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Transcriptional and Metabolic Response of Wine-Related *Lactiplantibacillus plantarum* to Different Conditions of Aeration and Nitrogen Availability

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Abstract: Lactic acid bacteria (LAB) perform the process of malolactic fermentation (MLF) in wine. Availability of oxygen and nitrogen nutrients could influence LAB growth, malolactic activity, and other metabolic pathways, impacting the subsequent wine quality. The impact of these two factors has received limited investigation within LAB, especially on a transcriptome level. The aim of this study was to evaluate metabolic changes in the strain *Lactiplantibacillus plantarum* IWBT B063, growing in synthetic grape juice medium (GJM) under different oxygen exposure conditions, and with low availability of nitrogen-based nutrients. Next-generation sequencing was used to analyze expression across the transcriptome (RNA-seq), in combination with conventional microbiological and chemical analysis. *L. plantarum* consumed the malic acid present in all the conditions evaluated, with a slight delay and impaired growth for nitrogen limitation and for anaerobiosis. Comparison of *L. plantarum* transcriptome during growth in GJM with and without O_2 revealed differential expression of 148 functionally annotated genes, which were mostly involved in carbohydrate metabolism, genetic information processing, and signaling and cellular processes. In particular, genes with a protective role against oxidative stress and genes related to amino acid metabolism were differentially expressed. This study confirms the suitability of *L. plantarum* IWBT B063 to carry out MLF in different environmental conditions due to its potential adaption to the stress conditions tested and provides a better understanding of the genetic background of an industrially relevant strain.

Keywords: malolactic fermentation; *L. plantarum*; anaerobic growth; nutrient availability; RNA-seq

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1. Introduction

Transformation of grape must in wine is a multifaceted process from a microbiological point of view, with a succession of different groups of microorganisms, more or less adapted to specific conditions throughout the course of winemaking. Lactic acid bacteria (LAB) are among the most relevant groups, mainly responsible for carrying out malolactic fermentation (MLF). The decarboxylation of L-malic acid to L-lactic acid is relevant in most red wines and some white wines as well, where it contributes to deacidification, microbial stability, and aroma complexity [1–3].

The main LAB species involved in MLF belong to the genera *Oenococcus*, *Lactobacillus sensu lato*, *Leuconostoc*, and *Pediococcus*. More recently, particularly *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum* [4]) has gained increased attention from researchers and winemakers, with proven suitability to drive this process [2,5]. The principal sensory effect of LAB is the conversion of tart malic acid into the softer and rounder lactic acid, but

other secondary metabolic reactions and enzymatic activities have also great impacts on wine aroma and flavor development [2,3].

Even with the inoculation of selected LAB starter cultures, the onset of MLF can be difficult to manage in some conditions. Multiple factors are held accountable for challenging the activity of LAB in winemaking conditions, of which the four best-studied are pH, sulfur dioxide (SO_2), ethanol, and temperature. From these, SO_2 in particular is added to must/wine due to its antioxidant and antimicrobial activity and is a potent inhibitor of LAB growth, especially the molecular form, in synergy with low pH and high temperature/ethanol. Nevertheless, less investigated factors have also to be considered, including yeast–LAB interactions, carbohydrates and organic acid content, phenolic compounds, inhibitory molecules, oxygen, and nitrogen availability [5,6].

LAB, usually developing in wine, are aerotolerant, fermentative bacteria, and MLF is minimally exposed to air to prevent oxidation and the growth of spoilage organisms. Availability of oxygen could influence bacterial growth, malolactic activity, carbon and nitrogen utilization, and the activity of other enzymes, impacting on wine properties. Certain compounds in wine can only be metabolized by LAB in the presence of oxygen, such as certain intermediates in the citric acid metabolic pathway [7,8]. Furthermore, research has demonstrated that some LAB strains could show advantageous industrial and biotechnological traits when exposed to conditions which promote aerobic and/or respiratory growth [9].

In addition, LAB need nitrogen for various vital functions. The most frequent sources are free amino acids and small peptides, present at varying concentrations depending on the grape variety and winemaking practices. Moreover, these nitrogen sources could be exhausted by yeasts during alcoholic fermentation and inhibit MLF. LAB are very fastidious in their amino acid requirements [1,2], although the exact quantities required for adequate growth and metabolism are still unclear and need further investigation [10].

Recent astounding progress in the -omics sciences made available a great level of knowledge regarding the diversity of LAB in winemaking and the mechanisms involved in many metabolic processes [11–13]. In particular, transcriptomics could help to elucidate the molecular basis behind the impacts of medium nutritional composition on growth and fermentation performance [14]. Among these approaches, RNA-seq uses high-efficiency sequencing of complementary DNAs (cDNAs) reverse-transcribed from RNAs to detect and compare whole-genome gene expression. It has many advantages compared to microarray technology, such as rapidness, high precision, and reproducibility, as RNA-seq does not require probe sequences and also has a greater dynamic range for measuring very low or very high gene expression levels [15,16]. The power of RNA-seq has been demonstrated in transcriptomics studies with a diverse array of bacteria [17,18], including *L. plantarum* [19–21], although none of these are related to MLF.

Regarding wine-associated microorganisms, RNA-seq was used to study *Saccharomyces cerevisiae* under different fermentation conditions [22–24], as well as *Torulaspora delbrueckii* [25], *Hanseniaspora vineae* [26], and *Brettanomyces bruxellensis* [27]. Studies focused on wine LAB are still scarce; a few investigations have dealt with *Oenococcus oeni* [16,28], but, to the best of our knowledge, this is the first report of RNA-seq analysis with wine-associated *L. plantarum* during MLF.

