, in vitro functional, and volatile characteristics of the apple beverage. Compared with the uninoculated control, total phenolic content, total flavonoid content, and ascorbic acid content increased by 73.08%, 32.97%, and 7.14%, respectively. These changes were accompanied by increases of 41.00%, 39.77%, and 37.86% in ABTS radical-scavenging activity, DPPH radical-scavenging activity, and ferric reducing antioxidant power, respectively. The Mp–Lp beverage also exhibited the highest α -glucosidase and α -amylase inhibitory activities, reaching 85.97% and 78.35%, respectively, as well as bile salt-binding capacities of 51.57% for sodium glycocholate and 54.97% for sodium taurocholate. Ethanol accumulation remained limited under the tested conditions, although concentrations below the measurement capability of the distillation–hydrometer method could not be excluded. A total of 95 volatile compounds were tentatively identified by HS-SPME-GC-MS. The sequentially fermented beverage contained a larger number of detected volatile compounds than the single-strain fermented beverages and showed treatment-associated changes in the relative distribution of esters, alcohols, and fatty acids. Exploratory PCA and OPLS-DA indicated differences in volatile composition among treatments, while nonanoic acid, ethyl hexanoate, n-decanoic acid, isophorone, and (E)-2-hexen-1-ol were identified as candidate compounds associated with group separation. These chemical changes should not be interpreted as direct evidence of improved aroma or sensory quality in the absence of sensory evaluation. Overall, Mp–Lp sequential fermentation represents a potentially useful bioprocessing



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strategy for modifying the bioactive properties, in vitro functional indicators, and volatile composition of fermented apple beverages.

Keywords: *Metschnikowia* sp.; *M. pulcherrima* clade; *Lactiplantibacillus plantarum*; apple beverages; sequential fermentation; bioactivity; antioxidant activity

1. Introduction

Fruit-based beverages are widely consumed because of their pleasant sensory properties and their contents of vitamins, minerals, and phytochemicals [1]. Among them, apple-based beverages are particularly popular worldwide because apples contain abundant phenolic compounds, including flavanols, phenolic acids, and flavonols [2], which are associated with antioxidant, anti-inflammatory, and antimicrobial activities [3]. However, a large proportion of these phenolics exists in bound or conjugated forms, limiting their bioaccessibility and biological functionality.

To improve the stability, functionality, and shelf life of fruit-based beverages, and to meet the growing demand for functional fruit beverages, various physical and chemical processing technologies have been explored. Common physical approaches include freezing, refrigeration, high-pressure processing (HPP), and irradiation. Freezing and refrigeration are cost-effective but may adversely affect texture and nutritional quality, whereas HPP better preserves flavor, nutrients, and microbial safety, although it is relatively expensive and energy-intensive. Irradiation can extend shelf life while maintaining product quality; however, its application is often limited by consumer acceptance and regulatory considerations [4]. Chemical additives, such as antioxidants, preservatives, and antimicrobial agents, are also widely used to extend shelf life, inhibit microbial growth, and reduce oxidative deterioration. For example, sodium benzoate effectively inhibits the growth of bacteria, fungi, and yeasts under acidic conditions [5]. Nevertheless, these approaches have certain limitations. Antioxidants may degrade during thermal processing or storage, while excessive use of preservatives may negatively affect product quality and consumer acceptance. In addition, growing concerns regarding the long-term health effects of synthetic additives have increased interest in natural and sustainable alternatives. Therefore, increasing attention has been directed toward natural and sustainable processing strategies that may enhance product characteristics while reducing reliance on synthetic additives.

In this context, increasing attention has been directed toward biological processing strategies capable of enhancing both the functional and sensory properties of fruit-based beverages. Fermentation represents a promising approach in this regard, as microbial metabolism can drive biochemical transformations of sugars, organic acids, and phenolic compounds [6]. During fermentation, microorganisms generate a wide range of metabolites, including organic acids, ethanol, esters, and other volatile compounds [7], which collectively influence physicochemical properties and sensory attributes. Moreover, microbial enzymatic activities, such as β -glucosidase- and esterase-mediated reactions, can promote the release of bound phenolics, thereby improving their bioaccessibility and antioxidant potential [8].

Lactiplantibacillus plantarum (Lp) has been widely applied in the fermentation of diverse fruit juices, including berries, tropical fruits, apples [9,10], and citrus fruits. Lp is characterized by metabolic versatility, enabling the conversion of sugars and organic acids into lactic acid and other metabolites, leading to acidification and enhanced microbial stability. In addition, Lp can transform phenolic glycosides and hydroxybenzoic- and hydroxycinnamic-acid derivatives through the activities of glycosidases, esterases, decarboxylases, and reductases,

thereby generating metabolites with altered bioactive and organoleptic properties [11]. These metabolic activities are associated with functional properties such as antioxidant, immunomodulatory, and cholesterol-lowering effects [12]. However, the effects of Lp monoculture fermentation on volatile composition are strain- and matrix-dependent and may differ from those achieved through mixed or sequential fermentation.

Non-*Saccharomyces* yeasts such as *Metschnikowia pulcherrima* are capable of alcoholic fermentation, converting glucose and fructose into ethanol and carbon dioxide, although typically with lower ethanol yields than *Saccharomyces cerevisiae*. *Metschnikowia pulcherrima*, which is commonly associated with fruits and flowers [13], has attracted increasing interest in beverage fermentation and is included in OIV guidance for specific winemaking applications (OIV-OENO 576B-2017). Beyond its fermentative role, *M. pulcherrima* has been reported to exhibit antimicrobial and antioxidant activities [14] and to produce extracellular enzymes, including β -glucosidase, which can hydrolyze glycosidically bound phenolic compounds into their free forms [15]. At winery scale, Perpetuini et al. [16] demonstrated that sequential inoculation with *M. pulcherrima* followed by *S. cerevisiae* increased the concentrations of esters and terpenes and modified the sensory profile of Pecorino wine. These findings further support the potential of *Metschnikowia*-containing sequential fermentation to modify the volatile characteristics of fermented beverages, although the effects may depend on the strain, fermentation matrix, and process conditions.

Recent studies have suggested that mixed fermentation involving *M. pulcherrima* and *L. plantarum* can influence phenolic transformation and volatile composition. In winemaking systems, co-inoculation of non-*Saccharomyces* yeasts and lactic acid bacteria has been shown to modify phenolic composition and volatile profiles [17]. Similarly, in fruit-based substrates, co-fermentation with *L. plantarum* and *M. pulcherrima* has been associated with increased total phenolic content and antioxidant capacity, as demonstrated in mango juice systems [18]. However, most previous studies have focused on co-inoculation strategies, whereas systematic investigations of sequential fermentation involving these microorganisms in apple-based beverages remain limited.

Recent studies have emphasized that sequential fermentation should be evaluated by integrating microbial culturability with changes in key fermentation products and functional components, including ethanol, phenolic compounds, antioxidant activity, and volatile compounds [19]. Such integrated monitoring may help characterize treatment-associated microbial and chemical changes during sequential fermentation.

Apples are widely consumed globally because of their availability, affordability, and nutritional value. According to FAOSTAT, China is the world's largest producer of apples [20]. However, postharvest losses remain significant because of the relatively short shelf life of fresh apples and their susceptibility to spoilage [21]. Conventional processing can extend shelf life, but thermal treatments may lead to losses of aroma compounds and reductions in bioactive components, and overall product stability remains a concern [22]. Meanwhile, consumer demand for natural, high-quality, and minimally processed apple-based beverages continues to increase.

Therefore, this study aimed to establish and optimize a sequential fermentation system for fermented apple beverages using *Metschnikowia* sp. Mp58 belonging to the *M. pulcherrima* clade (hereafter Mp) and *Lactiplantibacillus plantarum* Cro3-2 (hereafter Lp), with ABTS radical-scavenging activity as the response variable. The specific contribution of this work lies in the sequential application of Mp58 and Cro3-2 in an apple beverage matrix, together with the optimization of the inoculation interval, fermentation temperature, and total fermentation time. Microbial culturability was monitored, and the resulting beverages were characterized in terms of physicochemical properties, ethanol content, total phenolic and flavonoid contents, ascorbic acid content, antioxidant activity, in vitro α -glucosidase

and α -amylase inhibitory activities, bile salt-binding capacities, and volatile composition. However, strain-level genomic and phenotypic safety assessments of Mp58 and Cro3-2 were beyond the scope of the present study and remain necessary before practical food application. This study provides an integrated evaluation of how this specific strain combination and sequential inoculation strategy influence the chemical and in vitro functional characteristics of fermented apple beverages.

2. Materials and Methods

2.1. Strains

The thirteen *Metschnikowia* isolates and ten *Lactiplantibacillus* isolates used in this study were isolated from the surfaces of commercially available grapes collected in the Jieshi Mountain region. All isolates are preserved in the laboratory of the College of Food Science and Technology, Hebei Normal University of Science and Technology.

2.2. Strain Activation

The *Metschnikowia* and *Lactiplantibacillus* isolates were maintained at 4 °C on YPD and MRS agar slants, respectively. For activation, a single colony of each *Metschnikowia* isolate was inoculated into 10 mL of sterile YPD broth and incubated at 28 °C with shaking at 200 rpm for 24 h, whereas a single colony of each *Lactiplantibacillus* isolate was inoculated into 10 mL of sterile MRS broth and incubated at 37 °C under static conditions for 24 h. Subsequently, each activated culture was transferred at 3% (*v/v*) into 100 mL of the corresponding fresh medium and incubated under the same conditions for an additional 24–36 h to prepare the working cultures.

Cells were harvested by centrifugation at $2860 \times g$ for 10 min at 4 °C, washed twice with sterile physiological saline containing 0.85% (*w/v*) NaCl, and resuspended in sterile physiological saline to prepare the inoculum suspensions. The activation, subculturing, and cell-harvesting conditions were established in-house based on preliminary experiments and were applied consistently to all strains.

Cell concentration and viability were determined using a hemocytometer. Briefly, an aliquot of the cell suspension was appropriately diluted and mixed with 0.4% (*w/v*) trypan blue solution at a 1:1 ratio. Viable cells, which remained unstained, and non-viable cells, which were stained blue, were counted under a light microscope at $400\times$ magnification. Cell concentration was calculated using the standard hemocytometer equation, and viability was expressed as the percentage of viable cells relative to the total number of cells counted. The final inoculum volume was adjusted according to the microscopically estimated viable cell concentration to achieve the required initial cell concentration for fermentation.

2.3. Fermentation of Apple Beverage

Commercial pasteurized apple juice (Huabang Food Co., Ltd., Shanghai, China) was used as the fermentation substrate. The juice (100 mL) was aseptically dispensed into sterile 250 mL Erlenmeyer flasks.

The study was conducted in four sequential stages: (i) screening of thirteen *Metschnikowia* isolates and ten *Lactiplantibacillus* isolates based on ABTS radical-scavenging activity; (ii) comparison of fermentation strategies using the selected strains, *Metschnikowia* sp. Mp58 belonging to the *M. pulcherrima* clade (hereafter Mp) and *Lactiplantibacillus plantarum* Cro3-2 (hereafter Lp), including Mp monoculture, Lp monoculture, Mp–Lp co-inoculation, Mp–Lp sequential inoculation, and Lp–Mp sequential inoculation; (iii) ABTS-targeted optimization of the selected fermentation strategy; and (iv) characterization of the fermented apple beverage produced under the selected ABTS-targeted condition. Fermentation strategies were compared using ABTS radical-scavenging activity as the sole response

variable. The strategy showing the highest ABTS radical-scavenging activity was selected for subsequent optimization. TPC, TFC, ascorbic acid content, DPPH radical-scavenging activity, FRAP, in vitro functional properties, and volatile compounds were subsequently determined to characterize the beverage produced under the selected condition but were not included as optimization responses.

2.4. Strain Screening and Fermentation-Strategy Selection

2.4.1. Screening of *Metschnikowia* and *Lactiplantibacillus* Isolates Based on ABTS Radical-Scavenging Activity

Uninoculated apple juice was used as the control. Thirteen *Metschnikowia* isolates and ten *Lactiplantibacillus* isolates were activated and individually inoculated into apple juice at an initial concentration of 1.0×10^6 microscopically estimated viable cells/mL. Cell concentrations were determined using a Neubauer hemocytometer combined with trypan blue exclusion, as described above. Fermentation was conducted at 20 °C for 48 h under identical conditions. The activation temperatures of 28 °C for the *Metschnikowia* isolates and 37 °C for the *Lactiplantibacillus* isolates were used solely to prepare active inocula and were not intended to represent the fermentation temperatures used in the apple beverage matrix. A temperature of 20 °C was selected as a standardized low-temperature fermentation condition relevant to fruit-beverage fermentation and was subsequently used as the baseline temperature for fermentation-strategy comparison. This temperature was not intended to represent the optimal growth temperature of either microorganism.

