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ORIGINAL RESEARCH ARTICLE

The origin of the particular aroma of noble rot wines: various fungi contribute to the development of the aroma profile of botrytised grape berries

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ABSTRACT

During noble rot (NR), *B. cinerea,* together with other filamentous fungi and yeasts, play a role in developing the unique aromatic profile of botrytised wines. To gain more insight into the latter, we generated metatranscriptomic data representing the four NR stages (I-IV) from the Tokaj wine region of Hungary over three months. Since previous research has indicated that the most prevalent filamentous fungi and yeast include *Alternaria alternata*, *Botrytis cinerea*, *Epicoccum nigrum, Aureobasidium pullulans* and *Rhodotorula graminis*, RNAseq reads were aligned to the latter species. A weighted gene co-expression network analysis (WGCNA) followed by a non-metric multidimensional scaling (NMDS), eigengene ANOVA and enrichment analyses were performed. Amongst the ten generated gene module clusters, enriched pathways involved in synthesising aromatic compounds such as amino acid-, carbohydrate- and lipid metabolism co-jointly expressed by all filamentous fungi and yeast were identified within the turquoise module. Furthermore, it was found that the enzymes involved in the synthesis of aromatic compounds are expressed and up-regulated during the later stages (III-IV) of the NR process. This study has indicated that the unique aromatic profile of botrytised wines is due to the contributions of filamentous fungi and yeasts belonging to the NR grape microbiome, with the main aromatic contributions occurring during the later NR stages.

KEYWORDS: botrytised wines, wine aromas, metatranscriptomic analyse, filamentous fungi, noble rot stages

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INTRODUCTION

Noble rot (NR) of grape berries is a unique and natural process induced by *Botrytis cinerea*, which infects ripe berries which results in berries with a chocolate-brown with purple shade, raisin-like appearance and enriched with a superb and unique aromatic profile with citrus fruit, orange peel, crystallised fruit, caramel, honey, spicy-curry and walnut overtones (Reboredo-Rodríguez *et al*., 2015). These berries are used to produce botrytised wines, also known as "the wine of kings, the king of wines" (Ribéreau-Gayon *et al*., 2006), such as the Trockenbeerenauslese, Sauternes A.O.C. and Aszú of Germany, France and Hungary, respectively (Magyar, 2011; Blanco-Ulate *et al*., 2015). Noble rot only occurs under very specific microclimatic conditions, such as a short rainy period followed by a lengthy period of dry, sunny and warm weather during the day and humid and cool nights (Ribéreau-Gayon *et al*., 2006; Vannini and Chilosi, 2013). When environmental circumstances are not optimal, such as too high air humidity and the relatively low-temperature difference between day and night that continues for several days, causes *B. cinerea* to cause grey rot (Ribéreau-Gayon *et al*., 2006; Vannini and Chilosi, 2013), a condition leading to the soft decay of grape berries associated with vigorous fungal mycelium formation with an associated appalling smell and taste (Steel *et al*., 2013) or on several occasions causing a total loss.

In addition to *B. cinerea*, the grapevine contains many diverse microbiota, including bacteria, filamentous fungi and yeasts (Barata *et al*., 2012; Stefanini and Cavalieri, 2018). The complex microbial community found on grape berries play an important role in grape and wine production and quality (Barata *et al*., 2012). According to Liu *et al*. (2019), the microbial biogeography allows the production of exclusive agricultural products, referred to as 'terroir' in oenology and viticulture. The latter has been demonstrated for bacteria and fungi at a regional level (Bokulich *et al*., 2016) which are influenced by various environmental variables such as climate and vintage, cultivar, soil properties and vintage (Liu *et al*., 2019). The main fungus responsible for NR is *B. cinerea*, but several other filamentous fungi, such as *Alternaria alternata* and *Epicoccum nigrum*, appear on the surface of grape berries at the beginning of the NR process (Lorenzini *et al*., 2018), whereas yeasts, including *Saccharomyces* and *Aureobasidium*, appear only rarely (Bokulich *et al*., 2016).

During NR, several physiochemical changes occur on and within the grape berry, ultimately contributing to developing wines with a unique aromatic profile. Complex enzymatic conversions take place which yield a higher concentration of sugars, acids, glycerol, minerals and certain aroma compounds (Magyar, 2011) such as d-lactones, c-lactones (Miklósy *et al*., 2004), dihydro-3,5-dimethyl-2(3H)-furanone, dihydro-5-methyl-2(3H)-furanone, dihydro-5-ethoxy-2(3H)-furanone and dihydro-5-butyl-2(3H)-furanone which is specifically associated with *Tokaji Aszú* noble rot wines (Miklósy *et al*., 2004). Physically, the berry becomes more dehydrated due to digestion by weather conditions such as dry, sunny days and or morning dust/fog, as well as due to degradation by *B. cinerea* (Hegyi-Kaló *et al*., 2020) and other fungal and yeast species present on the grape berry surface. In addition to *Saccharomyces cerevisiae*, which is the main driver of NR fermentation and flavour formation (Goddard, 2008), the degraded grape berry skin allows the microbial community present on the grape berry skin access to the grape berry interior and, subsequently, the grape berry must undergoing fermentation which can influence the wine composition, flavour aroma and subsequently its quality (Barata *et al*., 2012; Morrison-Whittle and Goddard, 2018).