Hence, the aim of this study was to investigate transcriptional and metabolic changes in a *L. plantarum* strain during exposure to oxygen in medium with different nitrogen nutrient concentrations under controlled conditions using a bioreactor system. Besides the transcriptomic analysis through RNA-seq technology, cellular growth and malic acid degradation were determined during MLF in a synthetic grape juice medium. Data obtained will help to elucidate how aeration and nitrogen availability impact the gene expression level and how this could relate to changes in wine composition. Ultimately, the goal is to provide new tools to enhance the management of MLF in wine.

2. Materials and Methods

2.1. Strain and Inoculum Preparation

The strain *Lactiplantibacillus plantarum* IWBT B063 (South African Grape and Wine Research Institute (SAGWRI) culture collection, Stellenbosch University, Stellenbosch, South Africa), conserved at −80 °C and routinely maintained on MRS agar (50 g/L MRS broth with 15 g/L bacteriological agar (Biolab Diagnostics, Wadeville, South Africa)) at 4 °C, was cultured anaerobically at 30 °C without agitation in MRS broth (Biolab Diagnostics) for 48 h. Cells were harvested by centrifugation at 5000× *g* for 4 min, after reaching the stationary phase (approximately 1×10^9 CFU/mL). Prior to inoculation, the cell pellet was washed twice with 0.9 % NaCl solution to remove any residual medium.

2.2. MLF Conditions

MLF was carried out in the grape juice medium (GJM) formulated by Henschke and Jiranek [29], with the following modifications: 115 g/L glucose, 115 g/L fructose, 0.46 g/L NH_4Cl, 1 g/L cysteine. The composition of GJM is listed in Table 1. The pH was adjusted to 3.5 with KOH.

Table 1. Chemical composition of grape juice medium (GJM), simulating standard grape juice, slightly modified from previous descriptions [29].

Group	Component	Amount per Litre
Carbon Sources	Glucose	115 g
	Fructose	115 g
Acids	Potassium L-Tartrate Monobasic	2.5 g
	L-Malic acid	3 g
	Citric acid	0.2 g
Salts	Potassium phosphate dibasic (K_2HPO_4)	1.14 g
	Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	1.23 g
	Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.44 g
Nitrogen Sources	Ammonium chloride (NH_4Cl)	0.46 g
	Amino acids (prepared as 100X stock solution in 20 g/L $NaHCO_3$ buffer solution)	
	-Tyrosine	0.014 g
	-Tryptophane	0.137 g
	-Isoleucine	0.025 g
	-Aspartic acid	0.034 g
	-Glutamic acid	0.092 g
	-Arginine	0.286 g
	-Leucine	0.037 g
	-Threonine	0.058 g
	-Glycine	0.014 g
	-Glutamine	0.386 g
	-Alanine	0.111 g
	-Valine	0.034 g
	-Methionine	0.024 g
	-Phenylalanine	0.029 g
	-Serine	0.060 g
	-Histidine	0.025 g
	-Lysine	0.013 g
	-Cysteine	1 g
	-Proline	0.468 g

Table 1. *Cont.*

Group	Component	Amount per Litre
Trace Elements (prepared as 100X stock solution)	Manganese(II) chloride tetrahydrate ($MnCl_2.4H_2O$)	200 µg
	Zinc(II) chloride ($ZnCl_2$)	135 µg
	Iron(II) chloride ($FeCl_2$)	30 µg
	Copper(II) chloride ($CuCl_2$)	15 µg
	Boric acid (H_3BO_3)	5 µg
	Cobalt(II) nitrate hexahydrate ($Co(NO_3)_2.6H_2O$)	30 µg
	Sodium molybdate dihydrate ($NaMoO_4.2H_2O$)	25 µg
	Potassium iodate (KIO_3)	10 µg
Vitamins (prepared as 100X stock solution)	Myo-inositol	100 mg
	Pyridoxine hydrochloride	2 mg
	Nicotinic acid	2 mg
	Calcium pantothenate	1 mg
	Thiamin hydrochloride	0.5 mg
	PABA.K	0.2 mg
	Riboflavin	0.2 mg
	Biotin	0.125 mg
	Folic acid	0.2 mg
Anaerobic Factors (prepared as 10X stock solution in hot 96% EtOH)	Ergosterol	10 mg
	Tween 80	0.5 mL

Preliminary lab-scale fermentations were performed in 80-mL flasks fully filled with medium, fitted with rubber stoppers equipped with a tube for gas injection and a CO_2 outlet. IWBT B063 pre-culture was inoculated at 1 % v/v, to reach an initial viable population of 1×10^7 CFU/mL. The trials consisted of a control (CTRL) under semi-aerobic conditions without gas addition, in a fully complemented medium, and two treatments: the first (N2) was injected with filtered N_2 during the whole experiment to reach anaerobic conditions, in a fully complemented medium; the second (10%N) was semi-aerobic without gas addition, in a reduced medium containing 10% of the nitrogen nutrients cited in Table 1 (ammonium chloride and amino acid solution). All tests were conducted in duplicate, incubated at 25 °C under static conditions for 72 h. Uninoculated flasks were prepared to ensure the sterility of the trials and to monitor any changes in the medium.

A second set of MLF trials was carried out in 1.3-L fermenters (BioFlo 110; New Brunswick Scientific Company, Inc., Edison, NJ, USA), with a working volume of 1 L and with automated control of temperature, pH, and dissolved oxygen. They were filled with 2/3 volume and maintained at 25 °C under constant agitation at 200 rpm. Conditions regarding inoculation and medium preparation were the same as in the preliminary trials: CTRL, N2, and 10%N. Dissolved oxygen was maintained at 0% in the N2 condition, with sparging of filtered N_2 during the whole experiment. All trials were done in duplicate, for a period of 72 h.