Growth and viability were not monitored for all 23 isolates during the initial screening. Therefore, differences in ABTS radical-scavenging activity were interpreted as the overall performance of individual isolates under the standardized screening conditions, potentially reflecting both isolate-specific metabolic activity and adaptation to 20 °C, rather than as intrinsic antioxidant potential independent of microbial growth. After fermentation, ABTS radical-scavenging activity was measured, and the strains exhibiting the highest ABTS radical-scavenging activity under the standardized screening conditions were selected for subsequent ABTS-targeted fermentation optimization and product characterization.

The ABTS assay was selected as the primary antioxidant evaluation method because it is applicable to both hydrophilic and lipophilic antioxidant compounds and is suitable for complex fermented apple beverage matrices containing diverse antioxidant metabolites. The primary objective of the strain-screening and process-optimization stages was to identify isolates and fermentation conditions that maximized ABTS radical-scavenging activity. Therefore, ABTS radical-scavenging activity was used as the principal response variable as a rapid and widely applied indicator of antioxidant capacity. Accordingly, the resulting optimized conditions should be interpreted as ABTS-targeted conditions rather than as conditions optimized for overall product quality.

2.4.2. Single-Factor Experiments

Based on the strain-screening results, Mp58 and Cro3-2 were selected for fermentation-strategy evaluation and process optimization (Figure S1). Molecular identification results for the two selected strains are summarized in Table S1. Strain Mp58 was identified as a *Metschnikowia* sp. belonging to the *M. pulcherrima* clade based on sequencing of the D1/D2 domain of the 26S rRNA gene. The obtained 503 bp sequence showed 99.01% identity, with 498 of 503 nucleotides matching the closest reference sequence annotated as *Metschnikowia* aff. *pulcherrima* (GenBank accession no. JX188181.1). Strain Cro3-2 was identified as *Lactiplantibacillus plantarum* based on 16S rRNA gene sequencing. The obtained 1444 bp sequence showed 99.86% identity, with 1442 of 1444 nucleotides matching *L. plantarum* strain CIP 103151, with 100% query coverage and an E-value of 0.0 (GenBank accession no. NR_104573.1). JX188181.1 and NR_104573.1 are the accession numbers of the closest

matching reference sequences and are not strain-specific accession numbers assigned to Mp58 and Cro3-2, respectively. Accordingly, the D1/D2 and 16S rRNA gene sequence results support clade-level assignment of Mp58 and species-level identification of Cro3-2, respectively, but do not provide strain-level genomic resolution because whole-genome sequencing was not performed.

Unless otherwise specified, the baseline conditions used for fermentation-strategy comparison and single-factor experiments were an initial concentration of 1.0×10^6 microscopically estimated viable cells/mL for each inoculated microorganism, a fermentation temperature of 20 °C, and a total fermentation time of 48 h.

Six treatments were established to compare the fermentation strategies: (i) an uninoculated control (CK); (ii) Mp monoculture fermentation; (iii) Lp monoculture fermentation; (iv) Mp–Lp co-inoculation, in which Mp and Lp were inoculated simultaneously; (v) Mp–Lp sequential fermentation, in which Mp was inoculated first and Lp was inoculated after 24 h; and (vi) Lp–Mp sequential fermentation, in which Lp was inoculated first and Mp was inoculated after 24 h.

For monoculture fermentation, Mp or Lp was inoculated at an initial concentration of 1.0×10^6 microscopically estimated viable cells/mL. For co-inoculation, Mp and Lp were each inoculated at an initial concentration of 1.0×10^6 microscopically estimated viable cells/mL. Thus, the total initial inoculated cell concentration in the co-inoculation treatment was 2.0×10^6 microscopically estimated viable cells/mL. For sequential fermentation, the first microorganism was inoculated at an initial concentration of 1.0×10^6 microscopically estimated viable cells/mL and incubated for 24 h, after which the second microorganism was inoculated at the same initial concentration.

Samples were collected at 0, 24, and 48 h. In the sequential-fermentation treatments, the 24 h samples were collected immediately before inoculation of the second microorganism and therefore represented the end of the first fermentation stage, before the onset of the mixed-culture stage. Each treatment was performed in three independent biological replicates.

The effects of initial inoculum concentration were evaluated separately for Mp and Lp at 1.0×10^4 , 1.0×10^5 , and 1.0×10^6 microscopically estimated viable cells/mL. When the concentration of one microorganism was varied, the concentration of the other microorganism was maintained at 1.0×10^6 microscopically estimated viable cells/mL. The effects of total fermentation time and fermentation temperature were evaluated over the ranges of 12–72 h and 8–20 °C, respectively, while the other conditions were maintained constant. The temperature range of 8–20 °C was selected as an initial exploratory range to evaluate the performance of the sequential fermentation system under low-temperature conditions relevant to fruit-beverage fermentation, with 20 °C retained as the baseline temperature used for strain screening and fermentation-strategy comparison. This range was not selected on the assumption that the optimum necessarily occurred below 20 °C. Because ABTS radical-scavenging activity increased toward the upper end of this range, the subsequent response surface design was centered around 20 °C and extended to 22 °C, using temperature levels of 18, 20, and 22 °C. Cell growth and viability were not determined separately at each temperature during the single-factor experiment.

2.4.3. Orthogonal Experiment

Based on the single-factor results obtained for Mp–Lp sequential fermentation, four factors were selected for the orthogonal experiment: inoculation sequence (A), inoculation level (B), fermentation time (C), and fermentation temperature (D). Three levels were set for each factor. An L9 (3^4) orthogonal design was employed using ABTS radical scavenging

activity as the response variable. The levels of each factor and the experimental design are shown in Supplementary Table S2.

2.4.4. Response Surface Optimization

Based on the response trends observed in the preceding single-factor and L9 (3⁴) orthogonal experiments, fermentation time, fermentation temperature, and the inoculation interval between Mp inoculation and subsequent Lp inoculation were selected for further optimization. These factors were selected based on their observed effects on ABTS radical-scavenging activity and their practical relevance to the sequential fermentation process, rather than being formally identified as the three “most influential” factors through a separate factor-screening design. No Plackett–Burman or other formal factor-screening design was conducted. A three-factor, three-level Box–Behnken design (BBD) comprising 17 experimental runs, including five replicates at the center point, was used to optimize the Mp–Lp sequential fermentation process. The independent variables were fermentation time (36, 48, and 60 h), fermentation temperature (18, 20, and 22 °C), and inoculation interval (12, 24, and 36 h), with ABTS radical-scavenging activity as the response variable. The factors and corresponding levels are presented in Supplementary Table S3.

2.5. Microbial Growth and Differential Enumeration During Fermentation

Microbial culturability was evaluated under the baseline conditions used for fermentation-strategy comparison, namely fermentation at 20 °C for 48 h, with Lp inoculated at 24 h in the Mp–Lp sequential fermentation treatment. Apple juice from the same batch was subjected to the same pretreatment as described above. Four treatments were established: an uninoculated control (CK), Mp monoculture fermentation, Lp monoculture fermentation, and Mp–Lp sequential fermentation. Mp was inoculated at 0 h in the Mp and Mp–Lp treatments, whereas Lp was inoculated at 0 h in the Lp treatment and at 24 h in the Mp–Lp treatment. The initial culturable count of each inoculated strain was adjusted to approximately 1.0×10^6 CFU/mL and verified by plate counting immediately after inoculation. All fermentations were conducted statically at 20 °C for 48 h.

Samples were collected aseptically at 0, 6, 12, 24 h immediately before Lp inoculation, 24 h immediately after Lp inoculation, 36, and 48 h. Serial decimal dilutions were prepared using sterile 0.90% saline, and suitable dilutions were plated in duplicate. Presumptive Mp58 colonies were enumerated on yeast extract–peptone–dextrose (YPD) agar after aerobic incubation at 28 °C for 48 h. Presumptive Cro3-2 colonies were enumerated on de Man, Rogosa, and Sharpe (MRS) agar after anaerobic incubation at 37 °C for 48 h. No selective antimicrobial agents were added to either medium.

Plates containing 30–300 colonies were used for quantification. Samples yielding no colonies at the lowest dilution plated were reported as not detected under the assay conditions rather than as zero. Formal strain-specific validation of medium selectivity, recovery efficiency, and the method detection limit was not performed. Therefore, particularly during the mixed-culture stage of the Mp–Lp treatment, the plate-count results were interpreted as exploratory estimates of microbial culturability rather than as fully validated strain-specific differential counts. Three independent fermentation batches were analyzed, and culturable counts were expressed as mean $\pm$ SD log₁₀ CFU/mL.

This exploratory experiment was conducted under the baseline fermentation conditions rather than under the subsequently selected RSM conditions of 21.50 °C, a total fermentation time of 46 h, and Lp inoculation at 25 h. Although the two sets of process conditions were relatively close, the microbial-count results obtained under the baseline conditions cannot be directly extrapolated to microbial population dynamics under the final RSM-selected conditions.

2.6. Physicochemical Index Determination

All subsequent physicochemical analyses, bioactive compound determinations, antioxidant activity evaluations, *in vitro* enzyme-inhibitory and bile salt-binding assays, and volatile compound analyses were performed using samples produced under the RSM-selected ABTS-targeted fermentation conditions. Mp was inoculated at 0 h, followed by Lp at 25 h, and fermentation was continued for a total of 46 h at 21.50 °C. Samples were collected at 0, 23, and 46 h. The 23 h sample was collected 2 h before Lp inoculation to characterize the late stage of Mp monoculture fermentation, whereas the 46 h sample represented the completed Mp–Lp sequential fermentation. The 23 h sampling point also corresponded to the midpoint of the 46 h fermentation period.

Soluble solids content (SSC) was measured using a handheld digital refractometer and expressed as °Brix.

The pH was determined at room temperature (20–25 °C) using a calibrated pH meter.

Total acidity was measured by titration with 0.1 mol/L NaOH to an endpoint of pH 8.2 and expressed as malic acid equivalents (g/L).

Total sugar content was determined using Fehling's titration method with reference to GB/T 15038-2006 [23]. Briefly, the clarified sample was reacted with Fehling's A and B solutions under boiling conditions, and sugars were quantified by titration using methylene blue as an indicator. The results were expressed as glucose equivalents (g/L).

Ethanol content was estimated by distillation followed by alcohol hydrometer measurement with reference to GB/T 15038-2006 [24]. Briefly, fermented samples were distilled, and the distillate was collected and adjusted to a fixed volume. The apparent ethanol content was measured at 20 °C and expressed as % (*v/v*). The minimum scale division of the alcohol hydrometer was 0.1% (*v/v*). Formal limits of detection (LOD) and quantification (LOQ) were not established for this method; therefore, the measurement was used only as an approximate assessment of ethanol content, and ethanol concentrations below the measurement capability of the method could not be reliably quantified.

2.7. Determination of Bioactive Compounds

2.7.1. Total Phenolic Content

Total phenolic content (TPC) was determined using the Folin–Ciocalteu method with minor modifications [25]. Briefly, 1 mL of sample was mixed with 4 mL of distilled water. Then, 2 mL of Folin–Ciocalteu reagent and 3 mL of 20% (*w/v*) Na₂CO₃ solution were added. The mixture was incubated at 20 °C for 30 min, and the absorbance was measured at 760 nm. TPC was calculated from a gallic acid calibration curve and expressed as milligrams of gallic acid equivalents per liter (mg GAE/L).

2.7.2. Total Flavonoid Content

Total flavonoid content (TFC) was determined using the method of Zhishen et al. with minor modifications, using rutin as the standard [26]. Briefly, 0.5 mL of sample or rutin standard solution was mixed with 4.6 mL of 70% ethanol and 0.3 mL of 5% (*w/v*) NaNO₂ solution. After 6 min, 0.3 mL of 10% Al(NO₃)₃ solution was added and allowed to react for 6 min. Then, 4 mL of 1 mol/L NaOH was added, and the volume was adjusted to 10 mL with distilled water. After 15 min, the absorbance was measured at 510 nm. TFC was calculated from a rutin calibration curve and expressed as milligrams of rutin equivalents per liter (mg RE/L).