Understanding, controlling and manipulating the microbiota present during wine production is crucial to the industry and downstream stakeholders. Recent developments in rapidly developing and improving 'omics' methods are increasingly enabling us to understand the wine microbiome and its contribution to the development of sought-after flavours and aromas by forming precursors, organoleptically active, and oxidative or antioxidative compounds (Sirén *et al*., 2019). The current study aimed to investigate the role of the three highly abundant filamentous fungi *A. alternata*, *B. cinerea*, *E. nigrum* and the yeasts *A. pullulans* and *R. graminis* commonly associated with the NR process with regard to their contribution to the unique aromatic profile using metatranscriptomic data analyses and associated statistical and enrichment analyses. We hypothesised that in addition to *B. cinerea*, other filamentous fungi such as *A. alternata* and *E. nigrum,* as well as yeasts *A. pullulans* and *R. graminis* commonly associated with NR berries, also contribute to the aroma profile of NR wines during botrytisation which has not been studied to date. To identify the most significant impacts of the berry microbiome on the wine aroma during noble rot, the most relevant biochemical pathways and gene functions have been evaluated and analysed, all of which can contribute to a full understanding of the noble rot process through further investigation.

MATERIALS AND METHODS

1. Sampling, RNA extraction and data analysis

Healthy (H), noble rotten (NR) and grey rot (GR) grape berries cv. *Furmint* were randomly sampled in replicates of five from the same rot type in September, October and November 2017, respectively, from the Betsek vineyard located close to the village Mád (48° 11' 18.6" N, 21° 19' 01.8" E) within the Tokaj region of Hungary. In the case of NR, samples were collected at 3 different shriviling stages, representing phases II, III and IV of noble rot. The concept of the four phases of noble rot was introduced by Hegyi-Kaló *et al*. (2020). The phases were defined according to a protocol based on visual and textural differentiation as follows: phase I is the healthy berry (H); phase II represents the starting of botrytisation, berries with purple-brown spots; phase III berries are fully purple; finally, phase IV represents noble rotten raisin berries with latent mycelia. It is important to note that in vineyards, such as the

one sampled, which produce high quality, frequent and high quantities of berries with noble rot, these phases are often present in the bunches simultaneously, although in different proportions. The grape berries were immediately placed aseptically into sterile falcon tubes and were subsequently transferred to a flask containing liquid nitrogen. After this, samples were immediately taken to the analytical lab at the Food and Wine Research Centre at the Eszterházy Károly Catholic University, where they were stored at –80 °C until further analyses were conducted. The total RNA content of the grapevine tissue was extracted using an optimised protocol of Reid *et al*. (2006), as described in Otto *et al*. (2022). The extracted RNA was stored at –80 °C until downstream sequencing was performed using an Illumina HiScan sequencer at the UD GenoMed Medical Genomic Institute at the University of Debrecen. The sequencing produced 7.8–40.6 million reads per sample with a mean sample RNA length of 76 bp.

The quality of the RNAseq reads was evaluated using FastQC v0.11.5 (Babraham Bioinformatics, Cambridge, UK). Trimmomatic v0.36 was used to remove TruSeq2-PE adaptors using a simple clip threshold, an indome threshold as well as a seed mismatch threshold set at 10, 30 and 2, respectively (Bolger *et al*., 2014). The FASTX-TOOLKIT software was used to filter sequencing reads according to base call quality with the fastq_quality_filter parameters set at thresholds -Q33 -q30- and p50, respectively. The resulting reads were normalised according to Khamer v2.1.1. This was followed by removing low abundance fragments of high coverage reads using Khamer v2.1.1. The resulting reads were aligned to reference genomes *Alternaria alternata* (PRJNA239482), *Botrytis cinerea* (BioProject: PRJNA15632), *Aureobasidium pullulans* EXF-150 (BioProject: PRJNA207874) and *Epicoccum nigrum* ICMP 19927 (BioProject: PRJNA379853) and *Rhodotorula graminis* WP1 (PRJEB38623) and split into their respective genes with an in-house Python script with Salmon v 1.3.0 (Patro *et al*., 2017). Following alignment, transcript genes were linked to gene names with the GIF annotation for each genome with the *GenomicFeatures* package in R (Lawrence *et al*., 2013). DNA sequences have been deposited in the NCBI under BioSample SAMN19612984 and BioProject PRJNA736205. The transcriptomic data of the five microorganisms investigated carry a wealth of information; however, this study focuses on the correlations with wine flavours.