2.3. Analytical Methods

In the preliminary trials, cellular growth was followed throughout MLF by plating serial dilutions of samples on MRS agar medium (Biolab Diagnostics) and counting colonies after 4 days of anaerobic incubation at 30 °C using anaerobic jars with Anaerocult™ A (Merck, Darmstadt, Germany). L-malic acid concentrations were measured every 8 h with the automated enzymatic analyzer (Konelab Arena 20XT; Thermo Electron Corporation, Joensuu, Finland) using an L-malic acid enzymatic kit (Enzytec™ Liquid L-malate Id-No: E8280, Roche, R-Biopharm, Darmstadt, Germany). MLF was considered complete when L-malic acid concentrations decreased below 0.1 g/L.

In the fermenters, samples for viable cell counts and L-malic acid quantification were aseptically taken every 12 h, until the end of cultivation (72 h), and measured as described for the preliminary trials. L-lactic acid was quantified at 72 h, with the automated enzymatic analyzer (Konelab Arena 20XT) and specific kit (Enzytec™ Liquid L-lactate Id-No: E8260, Roche, R-Biopharm). Volatile aroma profile was analyzed through gas chromatography coupled to the flame ionization detection (GC–FID) method, after injection of samples prepared using liquid–liquid extraction, as described by Louw et al. [30]. Quantification was carried out using 4-methyl-2-pentanol (Fluka, Buchs, Switzerland) as internal standard.

2.4. RNA Sampling and Extraction

At the mid-exponential phase of cellular growth (24 h), samples for RNA extraction (20 mL) were collected from the fermenters. They were stabilized using RNAprotect®bacterial reagent (Qiagen, Hilden, Germany), following the manufacturer's instructions, and cells were rapidly harvested by centrifugation (8000× *g*, 4 °C, 10 min), flash-frozen in liquid nitrogen, and stored at −80 °C until RNA isolation. Total RNA extraction followed the protocol of Miller et al. [31]. RNA samples were then diluted on DEPC-treated water to a concentration of 100 ng/µL, purified with the Turbo DNA-free kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions, and maintained at −80 °C. Nucleic acid yield, purity, and integrity were preliminarily checked with the NanoDrop ND1000 UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and by electrophoresis run in a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Integrity was standardized with the RNA Integrity Number (RIN), in a range from 10 (intact) to 1 (totally degraded) [32].

2.5. RNA Sequencing and Data Processing

Quality check, cDNA preparation, library preparation, and sequencing were performed by BaseClear Group (Leiden, The Netherlands). Single-end sequence reads were generated using the Illumina HiSeq2500 system and quality of the FASTQ sequences was enhanced by trimming off low-quality bases using the "Trim sequences" option of the CLC Genomics Workbench version 9.5.1 (Qiagen, Hilden, Germany). Quality-filtered sequence reads were used for further analysis. Alignment against the reference genome of *L. plantarum* IWBT B063 and calculation of expression values were performed using the "RNA-Seq" option in CLC Genomics Workbench. Selected expression measure was the RPKM. It is defined as the reads per kilobase of exon model per million mapped reads [33] and seeks to normalize the difference in the number of mapped reads between samples as well as the transcript length. It is given by dividing the total number of exon reads by the number of mapped reads (in millions) times the exon length (in kilobases).

Differentially expressed genes annotated as coding hypothetical proteins on the reference genome of *L. plantarum* IWBT B063 were further manually annotated through targeted RAST [34] and BLASTx analysis.

2.6. Statistical Analysis

Growth functions and consumption kinetics were fitted to the cellular growth and malic acid consumption data of all experiments, for both lab-scale and fermenter trials. For the analysis of cellular growth in CFU, it was assumed that growth follows the logistics Equation (1):

$$\frac{dX_t}{dt} = \mu_{max} \times X_t(1 - X_t/K) \quad (1)$$

with X_t biomass in CFU, μ_{max} maximal specific growth rate that would be obtained at saturating substrate concentrations, and K the carrying capacity of the medium. The biomass data were fitted to the solution of the ordinary differential Equation (1) given in Equation (2):

$$X_t = \frac{K \times X_0}{X_0 + (K - X_0)E^{-\mu_{max} \times t}} \quad (2)$$

Consumption of malic acid was fitted to a kinetic rate equation. For this, two types of equations were used, a Michaelis–Menten type of equation with biomass as multiplier (3), or a mass action type of Equation (4), when not all parameters were identifiable:

$$\frac{dS_t}{dt} = X_t \times \frac{V_M \times S_t}{S_t + K_M} \quad (3)$$

$$\frac{dS_t}{dt} = X_t \times k \times S_t \quad (4)$$

with S_t the malic acid concentration (g/L), V_M the maximal specific malic acid consumption rate (g/L/10^8 CFU/h), K_M the Michaelis–Menten constant (g/L), k the specific rate constant (1/h/10^8 CFU).

Data from chemical analysis were compared in the software PAST [35] by one-way ANOVA (analysis of variance) and post-hoc Tukey's HSD (honestly significant difference) test, with a threshold for statistical significance set at p-value < 0.05.

Statistical analysis of RNA-seq data was performed by BaseClear Group and included the comparison of expression values, using Kal's Z-test [36] in the "Expression analysis" option with the CLC Genomics Workbench version 9.5.1 (Qiagen). Kal's Z-test is the choice in cases where a single sample is compared to another single sample (no biological replicates). The test relies on an approximation of the binomial distribution by the normal distribution. Considering proportions rather than raw counts, the test is also suitable in situations where the sum of counts is different between the samples. For all tests, the null hypothesis was that there would be no difference in expression between the two samples.