2.7.3. Ascorbic Acid Content

Ascorbic acid content was determined spectrophotometrically using the method of Güçlü et al. [27]. The complexing reagent contained 0.075% copper acetate and saturated

neocuproine in ethanol. Briefly, 2 mL of juice was transferred into a 10 mL volumetric flask, followed by the addition of 1 mL acetate buffer and 3 mL complexing reagent. The volume was adjusted to 10 mL with ethanol, mixed thoroughly, and allowed to stand for 5 min before absorbance measurement.

For calibration, to obtain a 0.112 mg/mL stock solution was dissolved in 1% citric acid and diluted to 100 mL to obtain a 0.1 mg/mL stock solution. Working solutions (0–12 µg/mL) were prepared by dilution with 1% citric acid. Absorbance was measured at 545 nm, and a calibration curve was plotted. Ascorbic acid content was calculated from the calibration curve.

2.8. Determination of the Antioxidant Activity

2.8.1. ABTS Radical Scavenging Activity

ABTS radical scavenging activity was measured using the method with minor modifications [28]. The ABTS stock solution was prepared by mixing 7.4 mM ABTS solution with 4.6 mM potassium persulfate solution at a ratio of 1:1 (v/v), followed by incubation in the dark at room temperature for 14 h to generate ABTS⁺• radicals.

The ABTS working solution was obtained by diluting the stock solution with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. For analysis, 2.8 mL of ABTS working solution was mixed with 0.7 mL of fermented apple beverage sample, incubated in the dark for 6 min, and the absorbance was measured at 734 nm (A). Ethanol was used as the control, and its absorbance was recorded as A₀.

ABTS radical scavenging activity was calculated according to Equation (1):

$$\text{ABTS radical scavenging activity (\%)} = [(A_0 - A)/A_0] \times 100 \quad (1)$$

where A represents the absorbance of the sample group and A₀ represents the absorbance of the control group.

2.8.2. DPPH Radical Scavenging Activity

DPPH radical scavenging activity was determined according to the method of Brand-Williams et al. [29] with minor modifications. Briefly, 0.5 mL of sample was mixed with 3.5 mL of DPPH solution in ethanol (0.1 mmol/L). The mixture was vortexed for 15 s at 20 °C and then incubated in the dark for 30 min. The absorbance was measured at 517 nm and recorded as A. Ethanol was used as the control, and its absorbance was recorded as A₀. DPPH radical scavenging activity was calculated using Equation (1).

2.8.3. Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing antioxidant power (FRAP) was determined using the method of Benzie and Strain [30] with minor modifications. The FRAP working reagent was freshly prepared by mixing 10 mmol/L TPTZ solution, 20 mmol/L FeCl₃ solution, and acetate buffer (pH 3.6) at a volume ratio of 1:1:10.

For analysis, 4 mL of FRAP working reagent was mixed with 0.2 mL of fermented apple beverage sample in a 10 mL test tube and thoroughly mixed. The mixture was incubated in a water bath at 37 °C for 20 min, and the absorbance was measured at 593 nm. FRAP values were calculated from a ferrous sulfate calibration curve and expressed as mmol Fe²⁺ equivalents/L.

2.9. Determination of In Vitro Enzyme Inhibitory and Bile Salt-Binding Activities

2.9.1. Determination of α-Glucosidase and α-Amylase Inhibitory Activities

Enzyme inhibitory activities of α-glucosidase and α-amylase were measured as described by Tadera et al. [31]. For α-glucosidase, sample solutions were incubated with

α -glucosidase (0.2 U/mL) in phosphate buffer (0.05 M, pH 6.8) at 37 °C for 20 min, followed by addition of p-nitrophenyl- α -D-glucopyranoside (0.02 M) for 20 min. The reaction was stopped with 1 M sodium carbonate, and absorbance was measured at 405 nm. For α -amylase, samples were incubated with α -amylase (5 U/mL) in phosphate buffer (pH 6.8) at 37 °C for 10 min, then soluble starch (1%, *w/v*) was added for 10 min. The reaction was stopped with 3,5-dinitrosalicylic acid and heated for 5 min. Absorbance was measured at 540 nm after cooling and dilution. Acarbose was used as positive control (0.1 mg/mL for α -glucosidase; 1 mg/mL for α -amylase).

The inhibitory activity of α -glucosidase and α -amylase was calculated according to Equation (2):

$$\text{Enzyme inhibitory activity (\%)} = [1 - (A_1 - A_2)/(A_0 - A_3)] \times 100 \quad (2)$$

where A_1 is the absorbance of the sample, A_2 is the absorbance of the sample control (enzyme replaced by buffer), A_0 is the absorbance of the blank (sample replaced by distilled water), and A_3 is the absorbance of the blank control.

2.9.2. Determination of Bile Salt-Binding Capacity

Bile salt-binding capacity was determined according to the method of Kahlon and Smith [32]. Two 3 mL aliquots of sample were subjected to simulated gastric digestion with pepsin (10 g/L) in 0.01 M HCl at 37 °C for 1 h, followed by adjustment of the pH to 6.3 using 0.1 M NaOH. Simulated intestinal digestion was then performed with trypsin (10 g/L) at 37 °C for 1 h. Sodium glycocholate (0.4 mM) or sodium taurocholate (0.5 mM) was added and incubated at 37 °C for 1 h. After centrifugation, 2 mL of the supernatant was mixed with 6 mL of 60% H_2SO_4 , incubated at 70 °C for 20 min, cooled on ice for 5 min, and the absorbance was measured at 387 nm. All enzyme and bile salt solutions were prepared in phosphate buffer (0.1 M, pH 6.3). Bile salt-binding capacity was calculated according to Equation (3).

$$\text{Bile salt-binding capacity (\%)} = [1 - (A_i - A_j)/A_0] \times 100 \quad (3)$$

where A_i is the absorbance of the sample, A_j is the absorbance of the control (in which the bile salt solution was replaced with phosphate buffer), and A_0 is the absorbance of the blank (in which the sample solution was replaced with distilled water).

2.10. HS-SPME-GC-MS Analysis of Volatile Compounds

Volatile compounds were analyzed using headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME-GC-MS). Apple beverage samples (3 mL) were transferred into 15 mL headspace vials containing 0.6 g NaCl and 5 μL of 4-octanol internal standard solution at a concentration of 100 $\mu\text{g/mL}$. The vials were equilibrated at 45 °C for 15 min with stirring. Volatile compounds were then extracted using a 65- μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber at 45 °C for 45 min, followed by thermal desorption in the GC injector.

Chromatographic separation was performed using an SH-Rxi-5Sil MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm), with helium as the carrier gas. The GC oven temperature was programmed from 40 to 270 °C. Mass spectrometric analysis was conducted in electron ionization mode at 70 eV over a scan range of m/z 30–450.

Volatile compounds were tentatively identified by comparing their mass spectra with those in the NIST 14 and NIST 20 mass spectral libraries and by comparing their calculated retention indices with literature values. Retention indices were determined using a homologous series of n-alkanes ranging from C7 to C30. Only compounds with a

mass spectral library match factor greater than 800 and retention indices consistent with available literature values were reported.

Semi-quantitative abundances were estimated using 4-octanol as the internal standard and were expressed as $\mu\text{g/L}$ 4-octanol equivalents. The semi-quantitative abundance of each compound was calculated according to Equation (4):

$$C_i = \frac{A_i}{A_{IS}} \times C_{IS} \quad (4)$$

where C_i represents the semi-quantitative abundance of compound i , expressed as $\mu\text{g/L}$ 4-octanol equivalents; A_i represents the peak area of compound i ; A_{IS} represents the peak area of 4-octanol; and C_{IS} represents the final concentration of 4-octanol added to the sample. An equivalent detector response between each volatile compound and 4-octanol was assumed.

The relative distribution of volatile compounds and chemical classes was calculated separately by peak-area normalization. The normalized relative proportion of each compound was calculated as its peak area divided by the summed peak areas of all tentatively identified volatile compounds, excluding the internal standard, and was expressed as a percentage. Thus, the values expressed as $\mu\text{g/L}$ 4-octanol equivalents and the normalized peak-area percentages represent two different measures and were used for semi-quantitative abundance comparison and relative-composition analysis, respectively.

Approximate odor activity values (approximate OAVs) were calculated by dividing the 4-octanol-based semi-quantitative abundance of each compound by its corresponding literature-derived odor threshold according to Equation (5):

$$\text{Approximate OAV}_i = \frac{C_i}{T_i} \quad (5)$$

where approximate OAV_i represents the approximate odor activity value of compound i ; C_i represents its semi-quantitative abundance, expressed as $\mu\text{g/L}$ 4-octanol equivalents; and T_i represents the corresponding literature-derived odor threshold expressed in the same units. The sources and matrices of the odor thresholds are provided in Supplementary Table S7. Compounds with approximate OAVs greater than 1 were considered candidate compounds with potential aroma relevance, whereas compounds with approximate OAVs of at least 0.1 were retained for subsequent exploratory multivariate analysis.

Because authentic standards, compound-specific calibration curves, response factors, recovery tests, matrix-effect evaluations, formal limits of detection and quantification, and formal SPME method validation were not established, the reported semi-quantitative abundances should not be interpreted as absolute concentrations. Furthermore, because the odor thresholds were obtained from different literature matrices and may not directly represent the apple beverage matrix, the calculated approximate OAVs were used only as preliminary screening indicators of potential aroma relevance and not as direct evidence of sensory impact.

The retention index was calculated according to Equation (6):

$$\text{RI} = 100n + 100 \times \frac{t_x - t_n}{t_{n+1} - t_n} \quad (6)$$

where n and $n + 1$ represent the numbers of carbon atoms in the n -alkanes eluting immediately before and after the target compound, respectively; t_n and t_{n+1} are the corresponding retention times (min) of these n -alkanes; and t_x is the retention time (min) of the target compound. The retention times satisfied the relationship $t_n < t_x < t_{n+1}$.

2.11. Statistical Analysis

All experiments were conducted in triplicate, and the data are expressed as mean $\pm$ standard deviation. Statistical analyses were performed using SPSS 27.0. Differences among treatments were evaluated by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test at a significance level of $p < 0.05$. Response surface methodology analysis was performed using Design-Expert 13. Bar and line plots, as well as exploratory principal component analysis (PCA) of the 27 volatile compounds with approximate OAVs ≥ 0.1 , were generated using Origin 2018. Exploratory orthogonal partial least-squares discriminant analysis (OPLS-DA) was performed using SIMCA 14.1.

3. Results

3.1. Strain Selection and ABTS-Targeted Optimization of Mp–Lp Sequential Fermentation

3.1.1. Screening of *Metschnikowia* and *Lactiplantibacillus* Isolates Based on ABTS Radical-Scavenging Activity

ABTS radical-scavenging activity was evaluated in apple beverages fermented with thirteen *Metschnikowia* isolates and ten *Lactiplantibacillus* isolates. All samples fermented with the *Metschnikowia* or *Lactiplantibacillus* isolates exhibited significantly higher ABTS radical-scavenging activity than the unfermented control (CK) (Figure S1; $p < 0.05$). Pronounced isolate-dependent differences were observed within both groups ($p < 0.05$). Among the tested isolates, Mp58 and Cro3-2 produced the highest ABTS radical-scavenging activity among the *Metschnikowia*- and *Lactiplantibacillus*-fermented apple beverages, respectively. These results indicate considerable isolate-level variability in the effects of fermentation on ABTS radical-scavenging activity. Therefore, Mp58 and Cro3-2 were selected for subsequent fermentation-strategy comparison and process-optimization experiments.

3.1.2. ABTS-Targeted Optimization of Mp–Lp Sequential Fermentation Conditions for Apple Beverages

Single-factor experiments were conducted to evaluate the effects of inoculation sequence, inoculation level, fermentation time, and fermentation temperature on the ABTS radical scavenging activity of apple beverages fermented with Mp58 and Cro3-2 (Figure 1). Sequential inoculation of Mp58 and Cro3-2 significantly increased ABTS radical scavenging activity compared with single-strain fermentation (Figure 1A). The inoculation sequence had a significant effect on antioxidant capacity ($p < 0.05$). The Mp–Lp sequential inoculation strategy, in which Lp was inoculated 24 h after Mp, exhibited the highest ABTS radical scavenging activity ($66.19 \pm 0.48\%$), followed by the Lp–Mp sequential inoculation strategy ($64.54 \pm 0.71\%$). Simultaneous co-inoculation (Mp + Lp) resulted in lower ABTS radical scavenging activity ($59.44 \pm 0.28\%$) than Lp monoculture fermentation ($62.15 \pm 0.46\%$), but higher activity than Mp monoculture fermentation ($53.96 \pm 0.75\%$). These results suggest that simultaneous co-inoculation may lead to competitive or antagonistic interactions between Mp and Lp, whereas sequential inoculation may alleviate such interactions and enhance antioxidant activity.