2. Statistical Analyses

All statistical analyses were carried out with R v4.1.0 (R Core Team, 2013). To understand the structure of the gene expression data, one-way ANOVAs combined with Tukey's HSD test were performed on the normalised abundance values (number of mapped transcripts reads) of the filamentous (*A. alternata*, *B. cinerea*, *E. nigrum*) and yeast species (*A. pullulans*, *R. graminis*) functional gene sets to find significant pairwise differences the different stages of noble rot (phase I, II, III and IV) and months (September, October, November), respectively. To visualise the trends in the functional genes between dates and phases, a non-metrical multidimensional scaling (NMDS) analysis was conducted on the combined September–October–November functional gene set between the respective phases of the microbes on median of ratios normalised abundance values with the vegan package (Oksanen *et al*., 2007). The data were subjected to 9999 iterations per run with the Bray-Curtis dissimilarity using a random starting number.

3. Weighted Gene Co-Expression Analyses

To identify co-expression patterns amongst the filamentous fungi and yeast, a weighted gene co-expression network analysis (WGCNA) was performed on the normalised abundance values on the combined month and phase functional gene set of all filamentous fungi and yeasts (Langfelder and Horvath, 2008). The analysis was conducted using 100 genes as a minimum cluster size with Pearson's correlation and a soft thresholding power and merging threshold set at 12 and 0.25, respectively. The clustering was visualised with a network cluster dendrogram. Pearson's correlation statistic was calculated to estimate significant abundance correlations of the functional transcripts of the respective filamentous fungi and yeasts. Eigengene expression values were calculated and visualised from the co-expressed merged modules (indicated by different colours) to determine connections to the NR process. The correlation and significance levels between transcript abundance and co-expression modules were calculated and visualised with a heat map. The resulting co-expressed modules were based on eigengene expression patterns resembling the NR process (Noble Rot Connected Modules, NRCM). An ANOVA test was performed with the eigengene expression values of the test result to identify significant changes between dates and neighbouring phases within the NRCMs. Tukey's test was performed to determine if the increasing or decreasing changes between phases were significant. Significant increases and decreases in the respective NRMSs were further used for enrichment analyses. To identify the hub genes in the interesting modules, a customised hub gene filtering method was used: the module membership (kME) value should be in the highest (lowest) 10 % of the genes and should be higher (lower) than 0.8 (–0.8).

4. Differential Gene Expression and Enrichment

To analyse the changes in functional gene expression composition between the NR phases, a serial differential expression (DE) analysis was, respectively, performed on the functional gene set between phases I and II, II and III, as well as III and IV for the combined months September, October and November for the filamentous fungi and the yeasts, respectively, with the DESq2 package in R (Love *et al*., 2014). In addition, a DE analysis was performed on the functional gene set of all phases between September and October and September and November for all filamentous fungi and yeasts, respectively. Differentially expressed genes were identified based on a *p*-value of 0.05 and a log2 (fold change) of 1, and those that belonged to neighbouring phases were assigned and grouped according to NRCM membership.

In the turquoise module, there was a significant increase in the eigengene expression between phases III and IV. Thus, all up-regulated genes between phases III and IV in the turquoise module were selected for enrichment analyses. Similarly, in the yellow module, there was a significant decrease between phases I and II; thus, all up-regulated genes between phases I and II in the yellow module were selected for enrichment analyses. Pathway enrichment was performed using the KOBAS v 2.0 software (Xie *et al*., 2011).

RESULTS

1. Statistical analyses

For all filamentous fungi and yeast, a significant increase was observed between the first (phase I) and the last phase (phase IV) whereas the harvest date showed no significant influence on the abundance values for any of the fungi. Specifically, the increase between phase I and II was significant for the filamentous fungi but not the yeast. The increase between phases II and III was only significant for

TABLE 1. Mean values of ten base logarithm of the sum of abundances and standard deviation in brackets of the four noble rot stages and three sampling months. For each column, different small letters indicate significant differences at $p = 0.05$ among phases or months.

Genus:	*Alternaria*	*Aureobasidium*	*Botrytis*	*Epicoccum*	*Rhodotorula*
Phase I	4.219 (0.160) [a]	4.626 (0.236) [a]	6.322 (0.077) [a]	3.980 (0.153) [a]	3.931 (0.196) [a]
Phase II	4.691 (0.146) [b]	4.857 (0.219) [ab]	6.470 (0.014) [b]	4.420 (0.141) [b]	3.950 (0.215) [ab]
Phase III	4.855 (0.151) [b]	5.100 (0.260) [b]	6.460 (0.021) [b]	4.599 (0.154) [c]	4.147 (0.193) [b]
Phase IV	5.171 (0.270) [c]	5.894 (0.418) [c]	6.513 (0.074) [b]	4.943 (0.162) [d]	4.385 (0.217) [c]
September	4.698 (0.443)	4.899 (0.495)	6.439 (0.129)	4.439 (0.429)	4.147 (0.323)
October	4.753 (0.364)	5.233 (0.548)	6.423 (0.060)	4.488 (0.365)	4.083 (0.221)
November	4.723 (0.386)	5.182 (0.590)	6.456 (0.067)	4.499 (0.364)	4.065 (0.269)