3. Results and Discussion

Among the many factors that knowingly affect the growth and activity of LAB in wine, in this study, the four most important were set at favorable conditions for growth: pH at 3.5, 25 °C, 0% ethanol, and without SO_2. Several studies considered the effects of these stress conditions and applied different recombinant and non-recombinant techniques to improve LAB tolerance to these harsh wine environments [37]. In contrast, the present investigation aimed to challenge a *L. plantarum* strain with two less-studied conditions, complete anaerobiosis and starvation of nitrogen-based nutrients, assayed separately. Medium simulated a co-inoculation strategy, when LAB are inoculated at the beginning of or during the first 48 h of alcoholic fermentation in grape must. Even selected well-studied starters, such as IWBT B063, could face problems during MLF. It is thus encouraged to test them in varying conditions to better understand the metabolism and propose new innovative strategies for fermentation management [6].

3.1. Lab-Scale Fermentations

The initial *L. plantarum* IWBT B063 population was around 4×10^7 CFU/mL (Figure 1A, solid lines). After the first 8 hours of growth, different growth curves were observed in the three conditions tested. In the control (CTRL), with semi-aerobic conditions and non-limiting nutrient concentration, IWBT B063 showed the best growth, reaching the stationary phase after 50 h. Strict anaerobic conditions (N2) resulted in a negative response for cell development, as growth was restrained compared with CTRL. However, cells were most affected in the ten-fold reduction of nitrogen nutrients (10%N), where a lower biomass concentration was observed throughout the whole experiment. Whereas the overall biomass formed is different for the three conditions, this is mostly due to a lower carrying capacity, i.e., the reduced growth rate is caused by the limiting substrate concentration in the medium, not by the maximal specific growth rate (Table 2). The effects of nitrogen on LAB metabolism have been reviewed by several authors, and the results obtained here confirm its importance [10,38,39].

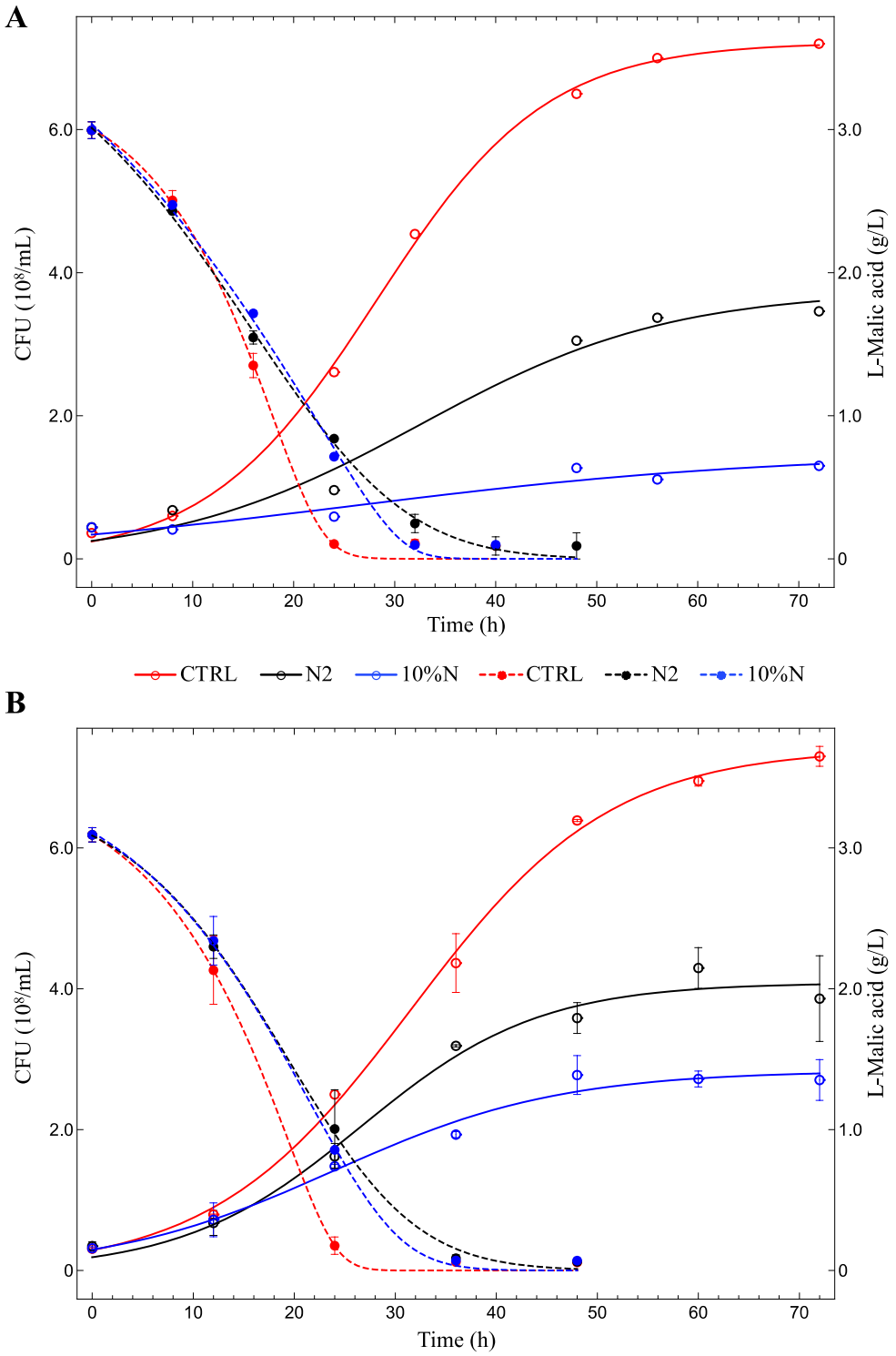

Figure 1. Fitted curves of the growth dynamics of *L. plantarum* IWBT B063 (solid lines) and malic acid evolution (dashed lines) during lab-scale (**A**) and fermenter (**B**) different MLF trials: CTRL, control; N2, anaerobiosis; 10%N, ten-fold reduction of nitrogen nutrients. Error bars are standard deviations of two independent replicates.