The highest ABTS radical-scavenging activity in the single-factor experiments was obtained under the following conditions: Mp–Lp sequential inoculation, an initial concentration of 1.0×10^6 viable cells/mL for each strain, a total fermentation time of 48 h, and a fermentation temperature of 20 °C (Figure 1B–D). Under these conditions, the ABTS radical-scavenging activity reached $71.48 \pm 0.27\%$. Cell growth and viability were not monitored across the tested temperature range of 8–20 °C. Therefore, the temperature-dependent ABTS responses, particularly those observed at 8 °C, may partly reflect changes in microbial growth, metabolic activity, or physiological stress rather than solely an antioxidant-related optimum. Accordingly, the selected temperature should be interpreted as an ABTS-targeted

process condition within the tested range rather than as the physiological growth optimum of either microorganism.

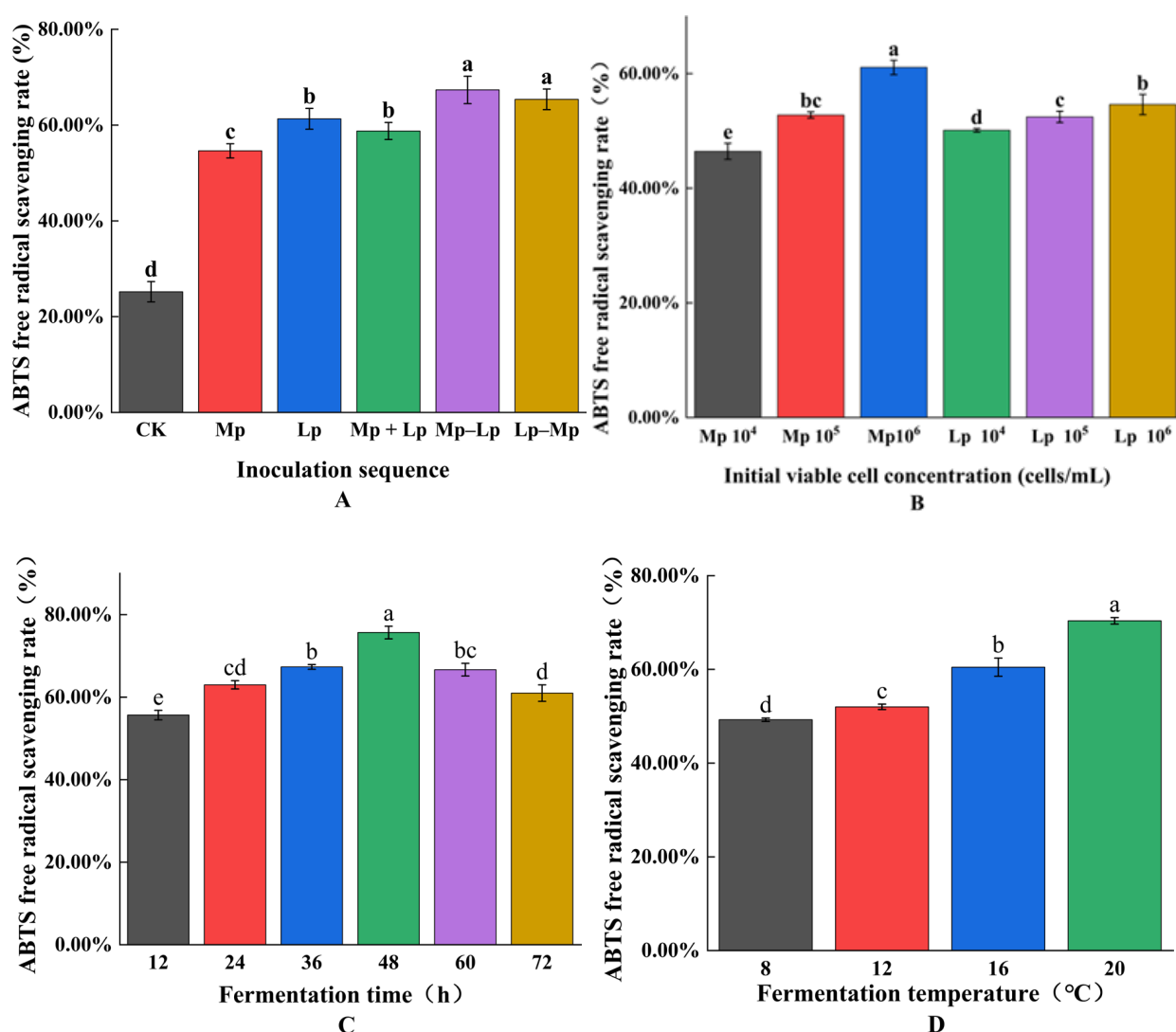


Figure 1. ABTS radical scavenging rate under different single-factor conditions. (A) Inoculation sequence; (B) Concentration; (C) Fermentation time; (D) Fermentation temperature. Different lowercase letters indicate significant differences among treatments at the same fermentation time ($p < 0.05$).

Based on the single-factor results, an L9 (3^4) orthogonal design was used to further evaluate the effects of the selected factors on ABTS radical-scavenging activity (Supplementary Table S4). The $A_1B_1C_1D_1$ treatment, corresponding to Mp-Lp sequential inoculation with a 24 h interval, an initial concentration of 1.0×10^6 viable cells/mL for each strain, a total fermentation time of 48 h, and a fermentation temperature of 20 °C, produced the highest ABTS radical-scavenging activity ($71.48 \pm 0.27\%$) and was therefore identified as the ABTS-targeted optimal condition in the orthogonal experiment.

Three independent experiments were subsequently performed under the optimal orthogonal-design conditions to validate the optimization results. The measured ABTS radical scavenging activity was $70.50 \pm 0.66\%$, which was close to the optimal value obtained from the orthogonal design. Range analysis showed that fermentation time (C, $R = 0.1272$) had the greatest effect on ABTS radical scavenging activity, followed

by fermentation temperature (D, $R = 0.0649$), inoculation sequence (A, $R = 0.0380$), and inoculation level (B, $R = 0.0047$).

Based on the single-factor and orthogonal experimental results, response surface methodology (RSM) was applied to further optimize the Mp–Lp sequential fermentation process, using ABTS radical scavenging activity as the response variable. In the RSM analysis, fermentation time (A), fermentation temperature (B), and inoculation interval (C) were selected as independent variables. The response variable Y was expressed as a decimal fraction for model fitting. The experimental design and results are presented in Supplementary Table S5. The data were fitted using Design-Expert 13.0, and the resulting regression model was as follows:

$$Y = 0.7646 - 0.0275A + 0.0074B + 0.0089C - 0.0007AB + 0.0028AC + 0.0010BC - 0.1091A^2 + 0.0012B^2 - 0.0318C^2$$

where Y represents ABTS radical scavenging activity expressed as a decimal fraction, and A, B, and C represent the coded variables of fermentation time, fermentation temperature, and inoculation interval, respectively.

Analysis of variance (ANOVA) was performed to evaluate the adequacy and significance of the fitted regression model (Supplementary Table S6). The model was highly significant ($p < 0.001$), indicating a strong regression relationship between the independent variables and the response. The lack-of-fit test was not significant ($p = 0.8097$), demonstrating that the model adequately described the experimental data and possessed good predictive ability. The coefficient of determination (R^2) and adjusted coefficient of determination (adjusted R^2) were 0.9960 and 0.9908, respectively, indicating that the model explained most of the variability in ABTS radical scavenging activity with minimal unexplained error.

In the RSM model, fermentation time (A), fermentation temperature (B), and inoculation interval (C) had significant effects on ABTS radical scavenging activity. Specifically, A, A^2 , and C^2 showed highly significant effects ($p < 0.001$), C showed a significant effect at $p < 0.01$, and B showed a significant effect at $p < 0.05$. In contrast, the interaction terms AB, AC, and BC, as well as the quadratic term B^2 , were not significant ($p > 0.05$) (Figure 2). Based on the F-values, the influence of the factors on ABTS radical scavenging activity was ranked as follows: fermentation time (A) > inoculation interval (C) > fermentation temperature (B).

The optimized conditions predicted by the RSM model were sequential inoculation with Mp followed by Lp after 25 h, a fermentation time of 46 h, and a fermentation temperature of 21.50 °C. The RSM model predicted an ABTS-targeted optimal temperature of 21.50 °C, which was within the experimental temperature range of 18–22 °C used in the response surface design. Under these conditions, the predicted ABTS radical scavenging activity was $77.26 \pm 0.78\%$.

To verify the reliability of the model, three independent experiments were performed under practical conditions corresponding to the predicted optimum: sequential Mp–Lp fermentation, with Mp inoculated first and followed by Lp after 25 h, a fermentation time of 46 h, and a fermentation temperature of 21.50 °C. The experimental ABTS radical scavenging activity reached $78.17 \pm 0.49\%$, which was in close agreement with the predicted value, confirming the validity of the model.

Notably, the ABTS radical-scavenging activity obtained under the RSM-selected conditions increased from 71.48% to 78.17%, representing a relative increase of 9.36% compared with the highest value obtained from the orthogonal design. This result indicates that RSM further improved the ABTS-targeted optimization of the fermentation conditions.

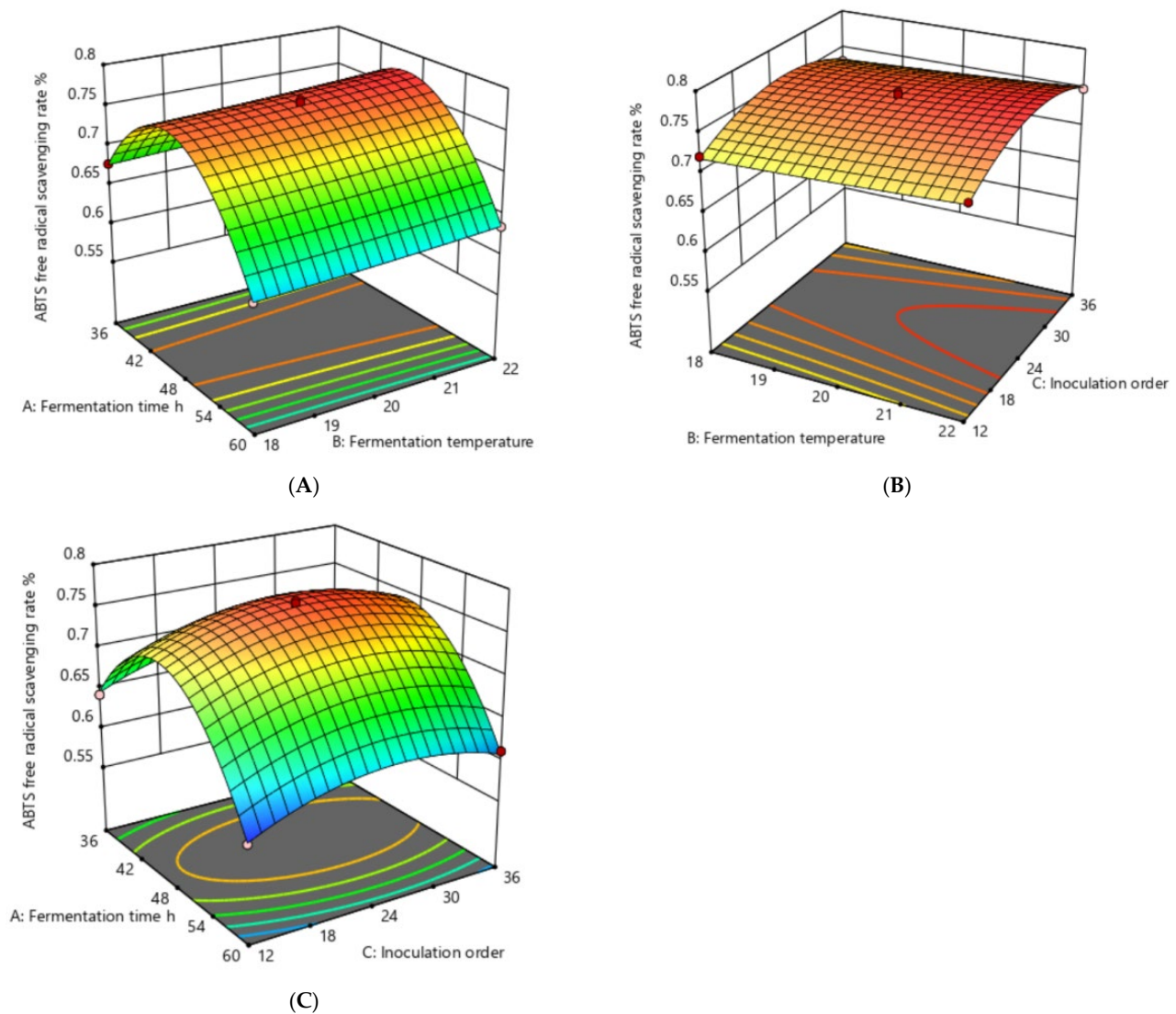


Figure 2. Response surface and contour plots showing the interaction effects of fermentation time, fermentation temperature, and inoculation interval on ABTS radical scavenging activity during Mp–Lp sequential fermentation of apple beverages. (A) Fermentation time and fermentation temperature; (B) fermentation temperature and inoculation interval; (C) fermentation time and inoculation interval. In the response-surface and contour plots, the color gradient from blue to red represents increasing predicted ABTS radical-scavenging activity.