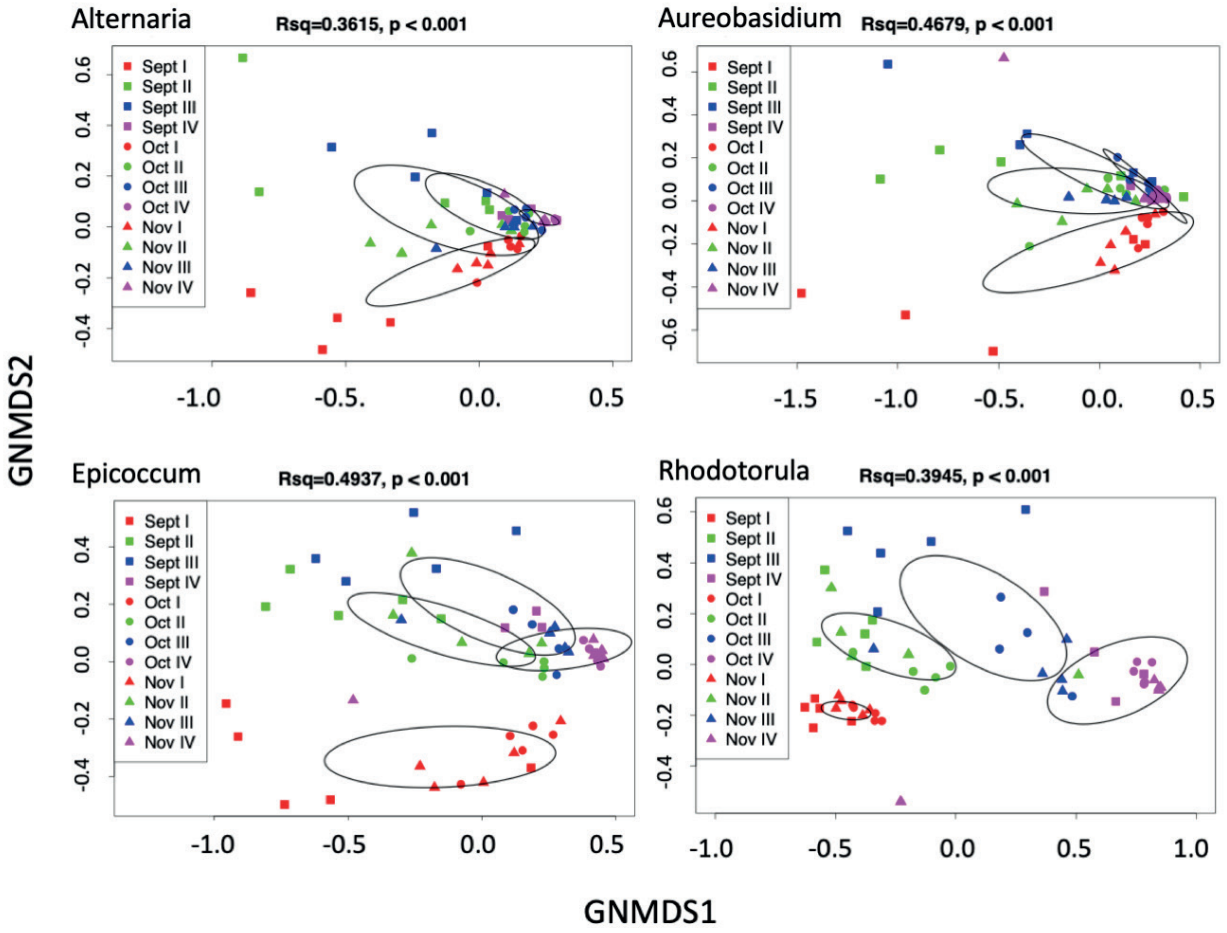

FIGURE 1. NMDS ordination plot of the observed genera.

E. nigrum, and finally, the increase between phases III and IV was significant for all filamentous fungi and yeast (Table 1).

From the ordination plots, in accordance with ANOVA, it could be observed that all the species gene expressions are separated by the botrytisation phase, but harvest time has less effect on the transcriptomic profile. In the gene expression distribution of the genera studied phase (*Alternaria, Aureobasidium, Botrytis, Epicoccum* and *Rhodotorula*), 36 %, 47 %, 26 %, 49 % and 39 % of the observed significant separation in the ordination plot can be explained by phase, respectively (Figure 1). As in the case of analysis of variance, it is noticeable in ordination plots that the harvest date determines the separation of samples to a much lesser degree than the phase (Figure 1).

2. Weighted Gene Co-Expression Analyses

The WGCNA cluster analyses generated ten gene module clusters (Figure 2). Amongst the created co-expression modules, the turquoise, blue, brown and yellow NRCMs showed an eigengene expression profile determined by the NR phases (Noble Rot Connected Modules, NRCM), whereas black, red, magenta, green, pink and purple had an unrelatable and unique eigengene expression profile (Figure 3). Amongst the NRCM gene sets belonging to all species, indicating similar expression abundance patterns were observed in the turquoise and yellow modules (Table 2), and hence these modules were the focus of the current study. From the ANOVA analyses on the eigengenes in the turquoise module, there was an increase in the over the four respective phases, but this increase was only significant between phases III and IV. In the yellow module, there was a significant decrease from phase I to phase II, followed by a further decrease to phase III and finally an increase to phase IV (Figure 3).