Table 2. Maximal specific growth rate (μ_{max}) and carrying capacity of the medium (K) for the fitted growth equations during lab-scale and fermenter different MLF trials with *L. plantarum* IWBT B063, under different conditions: CTRL, control; N2, anaerobiosis; 10%N, ten-fold reduction of nitrogen nutrients. Data reported as the average ± standard deviation of two independent replicates.

Parameter	Lab-Scale			Fermenter		
	CTRL	N2	10%N	CTRL	N2	10%N
μ_{max} (h^{-1})	0.12 ± 0.01	0.08 ± 0.02	0.05 ± 0.02	0.10 ± 0.01	0.11 ± 0.02	0.09 ± 0.02
K (10^8 CFU)	7.22 ± 0.10	3.75 ± 0.39	1.47 ± 0.37	7.41 ± 0.14	4.08 ± 0.18	2.84 ± 0.14

L-malic acid consumption was in accordance with cell growth results (Figure 1A, dashed lines). The concentration decreased more markedly in CTRL, and L-malic acid was completely consumed after 24 h. The other two conditions showed similar curves, albeit slower than the control.

Both experimental conditions tested, anaerobiosis and limitation of nitrogen nutrients, significantly affected the biomass production and this resulted in differences in the malic acid consumption of *L. plantarum* IWBT B063. The same conditions were replicated in 1.3-L fermenters to confirm the results at a larger scale with a higher overall biomass, which was necessary for RNA and transcriptome analysis.

3.2. Fermenters

Regarding cell populations (Figure 1B, solid lines), the two conditions displayed lower growth when compared with CTRL. The initial cell concentration in all fermenters was around 3.5×10^7 CFU/mL. Cells in CTRL were in the early stationary phase after 72 h at a concentration above 7×10^8 CFU/mL. The anaerobic condition (N2) was less difficult than the limitation of nitrogen nutrients (10%N), but the difference was less marked than in the preliminary trials. The IWBT B063 cell concentration in 10%N was slightly increased, reaching approximately 3×10^8 CFU/mL. In another study [40], a different wine-associated *L. plantarum* strain from the SAGWRI culture collection was inoculated in the same synthetic medium, but at an initial concentration ten times smaller than in the present study, around 4×10^6 CFU/mL. The authors also observed considerable growth, finding cell counts of approximately 4×10^7 CFU/mL after two days and above 10^8 CFU/mL after five days.

The only modifications of MLF settings in fermenters with respect to the preliminary trials were upscaling, from 80 to around 800 mL, and medium agitation, at 200 rpm. As expected, very similar results were obtained for the lab-scale and the fermenter set-ups. The growth functions could describe the growth data well, with the fitted parameter values obtained for the different conditions listed in Table 2. For the different media and aeration strategies, very similar results were obtained in terms of maximal specific growth rates. The strongest effect was observed for the carrying capacity parameter, i.e., the final biomass concentration reached, which was the highest for the control, intermediate for N2, and the lowest in the 10%N condition.

L-malic acid consumption (Figure 1B, dashed lines) confirmed the patterns observed in the preliminary lab-scale fermentations. A faster decrease occurred in CTRL, where the initial 3.0 g/L was almost completely exhausted after 24 h, while in N2 and 10%N, around 1.0 g/L was still present. After 36 h, L-malic acid concentrations dropped below 0.1 g/L in all conditions. Good fits were also found for the equations describing the malolactic conversion, and there was no significant effect of the different fermentation vessels and conditions in the preliminary trials compared with fermenters. The specific malic acid consumption rate, calculated with Equations (3) and (4), was normalized for the biomass concentration. Over the first 10 h of the fermentation, a fairly constant specific uptake rate was observed, independent of the culture conditions, i.e., -0.15 ± 0.01; -0.18 ± 0.03, and -0.16 ± 0.03 (g malic acid/L/h/(10^8 CFU)), respectively, for the control, N2, and 10%N conditions. Thus, it appears that the specific malic acid consumption rate was constant for the different conditions tested and the differences in the malolactic fermentations, as observed in Figure 1, can be related to the different biomass concentrations present.

L-lactic acid concentration and volatile compound profile were analyzed at the end of fermentation. A total of 32 volatile organic compounds were analyzed in GC–FID, but only six of them could be quantified above the detection limit in the trials. Nevertheless, concentrations of ethyl acetate (ranging between 2.918 and 2.882 mg/L), hexanoic acid (ranging between 0.154 and 0.143 mg/L), octanoic acid (ranging between 0.292 and 0.285 mg/L), and decanoic acid (ranging between 0.507 and 0.521 mg/L) were not significantly different between the trials. Only acetic acid, the volatile compound present at the highest concentration in all conditions, was produced in statistically different quantities. Acetoin levels were the same in CTRL and 10%N but could not be detected in N2. Therefore, these two metabolites are reported in Table 3 alongside L-lactic acid levels.

Table 3. Metabolite levels at the end of MLF trials in fermenters with *L. plantarum* IWBT B063, under different conditions: CTRL, control; N2, anaerobiosis; 10%N, ten-fold reduction of nitrogen nutrients. Data reported as the average ± standard deviation of two independent replicates. Different letters indicate statistically significant variation among growth conditions ($p < 0.05$).

Compound	Condition			Significance
	CTRL	N2	10%N	
L-lactic acid (g/L)	2.273 ± 0.001a	1.951 ± 0.077b	1.892 ± 0.055b	$p = 4.21 \times 10^{-6}$
Acetic acid (g/L)	0.137 ± 0.006a	0.073 ± 0.007c	0.087 ± 0.001b	$p = 0.01124$
Acetoin (mg/L)	15.492 ± 0.985	n.d.	15.499 ± 0.114	

n.d. = not detected.