3.2. Microbial Growth and Survival Dynamics During Fermentation

Figure 3 presents the exploratory culturable-count profiles of Mp58 and Cro3-2 during monoculture and sequential fermentation under the baseline conditions. No colonies assigned as presumptive Mp58 were recovered from the CK or Lp monoculture treatments on YPD agar, whereas no colonies assigned as presumptive Cro3-2 were recovered from the CK or Mp monoculture treatments on MRS agar throughout fermentation. Therefore, Figure 3A,B present only the treatments in which the corresponding microorganism was inoculated. However, because formal strain-specific validation of the media was not performed, the absence of colonies in these control treatments should not be interpreted as complete validation of medium selectivity.

As shown in Figure 3A, the presumptive culturable count of Mp58 increased progressively in both the Mp monoculture and Mp–Lp sequential fermentation treatments during the first 24 h. In the Mp monoculture, the count increased from $5.97 \pm 0.05 \log_{10}$ CFU/mL at 0 h to $7.13 \pm 0.07 \log_{10}$ CFU/mL at 24 h. A similar pro-

file was observed in the Mp–Lp treatment, in which the presumptive Mp58 count increased from 5.98 ± 0.07 to $7.01 \pm 0.08 \log_{10}$ CFU/mL over the same period. In the Mp monoculture, the count reached $7.58 \pm 0.07 \log_{10}$ CFU/mL at 36 h and subsequently decreased to $7.34 \pm 0.11 \log_{10}$ CFU/mL at 48 h. In the Mp–Lp treatment, following Cro3-2 inoculation at 24 h, the presumptive Mp58 count was $7.14 \pm 0.08 \log_{10}$ CFU/mL at 36 h and $6.93 \pm 0.05 \log_{10}$ CFU/mL at 48 h. These results indicate that colonies presumptively assigned to Mp58 remained recoverable on YPD agar after the introduction of Cro3-2, although the corresponding counts were lower than those observed in the Mp monoculture at the later sampling points.

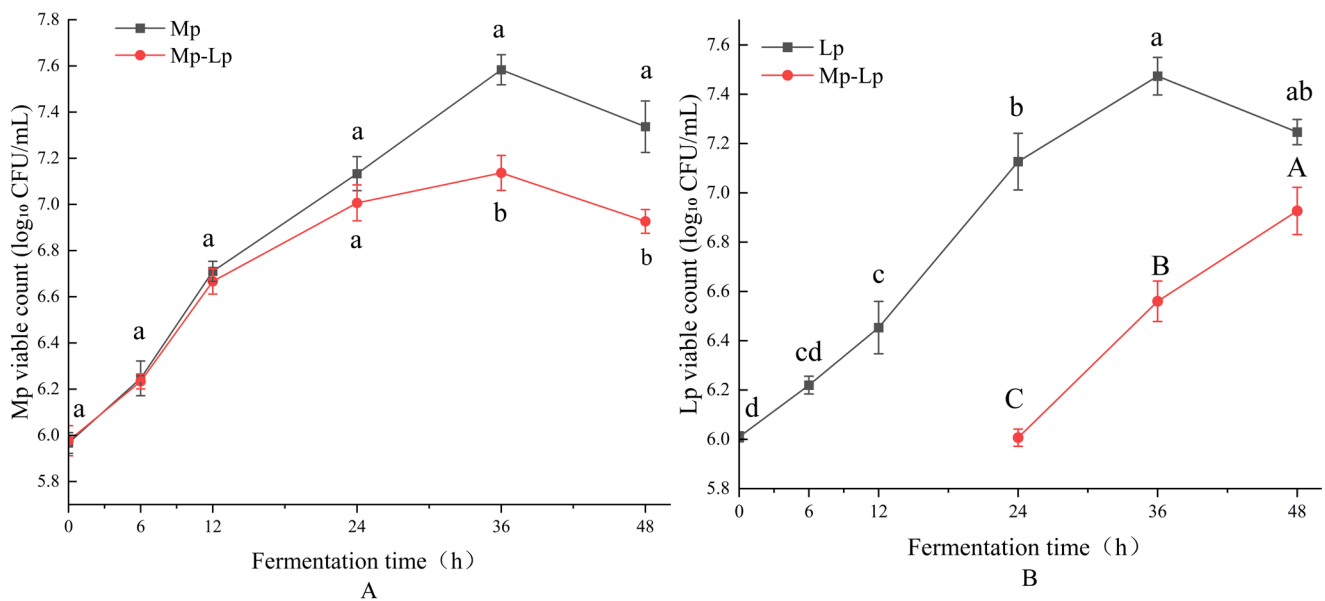


Figure 3. Exploratory culturable-count profiles of Mp58 and Cro3-2 during apple beverage fermentation under the baseline conditions. (A) Presumptive culturable counts of Mp58 recovered on YPD agar in the Mp monoculture and Mp–Lp sequential fermentation treatments; (B) presumptive culturable counts of Cro3-2 recovered on MRS agar in the Lp monoculture and Mp–Lp sequential fermentation treatments. Cro3-2 was inoculated into the Mp–Lp treatment at 24 h. Values are expressed as mean $\pm$ standard deviation of three independent fermentation experiments ($n = 3$). Different lowercase letters indicate significant differences among fermentation times within the monoculture treatments, whereas different uppercase letters indicate significant differences among sampling times within the Mp–Lp sequential fermentation treatment ($p < 0.05$). Because formal strain-specific validation of medium selectivity and recovery was not performed, counts obtained during the mixed-culture stage should be interpreted as exploratory estimates rather than as fully validated strain-specific counts.

As shown in Figure 3B, the presumptive culturable count of Cro3-2 in the monoculture increased from $6.01 \pm 0.02 \log_{10}$ CFU/mL at 0 h to $7.13 \pm 0.12 \log_{10}$ CFU/mL at 24 h. The count reached $7.47 \pm 0.08 \log_{10}$ CFU/mL at 36 h and subsequently decreased to $7.25 \pm 0.05 \log_{10}$ CFU/mL at 48 h. In the Mp–Lp sequential fermentation treatment, no colonies assigned as presumptive Cro3-2 were recovered before its inoculation at 24 h. Immediately after inoculation, the presumptive Cro3-2 count was $6.01 \pm 0.04 \log_{10}$ CFU/mL and subsequently increased to 6.56 ± 0.08 and $6.93 \pm 0.10 \log_{10}$ CFU/mL at 36 and 48 h, respectively. The increase in colonies recovered on MRS agar after 24 h was consistent with the successful introduction and subsequent culturability of Cro3-2 under the baseline sequential-fermentation conditions. Nevertheless, because the medium was not formally validated for strain-specific recovery from the mixed culture, these values should be interpreted as exploratory estimates.

Overall, colonies presumptively assigned to both Mp58 and Cro3-2 remained recoverable during the mixed-culture stage of Mp–Lp sequential fermentation under the baseline conditions of 20 °C for 48 h, with Cro3-2 inoculated at 24 h. Following Cro3-2 inoculation, presumptive Cro3-2 counts increased, whereas presumptive Mp58 counts at 36 and 48 h were lower than those observed in the corresponding Mp monoculture. However, these differences should not be interpreted as definitive evidence of direct competition, antagonism, dominance, or synergistic interaction because the microorganisms were inoculated at different times, experienced different growth durations, and were not enumerated using a formally validated strain-specific differential method.

In addition, this experiment was conducted under the baseline fermentation conditions rather than under the RSM-selected conditions of 21.50 °C, a total fermentation time of 46 h, and Cro3-2 inoculation at 25 h. Although the two sets of conditions were relatively close, the present culturable-count profiles cannot be directly extrapolated to microbial population dynamics under the final RSM-selected process.

3.3. Analysis of Physicochemical Properties

Uninoculated apple juice maintained under the same experimental conditions was used as the control (CK), while the Mp- and Lp-fermented beverages were used as single-strain fermentation controls. The apple beverage produced under the ABTS-targeted conditions selected by response surface methodology was designated as the Mp–Lp group. The physicochemical properties and ethanol contents of the different apple beverages at 46 h are summarized in Table 1.

Table 1. Physicochemical properties and ethanol contents of apple beverages at 46 h.

Treatment	pH	Titrateable Acidity (g/L Malic Acid)	Soluble Solids Content (°Brix)	Total Sugar (g/L)	Ethanol (% v/v)
CK	3.36 ± 0.01 ^a	2.99 ± 0.07 ^d	11.17 ± 0.06 ^a	97.33 ± 2.31 ^a	0.0
Mp	3.20 ± 0.02 ^b	3.17 ± 0.05 ^b	10.73 ± 0.06 ^c	94.67 ± 0.58 ^b	0.1
Lp	3.13 ± 0.02 ^c	3.26 ± 0.08 ^a	10.70 ± 0.10 ^d	92.00 ± 1.00 ^d	0.0
Mp–Lp	3.22 ± 0.02 ^b	3.12 ± 0.04 ^c	10.77 ± 0.06 ^b	93.33 ± 2.31 ^c	0.1

Physicochemical data are presented as mean ± standard deviation of three independent experiments. Different lowercase superscript letters within the same column indicate significant differences among treatments ($p < 0.05$). Ethanol values are hydrometer readings obtained using the distillation–hydrometer method, for which 0.1% (v/v) was the minimum positive graduation. A reading of 0.0% (v/v) indicates that no reading above the zero graduation was obtained and should not be interpreted as confirmation of the complete absence of ethanol. CK, uninoculated control; Mp, monoculture fermentation with *Metschnikowia* sp. Mp58 belonging to the *M. pulcherrima* clade; Lp, *Lactiplantibacillus plantarum* monoculture fermentation; Mp–Lp, sequential fermentation with Mp followed by Lp.

At 46 h, all fermented beverages exhibited significantly lower pH values and significantly higher titrateable acidity than CK ($p < 0.05$). Among the fermented groups, the Lp-fermented beverage had the lowest pH value (3.13 ± 0.02) and the highest titrateable acidity (3.26 ± 0.08 g malic acid/L), indicating the greatest degree of acidification. The pH values of the Mp- and Mp–Lp-fermented beverages were 3.20 ± 0.02 and 3.22 ± 0.02 , respectively, compared with 3.36 ± 0.01 in CK. Their corresponding titrateable acidity values were 3.17 ± 0.05 and 3.12 ± 0.04 g malic acid/L, respectively, compared with 2.99 ± 0.07 g malic acid/L in CK. These changes may be associated with the production and accumulation of organic acids during fermentation. However, because individual organic acids were not quantified, the specific acids responsible for the observed differences could not be determined.

The soluble solids content and total sugar content were also significantly lower in the fermented beverages than in CK ($p < 0.05$). CK retained the highest soluble solids content (11.17 ± 0.06 °Brix) and total sugar content (97.33 ± 2.31 g/L). Among the fer-

mented groups, the Lp-fermented beverage exhibited the lowest soluble solids content (10.70 ± 0.10 °Brix) and total sugar content (92.00 ± 1.00 g/L). The corresponding values were 10.73 ± 0.06 °Brix and 94.67 ± 0.58 g/L in the Mp-fermented beverage and 10.77 ± 0.06 °Brix and 93.33 ± 2.31 g/L in the Mp–Lp sequentially fermented beverage. These differences indicate treatment-dependent carbohydrate utilization under the tested fermentation conditions. However, they should not be attributed solely to microbial interactions because Mp and Lp were inoculated at different times in the sequential fermentation treatment.