3. Differential Gene Expression and Enrichment

Throughout the four NR stages, 1765, 348, 1599 and 293 genes were differentially expressed for the filamentous fungi *A. alternata*, *E. nigrum* and the yeast *A. pullulans* and *R. graminis,* respectively. Since the process of noble rot decay is essentially determined by botrytis, it has a much larger number of genes differentially expressed, quantitatively 11,246. With regards to the DE analyses between the respective phases of the NR process, genes were identified that were uniquely up- or down-regulated and shared between the respective phases. The -up and/or downregulated unique genes in the fourth phase mostly had the highest prevalence amongst all genes unique to a phase.

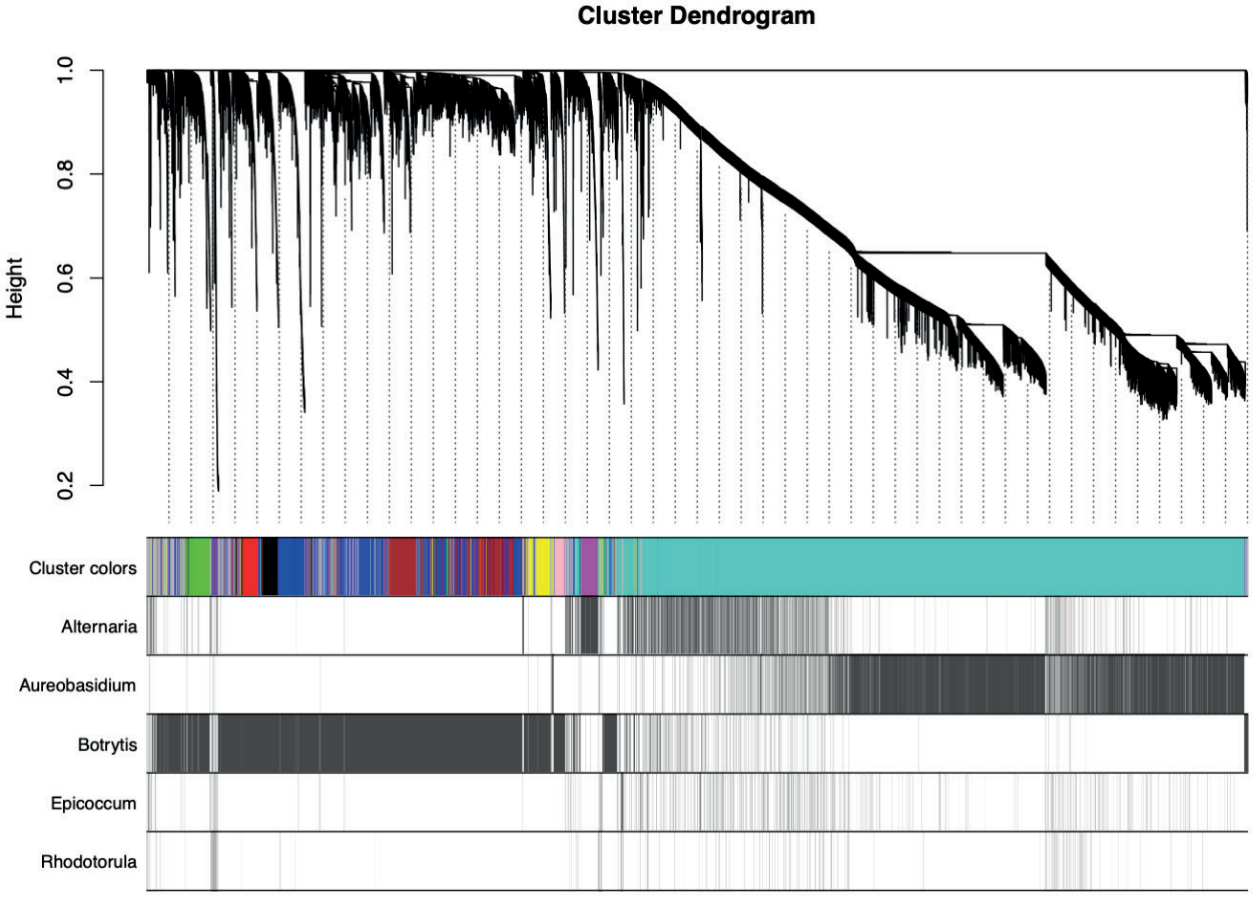

FIGURE 2. WGCNA cluster dendrogram of the aligned genes. Cluster colours are indicated under the dendrogram. The identified genus of each gene is indicated by grey lines at the bottom of the figure.