Production of L-lactic acid was significantly higher in CTRL than in N2 and 10%N, even though, in all conditions, the same initial L-malic acid concentration was completely consumed. Besides malolactic conversion, lactic acid can be produced from glucose and fructose present in the GJM in an energy-producing pathway. It is reasonable to expect a higher sugar consumption and consequent higher lactic acid production in CTRL, reflecting the higher growth observed in that condition, although, normally, only a small amount of sugar is fermented during MLF [8].

L. plantarum is an homofermentative LAB and thus metabolizes hexoses via the Embden–Meyerhof pathway, with pyruvate as the central branching point of metabolism [41]. In this metabolism, the fate of pyruvate is determined by the availability of oxygen and substrates. Lactate remains the major metabolite of homofermentative LAB growing aerobically or anaerobically, but aeration allows alternative routes, generating, for example, acetate and acetoin as minor products [41]. For instance, the presence of oxygen favors the conversion of pyruvate into acetoin and inhibits the conversion of acetoin to 2,3-butanediol [9], and it is well-established that the conversion of lactate to acetate can only occur during aerobic growth [42]. Indeed, besides lactate, the highest acetic acid level was found in CTRL, followed by 10%N and then N2, while acetoin was not produced at detectable amounts in N2, suggesting the importance of oxygen in this metabolic pathway.

Acetic acid, acetoin, 2,3-butanediol, and diacetyl could also be produced in *L. plantarum* metabolism from the breakdown of citrate. Although citric acid is present in smaller concentrations in must (0.1–1 g/L; 0.2 g/L in the GJM of the present study) compared to other organic acids, the metabolism of citrate can significantly impact the aromatic properties by releasing those carbonyl compounds, associated with the buttery aroma of wines [3,8,12]. Oxygen is among the several factors that can influence the conversion of diacetyl to acetoin and 2,3-butanediol in wine, alongside citrate and sugar concentration, temperature, SO_2 content, pH, inoculation strategy, and strain of malolactic bacteria used [12].

In summary, the results showed that, although a reduced final biomass and related delayed completion of malate degradation were observed, neither of the two conditions tested was detrimental to IWBT B063 performance in comparison with the control. The maximal specific growth rate and specific malic acid consumption rate were fairly constant, and there was no loss of cell viability. Active growth was present in all conditions, reaching higher final concentrations than initial, and cells had surpassed 10^8 CFU/mL already 24 h after inoculation.

Consumption of L-malic acid commenced immediately in all trials, as expected for inoculation with high numbers of bacteria (above 10^7 CFU/mL), since it is usually reported that a viable population of 10^6 CFU/mL is required for starting MLF. In agreement with this survey, Nielsen and Richelieu [7] reported continued bacterial growth after exhaustion of malic acid in semi-aerobic conditions, while cell count remained stable after MLF completion in anaerobiosis. Faster malate degradation, as in CTRL, is expected in rapidly growing cultures, but continued bacterial growth after the end of MLF could lead to acetic acid production if sugar is still available [8].

3.3. Transcriptional Response

RNA-seq technology was used to analyze the differential gene expression of *L. plantarum* IWBT B063 among MLF trials with different growing conditions. RNA samples, isolated at the mid-exponential phase of cellular growth, were firstly assessed for their quality before sequencing. The integrity of RNA has fundamental importance for the success of gene expression measurement techniques. To achieve a higher RNA concentration and integrity, it was necessary to prepare a pool with both replicates of each condition. RNA isolated from CTRL and N2 showed an RIN of 8.1 and 7.5, respectively. However, RIN for the condition 10%N was 3.3, which was insufficient for the collection of valid and meaningful results. Therefore, RNA-seq analysis was performed in the conditions of complete anaerobiosis (N2) and the control (CTRL).

The number of sequencing reads recovered was 32.6 million in CTRL and 34.8 million in N2, both with an average Phred quality score of 38.5, indicating approximately 99.99% base call accuracy for the sequencing. Reads were mapped into *L. plantarum* IWBT B063 genome, containing 3385 genes. Gene expression was quantified as RPKM, with a mean value of 468.94 in CTRL and 454.53 in N2. Considering a threshold of 1.5-fold for differential expression, 299 genes were selected (8.8% of total), 169 of them upregulated and 130 downregulated in N2 compared to CTRL. Annotation of differentially expressed genes (DEG) allowed us to classify 148 of them into functional categories, 86 up- and 62 downregulated (Figure 2). Among upregulated genes, the most represented categories were amino acid metabolism, genetic information processing, membrane transport, and signaling and cellular processes. Genes involved in these last three categories were also highly present in downregulated genes, but carbohydrate metabolism was predominant.

Narrowing the analysis to increased or decreased gene expression of at least twofold, 29 genes were upregulated (Table 4) and 28 downregulated (Table 5) in response to anaerobic conditions.

In aerobic conditions, it is expected to see an induction of genes that encode enzymes catalyzing the removal of toxic compounds formed by the presence of oxygen [43]. Previous studies with *L. plantarum* strains showed the presence of genes coding for NADH peroxidase (NPR; *npr1*, *npr2*) and for NADH oxidase (NOX; *nox1*, *nox2*, *nox3*, *nox4*, *nox5*, *nox6*). The NOX/NPR system is involved in oxygen tolerance mechanisms by preventing oxygen accumulation and contributes to the maintenance of the $NADH/NAD^+$ balance by promoting cofactor regeneration [9]. It was shown that *npr2* and *nox5* are the principal genes from this system that are upregulated during aerobic growth [42,44]. In the present study, only *npr2* and *nox6* were downregulated in *L. plantarum* IWBT B063 in the anaerobic growth condition.