Ethanol readings were 0.0% (v/v) in the CK and Lp groups and 0.1% (v/v) in the Mp and Mp–Lp groups. Because 0.1% (v/v) was the minimum positive graduation of the alcohol hydrometer, these results indicate limited ethanol accumulation under the tested fermentation conditions. A reading of 0.0% (v/v) indicates only that no reading above the zero graduation was obtained and should not be interpreted as confirmation of the complete absence of ethanol. Because formal limits of detection and quantification were not established, ethanol concentrations below the measurement capability of the method cannot be excluded.

3.4. Analysis of Bioactive Compounds

Phenolic compounds are important bioactive constituents of fruits and are widely recognized for their antioxidant properties [33]. The predominant phenolic compounds in fermented apple beverages include flavonoids, catechins, hydroxycinnamic acids, and dihydrochalcones [34]. Among these compounds, flavonoids are abundant in apples and generally exhibit stronger antioxidant activity than other phenolic subclasses. Therefore, increasing total phenolic and flavonoid contents is considered an important strategy for improving the nutritional and functional quality of fruit beverages.

The total phenolic content (TPC) and total flavonoid content (TFC) of fermented apple beverages from different fermentation groups are shown in Figure 4A,B. During fermentation (0–46 h), the TPC and TFC of the Mp-, Lp-, and Mp–Lp-fermented beverages generally increased, whereas a decreasing trend was observed in the unfermented control (CK). After 46 h, the Mp–Lp sequentially fermented beverage exhibited the highest TPC (253.95 ± 3.20 mg GAE/L), which was significantly higher than those of the Lp- and Mp-fermented beverages ($p < 0.05$). The TPC values of all fermented groups were significantly higher than that of the unfermented control (CK) (146.72 ± 4.42 mg GAE/L; $p < 0.05$). At the same time point, TFC was also highest in the Mp–Lp group (109.71 ± 1.84 mg RE/L), slightly higher than that in the Mp group, although the difference was not significant ($p > 0.05$), and significantly higher than those in the Lp and CK groups ($p < 0.05$).

These results indicate that fermentation with both Mp and Lp effectively enhanced the accumulation of phenolic and flavonoid compounds in fermented apple beverages. The increase observed during Mp fermentation may be associated with its extracellular enzyme activities, including β -glucosidase and pectinase [13]. The enhancement of TPC and TFC during Lp fermentation may be related to acid production, which facilitates the release, transformation, and enzymatic depolymerization of bound phenolic compounds [35]. Notably, Mp–Lp sequential fermentation resulted in higher TPC and TFC than single-strain fermentation, consistent with previous findings in Mp–Lp-fermented mango juice [18], highlighting the potential advantage of sequential fermentation for phenolic enrichment.

Ascorbic acid contributes to human health primarily through its antioxidant activity as an electron donor [36]. As shown in Figure 4C, after 46 h of fermentation, the ascorbic acid content of fermented apple beverages did not decrease significantly and remained slightly higher than the initial level, whereas a significant reduction was observed in the unfermented control (CK) ($p < 0.05$).

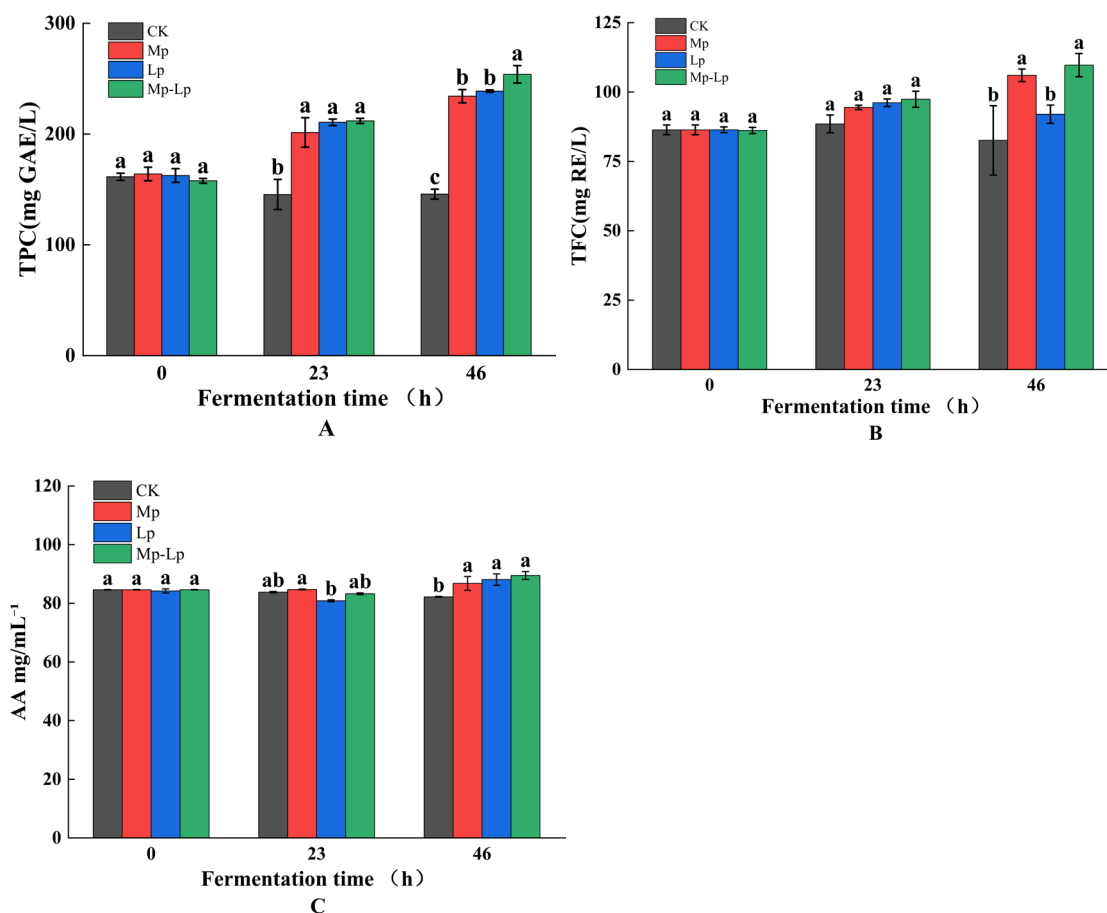


Figure 4. Bioactive compounds of apple beverages before and after fermentation. (A) Total phenolic content; (B) total flavonoid content; (C) ascorbic acid content. Values are expressed as mean $\pm$ standard deviation ($n = 3$). Different lowercase letters indicate significant differences among treatments at the same fermentation time ($p < 0.05$).

These results suggest that fermentation contributes to the retention of ascorbic acid in fermented apple beverages. Previous studies have reported that Lp fermentation can enhance or preserve ascorbic acid levels in fruit and vegetable beverages [37], possibly through the production of specific metabolites and pH modulation, which create a more favorable environment for ascorbic acid stability. In addition, Mp fermentation has been reported to promote ascorbic acid retention, potentially because of its high oxygen consumption, which may limit polyphenol oxidase activity and reduce oxidative degradation [38].

Overall, Mp–Lp sequential fermentation was more effective in enhancing and preserving key bioactive compounds than single-strain fermentation.

3.5. Analysis of Antioxidant Activity

Antioxidant activities of fermented apple beverages were evaluated using ABTS, DPPH, and FRAP assays (Figure 5). During fermentation, ABTS radical scavenging activity, DPPH radical scavenging activity, and ferric reducing antioxidant power (FRAP) increased in all fermented beverages, whereas a slight decrease was observed in the unfermented control. After 46 h, the Mp–Lp sequentially fermented beverage exhibited the highest ABTS radical scavenging activity, DPPH radical scavenging activity, and FRAP value, followed by the Lp- and Mp-fermented beverages. All fermented beverages showed significantly higher antioxidant activities than the control ($p < 0.05$). These results indicate that fermentation with Mp and Lp enhanced antioxidant capacity, which was consistent with the increases in

total phenolic and flavonoid contents. Notably, Mp–Lp sequential fermentation exerted a stronger effect than single-strain fermentation.

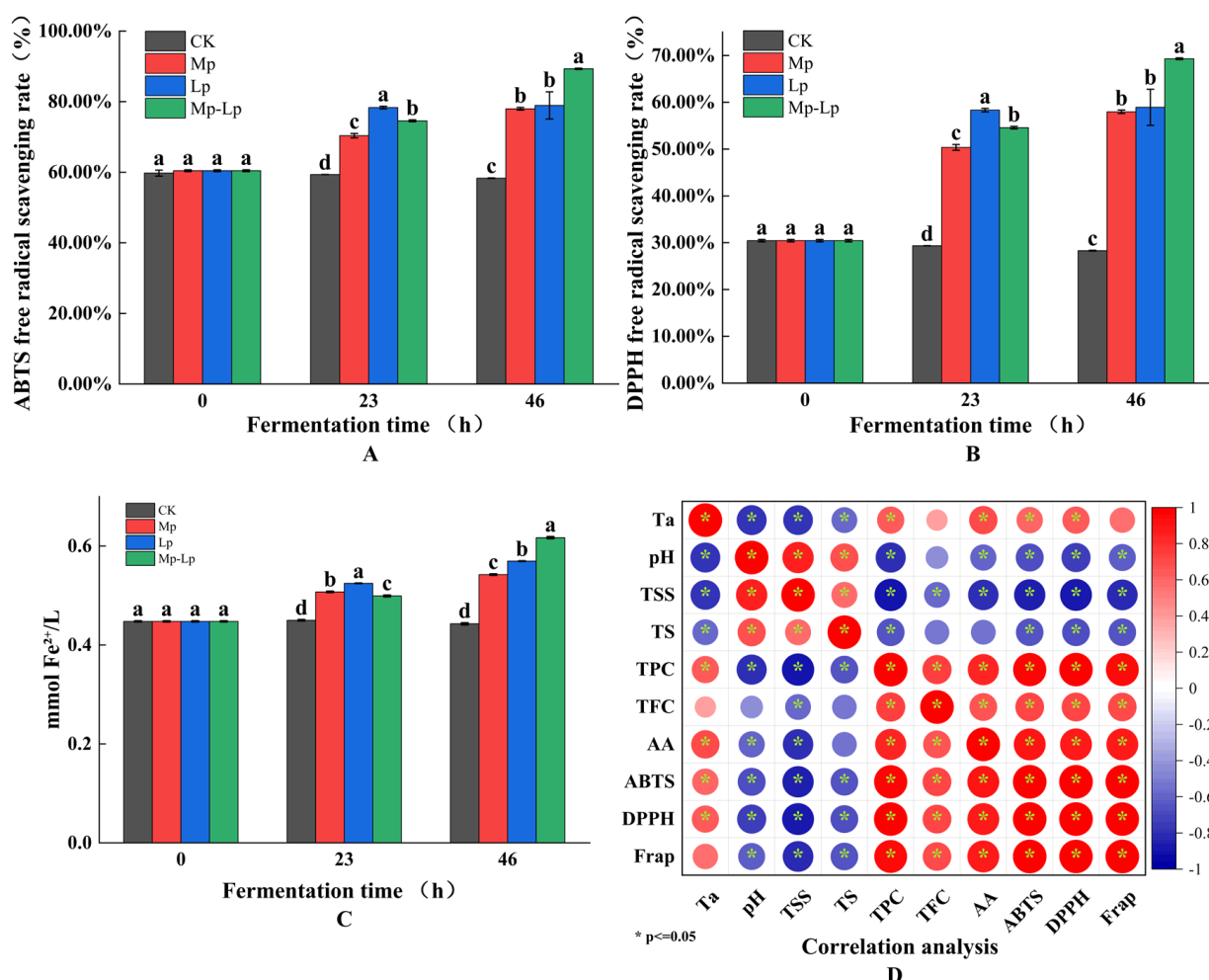


Figure 5. Antioxidant activity and correlation analysis of apple beverages before and after fermentation. (A) ABTS radical scavenging activity; (B) DPPH radical scavenging activity; (C) ferric reducing antioxidant power (FRAP); (D) correlation analysis among physicochemical properties, bioactive compounds, and antioxidant activities. Values are expressed as mean $\pm$ standard deviation ($n = 3$). Different lowercase letters indicate significant differences among treatments at the same fermentation time ($p < 0.05$). Asterisks in the correlation plot indicate significant correlations ($* p < 0.05$).