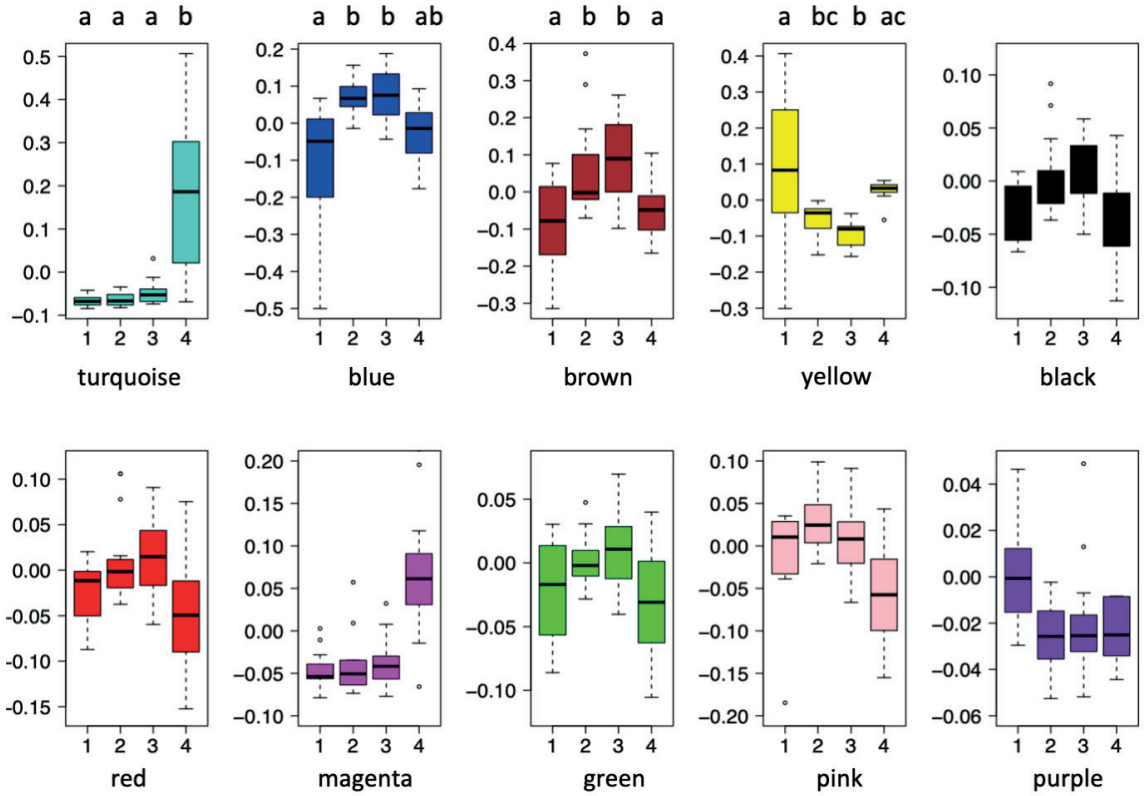

FIGURE 3. The phase distribution of the different gene expression modules; significant differences are marked by small letters, and module colours are mentioned below each plot.

TABLE 2. Distribution of the expressed genes of each WGCNA module between the observed genera.

Module	*Alternaria*	*Aureobasidium*	*Botrytis*	*Epicoccum*	*Rhodotorula*
red	1	0	520	0	1
purple	22	7	42	25	57
pink	0	0	452	0	0
magenta	437	2	3	1	0
black	1	0	484	0	0
turquoise	3191	9914	1013	888	279
green	14	1	592	1	0
blue	4	0	2660	4	4
brown	0	0	2148	1	2
yellow	22	40	682	6	4

For *B. cinerea*, the proportion of the up-regulated number of fourth-phase unique genes (874) was 58 % of the total number of up-regulated DEed genes unique to a phase, whereas the proportion of the down-regulated number of fourth-phase unique genes (800) was 73 % of the total number of down-regulated DEed genes unique to a phase. This trend was also observed in *A. alternata* [(54) 90 %; (648) 78 %], *E. nigrum* [(79) 96 %; (97) 72 %], *A. pullulans* [(384) 89 %; (467) 92 %] and *R. graminis* [(136) 97 %; (10) 50 %].

With regards to the DE analyses between the months, the -up and or downregulated genes unique to November mostly had the highest prevalence amongst all genes unique to a month. For *B. cinerea*, the proportion of the up-regulated number of genes unique to November (553) was 86 % of the total number of up-regulated DEed genes unique to a month, whereas the proportion of the down-regulated number of genes unique to November (867) was 82 % of the total number of down-regulated DEed genes unique to a month. This trend was also observed in *A. alternata* [(553) 86 %; (3) 33 %], *E. nigrum* [(36) 77 %; (3) 43 %], *A. pullulans* [(195) 37 %; (250) 60 %] and no gene was up or down-regulated unique to November regarding *R. graminis*.

In the turquoise modules 47, 7, 50, 21 and 21, enriched pathways were identified in *A. alternata*, *B. cinerea*, *E. nigrum*, *A. pullulans* and *R. graminis*. Amongst these pathways, several were identified which contain enzymes that

contribute to the wine stability and quality and those which are precursors for the formation of aromatic compounds in wine, particularly those pertaining to amino acid metabolism (Callejón *et al*., 2010), carbohydrate metabolism (Han *et al*., 2020), lipid metabolism as well as those involved in the formation of secondary metabolites. With the exception of *B. cinerea,* for which only one 'Secondary metabolites' pathway was identified, pathways pertaining to amino acid metabolism were the most prevalent, with 11, 14, 6 and 4 pathways being identified in *A. alternata*, *E. nigrum*, *A. pullulans* and *R. graminis* respectively. This was followed by carbohydrate metabolism, with 4, 9, 5 and 6 enriched pathways identified in the latter species. The 'secondary metabolites' pathway was the least prevalent in *A. alternata* (4), *E. nigrum* (3), A. *pullulans* (2) and *R. graminis* (1). Genes contributing to the sensory and quality parameters of wines were extracted from these enriched pathways; furthermore, hub genes with similar functions, which belong to enriched pathways, were extracted from the WGCNA clustered modules and are presented in Supplementary Table 1.