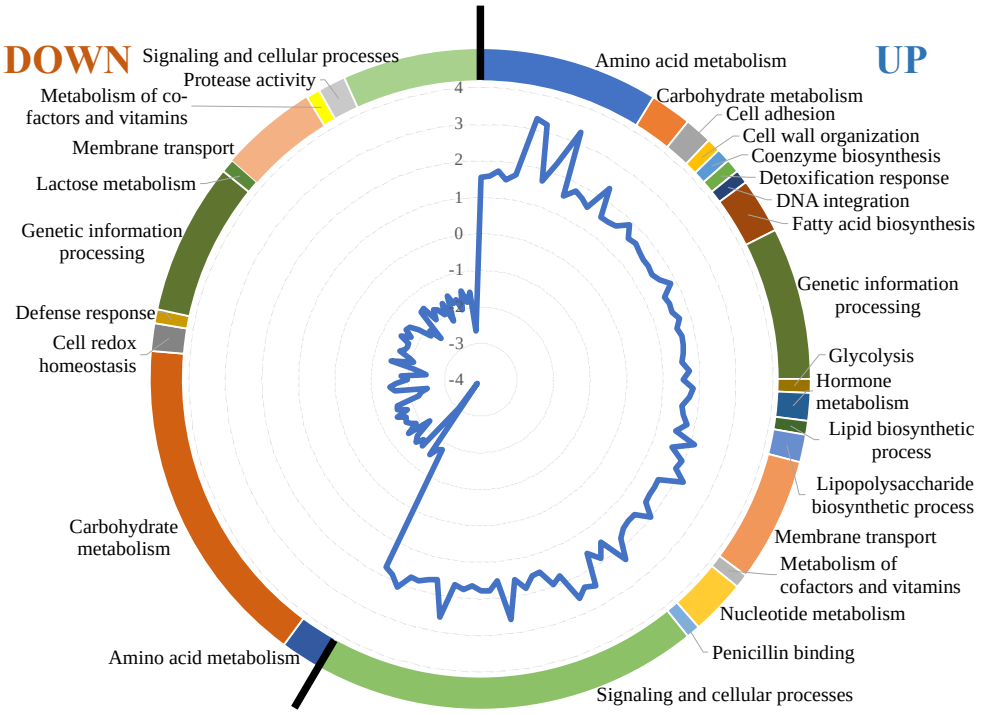

Figure 2. Functional annotation and fold-change of differentially expressed genes (DEG; |fold-change| > 1.5) in *L. plantarum* IWBT B063 at mid-exponential growth in MLF fermenter trials, under anaerobic conditions (N2), compared to the control (CTRL).

Table 4. Upregulated genes (fold-change > 2.0) in *L. plantarum* IWBT B063 at mid-exponential growth in MLF fermenter trials, under anaerobic conditions (N2), compared to the control (CTRL).

Gene	Function	Category	Fold-Change
adeC	Adenine deaminase	Nucleotide metabolism	2.00
argO	arginine transmembrane transporter activity	Signaling and cellular processes	2.31
csbC_3	Major facilitator superfamily protein	Membrane transport	2.11
cycA_2	Cyclin-A2-4	Signaling and cellular processes	2.46
cysE	Serine acetyltransferase	Amino acid metabolism	3.33
cysM	Cysteine synthase B	Amino acid metabolism	3.23
dtpT_1	Amino acid/peptide transporter (Peptide:H+ symporter)	Signaling and cellular processes	2.35
dtpT_2	Di-/tripeptide transporter	Signaling and cellular processes	2.56
licB_1	Protein LicB	Carbohydrate metabolism	2.31
lysN	LysN	Amino acid metabolism	2.28
metB	metB	Amino acid metabolism	3.31
oppA_4	Oligopeptide-binding protein	Signaling and cellular processes	2.61
sdcS	Sodium-dependent dicarboxylate transporter SdcS	Signaling and cellular processes	2.57
yjeM	-	Membrane transport	2.20
ywqD_2	Cytokinin riboside 5′-monophosphate phosphoribohydrolase	Signaling and cellular processes	2.11
S_00258	Putative protein of unknown function	NrdR-regulated deoxyribonucleotide transporter, PnuC-like	2.33
S_00271	Putative protein of unknown function		3.81
S_00272	Extracellular protein		3.83
S_00273	Putative protein of unknown function		2.80
S_00282	Putative protein of unknown function		2.11
S_01162	Sulfotransferase/hypothetical protein		2.33

Table 4. *Cont.*

Gene	Function	Category	Fold-Change
S_01534	Putative protein of unknown function		2.09
S_01773	Putative protein of unknown function		2.44
S_01864	Putative protein of unknown function		2.50
S_02063	Prophage protein	Mobilome: prophages and transposons	2.11
S_02789	Hydrolases of the alpha/beta superfamily		2.24
S_02790	HTH_MerR-SF transcriptional regulator	Transcription	2.33
S_02820	Glycopeptide antibiotic-resistance protein		2.06
S_03325	Putative protein of unknown function		3.06

Table 5. Downregulated genes (fold-change < −2.0) in *L. plantarum* IWBT B063 at mid-exponential growth in MLF fermenter trials, under anaerobic conditions (N2), compared to the control (CTRL).