Correlation analysis among DPPH radical scavenging activity, ABTS radical scavenging activity, ferric reducing antioxidant power (FRAP), total phenolic content, and total flavonoid content in fermented apple beverages (Figure 5D) revealed significant positive correlations between antioxidant activity and both phenolic and flavonoid contents ($p < 0.05$), with flavonoids showing a stronger association. The Mp–Lp sequentially fermented beverage exhibited the highest total phenolic content (Figure 4A) and total flavonoid content (Figure 4B), which may partially explain its superior ABTS radical scavenging activity, DPPH radical scavenging activity, and FRAP value (Figure 5).

Previous studies have shown that Lp can enhance antioxidant capacity by transforming phenolic compounds during fermentation, thereby increasing the availability of more active antioxidant metabolites [39,40]. Similarly, the enhanced antioxidant activity observed in Mp-fermented beverages may be associated with β -glucosidase activity, which promotes the release of polyphenols and terpene derivatives and consequently increases overall antioxidant capacity [41].

Overall, both single-strain fermentation and Mp–Lp sequential fermentation enhanced the antioxidant capacity of apple beverages, with sequential fermentation producing the greatest improvement.

3.6. Analysis of In Vitro Enzyme Inhibitory and Bile Salt-Binding Capacities

Both α -glucosidase and α -amylase inhibitory activities of fermented apple beverages are shown in Figure 6A,B. All three fermentation treatments significantly increased enzyme inhibitory activities ($p < 0.05$). The Mp–Lp sequentially fermented beverage exhibited the highest inhibitory activities toward both enzymes (85.97% and 78.35%, respectively), followed by the Lp-fermented beverage (66.73% and 62.50%) and the Mp-fermented beverage (52.73% and 57.10%). All fermented beverages showed significantly higher inhibitory activities than the unfermented control (CK; 27.27% and 31.45%) ($p < 0.05$).

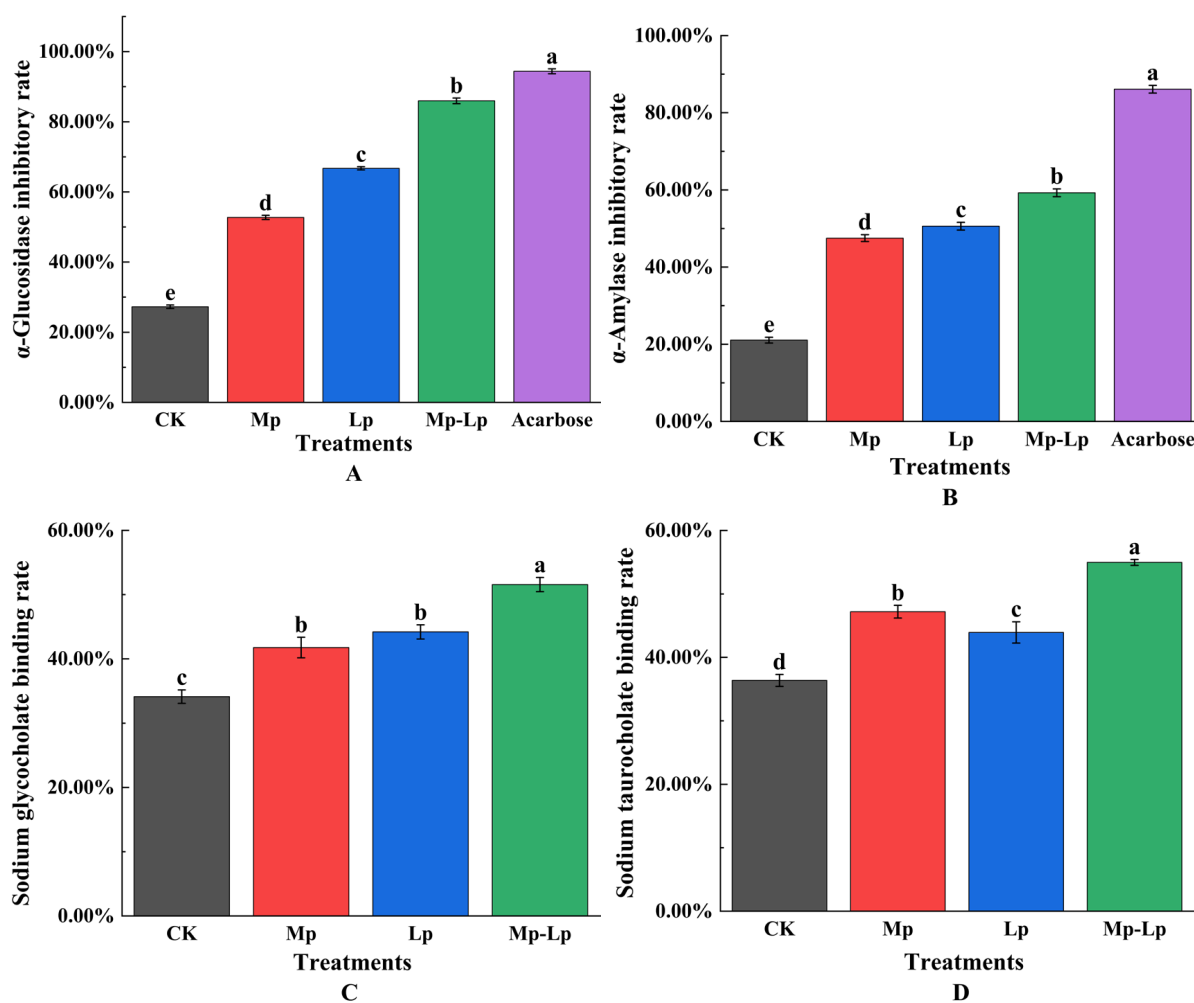


Figure 6. In vitro enzyme inhibitory and bile salt-binding capacities of fermented apple beverages. (A) α -Glucosidase inhibitory activity; (B) α -amylase inhibitory activity; (C) sodium glycocholate-binding capacity; (D) sodium taurocholate-binding capacity. Values are expressed as mean $\pm$ standard deviation ($n = 3$). Different lowercase letters indicate significant differences among treatments ($p < 0.05$).

Starch hydrolysis, mainly mediated by α -amylase and α -glucosidase, contributes to glucose release during carbohydrate digestion [42]. α -Glucosidase catalyzes the final step of carbohydrate digestion by converting oligosaccharides into absorbable glucose. Previous studies have shown that bioactive compounds in fruit beverages, particularly phenolics and polysaccharides, can inhibit α -glucosidase activity [43]. During digestion,

salivary and pancreatic α -amylases hydrolyze starch by cleaving α -1,4-glycosidic bonds to produce maltose and short-chain oligosaccharides [44]. These products are subsequently hydrolyzed by α -glucosidase located on the intestinal brush border [45].

In the present study, fermentation with Mp and Lp, particularly Mp–Lp sequential fermentation, significantly enhanced α -glucosidase and α -amylase inhibitory activities. This enhancement was accompanied by increased levels of phenolics and polysaccharides. Mp has been reported to alter carbohydrate metabolism by channeling starch hydrolysates into lipid biosynthesis [46]. Such metabolic changes may contribute to the enhanced enzyme inhibitory activities observed in the fermented beverages.

To evaluate bile salt-binding capacity, sodium glycocholate and sodium taurocholate were used as model bile salts in simulated gastrointestinal digestion. The binding capacities toward the two bile salts are shown in Figure 6C,D. Fermented apple beverages exhibited significantly higher bile salt-binding capacities than the control ($p < 0.05$). Binding to sodium taurocholate was greater than that to sodium glycocholate. Among all treatments, the Mp–Lp sequentially fermented beverage showed the highest binding capacity, reaching $51.57 \pm 0.46\%$ for sodium glycocholate and $54.97 \pm 0.32\%$ for sodium taurocholate.

Bile salt-binding capacity is commonly used as an in vitro indicator associated with potential cholesterol-lowering properties because bile acid binding may influence bile acid availability during intestinal digestion. However, the present results demonstrate only physicochemical bile salt binding under simulated gastrointestinal conditions and do not establish bile acid excretion, cholesterol-lowering activity, or hypolipidemic effects in vivo. More broadly, fermented milk products containing *Bifidobacterium longum* have been investigated for their effects on blood lipid profiles in animal and human models [47]. However, because these findings were obtained using a dairy matrix and in vivo experimental models, they cannot be directly extrapolated to the present fermented apple beverage.

3.7. Analysis of Volatile Compounds

A total of 95 volatile compounds were tentatively identified across all apple beverage samples based on mass spectral library matching and retention-index comparison, with esters, alcohols, terpenes, and fatty acids representing the major detected classes. Detailed information on the semi-quantitative abundances, expressed as $\mu\text{g/L}$ 4-octanol equivalents, literature-derived odor thresholds, threshold sources and matrices, and approximate odor activity values is provided in Supplementary Table S7. The approximate odor activity value of each compound was calculated as the ratio of its 4-octanol-based semi-quantitative abundance to the corresponding literature-derived odor threshold. Because compound-specific calibration curves, response factors, recoveries, matrix effects, and formal LOD and LOQ values were not established, the reported abundances should not be interpreted as absolute concentrations. In addition, because odor thresholds may vary substantially among matrices, the calculated approximate OAVs were used only as preliminary screening indicators of potential aroma relevance and not as direct evidence of sensory impact. Compounds with approximate OAVs greater than 1 were considered candidate compounds with potential aroma relevance, whereas compounds with approximate OAVs of at least 0.1 were retained for subsequent exploratory multivariate analysis. In total, 27 volatile compounds met the latter criterion.

As shown in Figure 7A, 59 volatile compounds were detected in each of the CK, Mp-fermented, and Lp-fermented beverages, whereas 69 volatile compounds were detected in the Mp–Lp sequentially fermented beverage, indicating a larger number of detected volatile compounds following sequential fermentation. However, the total semi-quantitative volatile abundance decreased after fermentation and was lower in the fermented beverages than in the unfermented control. The total semi-quantitative volatile abundance was

2775.41 µg/L 4-octanol equivalents in CK and ranged from 822.82 to 959.65 µg/L 4-octanol equivalents in the fermented beverages. These results suggest that both single-strain and sequential fermentation reduced the semi-quantitative abundances of several native apple volatile compounds, particularly esters. This decrease may be associated with volatilization, microbial transformation, or metabolic conversion during fermentation. Similarly, Ji et al. [48] reported a decrease in esters and an increase in alcohols during Lp fermentation, resulting in alcohols becoming the predominant volatile class, which is consistent with the present findings.

The relative composition of volatile classes, expressed as normalized peak-area percentages, changed markedly after fermentation (Figure 7B). Before fermentation, esters were the predominant volatile class, accounting for 76.96% of the total normalized volatile peak area. After fermentation, the normalized relative proportion of esters decreased, whereas those of higher alcohols and fatty acids increased. Within the subset of compounds with approximate OAVs ≥ 0.1 , esters accounted for 26.51% of the normalized peak area in the Mp–Lp sequentially fermented beverage, compared with 21.61% and 17.86% in the Mp- and Lp-fermented beverages, respectively, although alcohols remained the predominant volatile class. Among the fermented samples, the Mp–Lp beverage exhibited the highest summed semi-quantified abundance of fatty acids, expressed as µg/L 4-octanol equivalents, mainly attributable to decanoic acid and nonanoic acid. Fatty acids have been reported to influence the fruity and complex aroma characteristics of fermented apple beverages, depending on their identities and relative abundances [49]. These results indicate that Mp–Lp sequential fermentation altered the relative peak-area distribution of esters and higher alcohols, as well as the semi-quantified fatty acid profile, compared with the single-strain fermentations. In contrast, the unfermented control contained a higher normalized relative proportion of esters (76.96%) and a lower proportion of alcohols (7.13%), reflecting its distinct non-fermented volatile composition. However, these compositional differences should not be interpreted as evidence of improved aroma balance or sensory quality in the absence of sensory evaluation.