DISCUSSION

The current paper, to our knowledge, is the first study on the active metabolic genes of filamentous and yeast fungi during NR, other than *B. cinerea*, and on how these potentially contribute to the NR process, specifically to the formation of aromas, aroma-related compounds, and other parameters that could influence the organoleptic perception, and overall wine quality. We provide novel insights into how the NR-associated filamentous and yeast fungal functional gene expression profiles change over the course of NR as influenced by phase and months, with crucial implications for winemakers.

The increase in transcript abundance of both the filamentous and yeast fungi over the course of NR contrasts with what is known from the literature, namely that filamentous fungi appear at the beginning of the NR, contrary to yeasts which dominate the latter stages (Li *et al*., 2021). Furthermore, it is most likely a reflection of the increasing functional contribution over the four stages of NR, particularly in the current study, the contribution of aroma- and aroma-related precursors. The significant increase in the eigengenes in the WGCNA clustered turquoise module from the NR phases III to IV, as well as the prevalence of the highest number of uniquely up-regulated genes in all filamentous and yeast fungi in the fourth NR phase, quantitatively indicates that co-joint functional contributions in terms of wine flavour and aroma of the NR microbiome other take place during the later phases of NR. The prevalence of several enriched pathways and hub genes pertaining to aroma development, for example, pathways involved in amino acid and lipid metabolism, qualitatively indicated their role in aroma development both directly and indirectly. The identification of several enzymes from NR-associated filamentous and yeast fungi other than *B. cinerea* suggests the minor role of *B. cinerea* in the latter aspect. Even though *B. cinerea* is the predominant fungus during all four stages of NR; its main functional role appears to be inciting structural changes to the berry skin during the early stages of NR (phases I and II), as was shown by Hegyi *et al*. (2022). The lack of influence (not significant) of the month on the expression profile of the filamentous and yeast fungi, as shown by the NMDS analyses, corresponds to Hegyi *et al*. (2022), who found similar results when analysing the functional gene expression profile for *B. cinerea* over the course of three months. This shows that the functional gene profile of the associated filamentous and yeast fungi predominantly associated with *B. cinerea* is relatively stable and has a degree of predictability which is of importance to winemakers.

With regards to aroma-related amino acid metabolism enzymes, most of the identified enzymes pertained to the synthesis of amino acids, some of which are aromatic as well as amino acid-derived conjugates. Subsequent degradation or further metabolic modification of these amino acids is linked to the formation of aroma compounds (Ardö, 2006). For example, a pentafunctional AROM polypeptide was identified from *E. nigrum* (B5807_03777), which is involved in the synthesis of aromatic amino acids (Arora Verasztó *et al*., 2020). In addition, an aromatic amino acid transferase (H), which catalyses the transamination of aromatic amino acids to corresponding alpha-keto acids, was identified in *A. pullulans* (M438DRAFT_343791). Specifically, a tyrosine: phenylpyruvate aminotransferase was identified in *A. alternata* (CC77DRAFT_1066078), and an aspartate aminotransferase was found in *A. pullulans* (M438DRAFT_279163) which are, respectively, involved in the biosynthesis of the aromatic amino acids phenylalanine (Qian *et al*., 2019) and tyrosine. Phenylalanine is a precursor for the synthesis of aroma compounds that have several pleasant tones, such as 2-phenylacetate, which has a flower tone. Furthermore, phenylalanine is a precursor for phenylpropanoids, and the cynnamyl alcohol dehydrogenase found in *A. alternata* (CC77DRAFT_404246) converts cinnamyl alcohol to cinnamaldehyde which has a sweet cherry type of aroma (Burdock, 2016). The degradation of tyrosine can lead to the formation of pleasant aromas, such as floral flavours as well as unpleasant flavours, including chemically associated flavours (Ardö, 2006). In addition to the synthesis of phenylalanine and its aromatic derivatives, the enzyme is also responsible for the reversible interconversion between oxaloacetate and aspartate. Furthermore, a 3-isopropylmalate dehydrogenase (M438DRAFT_313770) and a saccharopine dehydrogenase (M438_DRAFT_353262) were found in *A. pullulans* which are, respectively, involved in leucine and lysine biosynthesis (Herzan *et al*., 2020). Leucine is known to provide wine and other alcoholic beverages with a fruity. The saccharopine dehydrogenase is specifically responsible for the formation of 2-oxoglutarate, which is the main carbonyl compound in wine (Herzan *et al*., 2020).