Gene	Function	Category	Fold-Change
adhE_1	Aldehyde-alcohol dehydrogenase	Carbohydrate metabolism	−3.87
adhE_2	Aldehyde-alcohol dehydrogenase	Carbohydrate metabolism	−3.01
asnS_1	Asparagine–tRNA ligase	Genetic information processing	−2.10
bceA_2	Bacitracin export ATP-binding protein BceA	Signaling and cellular processes	−2.23
copA_1	Metal transporting atpase Mta72	Membrane transport	−2.19
copB	CopB	Membrane transport	−2.06
davT	5-aminovalerate aminotransferase DavT	Carbohydrate metabolism	−2.22
dppE_2	Dipeptide-binding protein DppE	Membrane transport	−2.41
lrgB	Antiholin-like protein LrgB	Signaling and cellular processes	−2.03
manX_1	EIIAB-Man	Carbohydrate metabolism	−2.23
manY	-	Carbohydrate metabolism	−2.32
manZ	ManZ protein	Carbohydrate metabolism	−2.51
nox_6	-	Cell redox homeostasis	−2.50
npr_2	Regulatory protein NPR2	Defense response	−2.11
yhdG_3	Putative amino acid permease YhdG	Signaling and cellular processes	−2.64
yodC_3	-	Cell redox homeostasis	−2.34
S_00347	L-lactate dehydrogenase	Carbohydrate metabolism	−2.24
S_02279	MSF transporter		−2.27
S_02428	Putative protein of unknown function		−2.04
S_02979	MSF sugar transporters		−2.20
S_03213	Putative protein of unknown function		−2.03
S_03235	LtrC-like protein, unknown function		−2.01
S_03252	Putative protein of unknown function		−2.04
S_03253	ATP-binding protein		−2.13
S_03254	Putative protein of unknown function		−2.11
S_03255	Putative protein of unknown function		−2.14
S_03256	Putative protein of unknown function		−2.43
S_03399	DNA topoisomerase III	Replication, recombination, and repair	−2.12

During anaerobic fermentation, cofactor recycling (NADH/NAD^+) to maintain the redox balance could be provided through acetaldehyde/ethanol production from acetyl-CoA via aldehyde-alcohol dehydrogenases (ADH) [9]. In the presence of oxygen, this pathway is inhibited and *adh* is downregulated [42]. However, here, the opposite was observed, where *adhE1* and *adhE2* were both significantly less expressed in the anaerobic growth condition. This could be related to the timing of sample collection for RNA-seq analysis. At 24 h, MLF was almost finished in the aerobic conditions, while the cells in the anaerobic conditions were still using malic acid. Thus, glucose was not being metabolized in N2. Moreover, Echave et al. [45] proposed that, even if ADHE activity is much lower in aerobic conditions than anaerobiosis, the enzyme is also present in the latter and, in *Escherichia coli*, has a protective role against oxidative stress.

Besides *adhE*, also the genes *manXYZ*, related to the mannose phosphotransferase system (PTS) putatively involved in glucose transport, were downregulated in N2, reinforcing the idea that glucose-induced overexpression did not occur during the mid-exponential

phase of growth in the anaerobic fermentation [46]. Similarly to *adhE*, it has also been previously indicated that the mannose PTS can enhance oxidative stress tolerance, observed in *L. plantarum* [47]. A further group of genes that can improve oxidative stress tolerance includes *copA* and *copB*, which modulate copper homeostasis in *L. plantarum* cells and play a role in H_2O_2-detoxifying mechanisms [48]. They were both downregulated in N2 compared to CTRL. Anaerobic conditions were also characterized by a downregulation of a putative L-lactate dehydrogenase, which could be related, upon validation, to the diacetyl production within this environment: in fact, previous studies revealed that upregulated L-lactate dehydrogenase in *O. oeni* could enable L-lactic acid to be utilized as a precursor for the production of diacetyl during malolactic fermentation in Cabernet Sauvignon wine [28].

Among the upregulated genes in the anaerobic conditions, it is interesting to note four genes related to amino acid metabolism (*cysE*, *cysM*, *lysN*, *metB*). Overexpression of genes implicated in amino acid biosynthesis—in this case, the serine family and aspartate family—might have a positive influence on the pathways leading to the production of volatile flavor compounds by increasing the generation of aromatic precursors [43]. Moreover, genes associated with the oligopeptide ABC transport system (*oppA* and *dtpT*) were also overexpressed, strongly suggesting active peptide uptake [49]. Thus, the oxygen levels during MLF could not only influence LAB growth and malolactic activity but also impact the flavor formation in wine, which could be further investigated through sensory analysis.

As observed in the curves of biomass growth and malic acid consumption, the differences in MLF activity are related to the different biomass concentrations, rather than the specific malic acid consumption rate. Indeed, the gene *mle* coding for the malolactic enzyme was not differentially expressed between the aerobic and anaerobic conditions. In another study, Miller et al. [31] described that *mle* expression was modulated by the pH and ethanol content of the fermentation media, increasing at lower pH and decreasing in the presence of ethanol.

Interestingly, upregulated genes include also putative prophage proteins and traits encoding putative defense proteins (i.e., resistance protein to glycopeptides). In this framework, it has been observed that *L. plantarum* strains can adapt to MRS medium via the power of genes coding for phage- and prophage-related proteins, which can help the bacterial response to various stressors as active loci [50,51]. Further, the overexpression of putative hydrolases in anaerobic conditions compared to aerobic growth confirmed the ability of *L. plantarum* to utilize several carbohydrate sources in different environments, thus remarkably reflecting the adaptation capacity of this species [52].

4. Conclusions

Aeration control and grape must composition are of major importance for successful completion of MLF, due to their relevant impact on LAB development. *L. plantarum* IWBT B063 was able to carry out MLF under challenging conditions, but anaerobic conditions and limitation of nitrogen nutrients reduced its growth in grape juice medium, delaying the completion of MLF.

Using RNA-seq for the first time to monitor a *L. plantarum* strain during MLF, it was possible to identify 86 upregulated and 62 downregulated genes comparing the absence vs. presence of oxygen in grape juice medium. In particular, genes with a protective role against oxidative stress and genes related to amino acid metabolism were differentially expressed.

This study highlights the usefulness of RNA-seq technology to detect the transcriptional response of LAB and the genetic impact under specific stressful or suboptimal environmental conditions, which otherwise would not be underlined by the standard wine parameters. The results could give insights for further studies aiming to understand the fine-tuning of important parameters affecting the success of LAB starter cultures and malolactic activity in wine.

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