Exploratory PCA was performed using the 27 volatile compounds with approximate OAVs ≥ 0.1 (Figure 7C). PC1 and PC2 explained 76.2% and 13.9% of the total variance, respectively, accounting for a cumulative variance of 90.1%. The CK samples were located on the positive side of PC1 and were visually separated from the Mp-fermented, Lp-fermented, and Mp–Lp sequentially fermented beverages in the score plot, indicating treatment-associated differences in volatile composition between the unfermented and fermented samples. The Mp–Lp sequentially fermented beverage was positioned between CK and the single-strain fermented beverages along PC1, suggesting an intermediate chemical profile within the present dataset. This distribution reflects differences in volatile composition but does not demonstrate retention of fruity aroma, perceived sensory similarity, or improved aroma balance.

The volatile profile of CK was characterized by relatively high semi-quantitative abundances of several esters and terpenes. Butyl acetate, hexyl acetate, isoamyl acetate, and β -damascenone have been associated in the literature with fruity, pear-like, banana-like, and floral odor descriptors, respectively. These descriptors are provided only as literature-based chemical context and do not represent sensory observations from the present study. Compared with CK, several higher alcohols and fatty acids showed higher semi-quantitative abundances and/or approximate OAVs in the fermented beverages. Isoamyl acetate, phenylethyl alcohol, and isobutyl alcohol were among the candidate compounds with potential aroma relevance and have been associated in the literature with banana-like, floral, honey-like, or alcoholic odor descriptors.

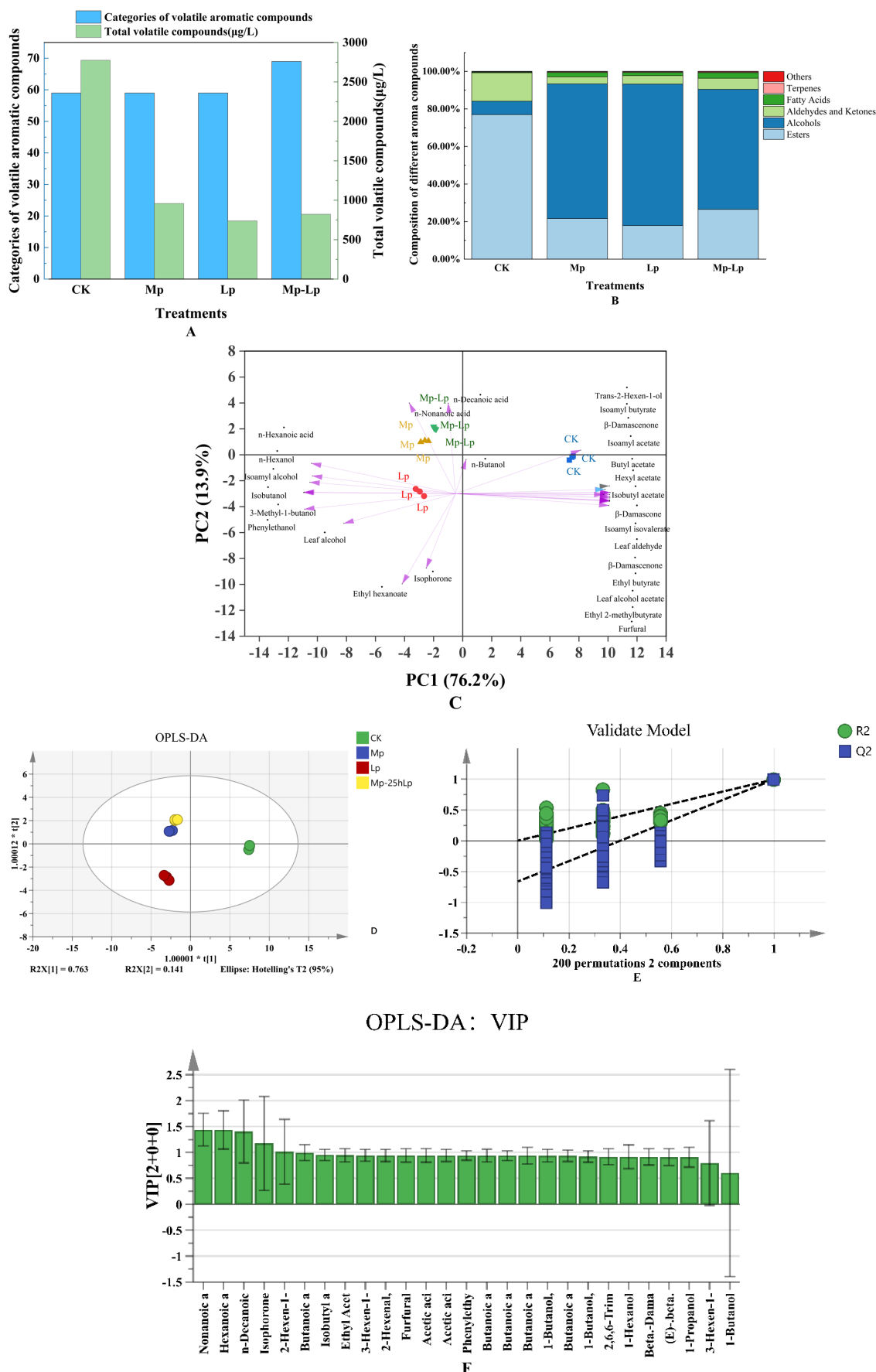


Figure 7. Volatile aroma profiles of apple beverages subjected to different fermentation treatments. (A) Number and total semi-quantified abundance of volatile compounds; (B) composition of major

volatile compound classes; (C) principal component analysis (PCA) based on candidate compounds with potential aroma relevance with odor activity values (approximate OAVs) ≥ 0.1 ; (D) orthogonal partial least squares discriminant analysis (OPLS-DA) score plot based on candidate compounds with potential aroma relevance with approximate OAVs ≥ 0.1 ; (E) permutation test of the OPLS-DA model with 200 permutations; (F) variable importance in projection (VIP) scores of volatile variables associated with group separation. The dashed line indicates the VIP threshold of 1.0.

In particular, the Mp–Lp sequentially fermented beverage showed relatively high semi-quantitative abundances of nonanoic acid and n-decanoic acid. These compounds have been associated in the literature with fatty, waxy, or creamy odor descriptors. Their relatively high semi-quantitative abundances and approximate OAVs suggest that they may be associated with the altered volatile characteristics of the sequentially fermented beverage. However, their actual sensory contributions cannot be determined from chemical analysis alone and would require further confirmation using sensory evaluation, gas chromatography–olfactometry, aroma recombination, or omission experiments.

Exploratory OPLS-DA was further performed using the 27 volatile compounds with approximate OAVs ≥ 0.1 to examine treatment-associated differences in volatile composition. The two-component model yielded cumulative R^2X , R^2Y , and Q^2 values of 0.905, 0.665, and 0.506, respectively (Figure 7D). The data were mean-centered and unit-variance scaled before modeling, and internal model assessment was performed using seven-group cross-validation and a 200-permutation test. The regression-line intercepts obtained from the permutation test were approximately 0.001 for R^2 and -0.660 for Q^2 (Figure 7E).

Although these internal assessment results indicated that the original model performed better than models generated after class-label permutation within the present dataset, the limited number of biological replicates—three per treatment—substantially restricts the robustness and generalizability of the supervised model. In addition, neither external validation nor bootstrap analysis was performed. The OPLS-DA results were therefore interpreted as exploratory and were used only to identify candidate volatile variables associated with group separation rather than to establish validated discriminant biomarkers.

Variable importance in projection scores was combined with univariate statistical analysis to screen candidate volatile variables associated with group separation. According to Supplementary Table S8, five compounds met the criteria of $VIP > 1$ and $p < 0.001$: nonanoic acid, ethyl hexanoate, n-decanoic acid, isophorone, and (E)-2-hexen-1-ol. Nonanoic acid had the highest VIP value of 1.439, followed by ethyl hexanoate at 1.434 and n-decanoic acid at 1.402. These compounds should be regarded as candidate variables associated with separation in the present dataset rather than as independently validated biomarkers.

Nonanoic acid and n-decanoic acid showed higher semi-quantitative abundances in the Mp–Lp sequentially fermented beverage and also exhibited relatively high approximate OAVs, indicating an association between sequential fermentation and changes in fatty-acid-related volatile compounds. In addition, (E)-2-hexen-1-ol is a C6 unsaturated alcohol that has been detected in apple cider and has been associated with green and leafy odor descriptors [50]. Its higher semi-quantitative abundance in the Mp-fermented and Mp–Lp sequentially fermented beverages suggests an association between Mp-containing fermentation treatments and changes in this compound. However, its contribution to perceived freshness or green aroma cannot be established without sensory or olfactometric validation.

Perpetuini et al. [16] similarly reported that sequential inoculation involving *Metschnikowia pulcherrima* modified the volatile composition of wine. Although the fermentation matrix and microbial combination differed from those used in the present study, their findings support the potential influence of *Metschnikowia*-containing sequential fermentation on volatile profiles.

The observed changes in volatile composition may be related to microbial metabolism and extracellular enzymatic activities during fermentation. Mp has been reported to exhibit extracellular hydrolytic activities, including β -glucosidase, pectinase, and protease activities, although the occurrence and intensity of these activities are strain dependent [13,51]. Such activities could potentially facilitate the release or transformation of volatile precursors and other metabolites. However, because the relevant enzymatic activities and metabolic pathways were not directly measured in the present study, these explanations remain hypothetical and require further experimental verification.

Overall, fermentation substantially altered the semi-quantitative abundance and relative distribution of volatile compounds in the apple beverages. Although several native apple volatile compounds decreased following fermentation, the Mp–Lp sequentially fermented beverage contained a larger number of detected volatile compounds and a higher normalized peak-area proportion of esters than the single-strain fermented beverages. The combined semi-quantitative analysis, approximate OAV screening, PCA, and exploratory OPLS-DA indicated treatment-associated modifications of the volatile profile following Mp–Lp sequential fermentation. Nevertheless, these chemical results do not constitute direct evidence of improved aroma quality, aroma balance, perceived complexity, or consumer acceptance.

4. Conclusions

This study established and optimized an ABTS-targeted sequential fermentation strategy for fermented apple beverages using *Metschnikowia* sp. Mp58 belonging to the *M. pulcherrima* clade (Mp) and *Lactiplantibacillus plantarum* Cro3-2 (Lp). The selected conditions consisted of Mp inoculation at 0 h, Lp inoculation after 25 h, and fermentation for a total of 46 h at 21.50 °C. These conditions were selected exclusively on the basis of ABTS radical-scavenging activity and should therefore be interpreted as ABTS-targeted process conditions rather than as conditions optimized for overall product quality.

Compared with the uninoculated control, the Mp–Lp sequentially fermented beverage exhibited higher total phenolic, total flavonoid, and ascorbic acid contents, together with higher ABTS and DPPH radical-scavenging activities and ferric reducing antioxidant power. The Mp–Lp treatment also exhibited the highest in vitro α -glucosidase and α -amylase inhibitory activities among the tested treatments and showed increased bile salt-binding capacities relative to the uninoculated control. These findings represent in vitro functional indicators and should not be interpreted as evidence of physiological or health effects without further bioaccessibility and in vivo validation.

Mp–Lp sequential fermentation also altered the semi-quantitative abundance and relative distribution of volatile compounds. However, these chemical differences do not demonstrate improved aroma balance, aroma complexity, sensory quality, or consumer acceptance because sensory evaluation was not conducted. In addition, whole-genome sequencing and strain-level genomic and phenotypic safety assessments of Mp58 and Cro3-2 were not performed. Further studies involving sensory evaluation, strain-level safety assessment, microbial dynamics under the RSM-selected conditions, storage stability, bioaccessibility, in vivo validation, and scale-up fermentation are required before practical food application.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation12080350/s1>. Figure S1: ABTS radical scavenging activity of apple beverages fermented with individual strains of *Metschnikowia pulcherrima* (A) and *Lactiplantibacillus plantarum* (B); Table S1: Molecular identification of strains Mp58 and Cro3-2 based on rRNA gene sequencing; Table S2: Factors and levels of the orthogonal experiment; Table S3: Factors and levels used in the response surface methodology design; Table S4: Results of the orthogonal

experiment; Table S5: Experimental design and results of response surface methodology optimization; Table S6: Analysis of variance (ANOVA) for the fitted response surface methodology model; Table S7: Semi-quantified abundances and odor activity values (approximate OAVs) of volatile compounds in apple beverage samples [52–68]; Table S8: VIP and *p*-values of volatile compounds analyzed by OPLS-DA.

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