With regards to amino acid-related derivatives, a glutathione hydrolase (H) (M438DRAFT_280042) and a lactoylglutathione lyase (B5807_09934) were present in the gene expression profile of *A. pullulans* and *E. nigrum*, respectively. The glutathione hydrolase is a precursor of

3-mercaptohexan-1-ol, which is associated with the tropical or citrus fruity aroma of the wine. The lactolylglutathione lyase detoxifies methylglyoxal in conjugation with an aldo-keto reductase. The latter compound is involved in the synthesis of methylbutanal, which is responsible for a moderate amylic aroma (Howell *et al*., 2006). Finally, an anthranilate synthase (H) (M438DRAFT_335458) involved in anthranilate metabolism was identified in *A. pullulans* which synthesises methyl anthranilate, a molecule with an orange blossom aroma (Lin *et al*., 2019).

The majority of enzymes associated with lipid metabolism, just like the nitrogen metabolism-related enzymes, pertained to the synthesis of aroma compound precursors, namely fatty acids, their derivatives (esters) as well as sterols. With regards to fatty acid and associated derivatives synthesis, a long-chain-fatty-acid-CoA ligase (CC77DRAFT_1033357) and fatty acid synthase beta subunit (CC77DRAFT_928877) was found in *A. alternata,* whereas a fatty acid synthase alpha subunit was identified in *E. nigrum* (B5807_00377) (Baekdal *et al*., 1997). Furthermore, a thiol ester dehydratase-isomerase (M438DRAFT_310991), a pantothenate kinase (M438DRAFT_366935) responsible for the synthesis of esters (Cordente *et al*., 2010), a carboxylic ester hydrolase (H) (M438DRAFT_363842) responsible for ester degradation (Pérez-Jiménez *et al*., 2019), and a feruloyl esterase (H) (M438DRAFT_347279), involved in the release of hydroxycinnamic acids from their esterified forms (Collombel *et al*., 2019) subsequently transformation into volatile phenols and thus contributing to the aroma profile (Pérez-Jiménez *et al*., 2019) was identified in *A. pullulans*. The mevalonate kinase found in *A. alternata* (CC77DRAFT_1019990) and *A. pullulans* (M438DRAFT_265955) is linked to the synthesis of sterols as well as terpenes such as geranyl diphosphate, farnesyl diphosphate and diterpenes geranylgeranyl diphosphate (Wedler *et al*., 2015). Similarly, a geranyl diphosphate synthase (M438DRAFT_273287), maleylacetate reductase (M438DRAFT_268034) and monoterpenyl glycosylteransferase (H) (M438DRAFT_316845) found in *A. pullulans*, is also involved in the synthesis of terpenes (Li *et al*., 2017). Terpenes are one of the most important groups of volatile compounds contributing to the aroma profile of wine grapes (Strauss *et al*., 1986). Only one enzyme, amongst all the lipid metabolic associated enzymes, namely an ERG20 farnesyl diphosphate synthase from *A. pullulans* (M438DRAFT_269366), directly contributes to aroma by the formation of rotundone, a compound associated with a pepper-like aroma (Siebert *et al*., 2008).

As for the amino acid- and lipid metabolism-related enzymes, carbohydrate metabolism-related enzymes that have an influence on the formation of precursor aroma compounds were identified, namely a glycoside hydrolase (H) (M438DRAFT_317847), a pyruvate decarboxylase (M438DRAFT_346971) and pyruvate dehydrogenase (H) (M438DRAFT_348965) and prephenate dehydratase (M438DRAFT_326681) in *A. pullulans* as well as a phosphoglucomutase (H) (B5807_00578) in *E. nigrum.* The glycoside hydrolase forms glycosides, which make up a reserve of aroma (Sarry and Günata, 2004). The pyruvate dehydrogenase forms acetate, which is converted to acetaldehyde by pyruvate decarboxylase (Neuser *et al*., 2000), a compound which makes up 90 % of the total aldehyde content of wine and is associated with a fruity aroma. The prephenate dehydratase converts prephenate to phenylpyruvate which is subsequently converted to 2-phenylethanol, responsible for a floral and honey aroma in fermented foods (Synos *et al*., 2015). It has been shown that in the case of *Saccharomyces cerevisiae* gene expression during fermentation, phosphoglucomutase catalyses a key step in hexose metabolism, which can form precursors of important volatiles that might have important contributions to wine aroma during alcoholic fermentation (Reiter *et al*., 2021). The drawback of volatile acids in excessive quantities is, however, that the development causes an unpleasant vinegar-like aroma (Boulton *et al*., 2013).

Amongst the carbohydrate metabolism aroma-related enzymes identified in the current study, several were identified which are involved in citrate metabolism and associated malolactic fermentation, namely a citrate synthase in *A. alternata* (CC77DRAFT_250650), *A. pullulans* (M438DRAFT_294441), *E. nigrum* (B5807_06716) (H), and *A. pullulans* (M438DRAFT_284526), an l-lactate dehydrogenase in *A. alternata* (CC77DRAFT_285449) and *A. pullulans* (M438DRAFT_292234), and a malate dehydrogenase (B5807_02946), malic enzyme (B5807_05369) acetolactate synthase in *A. pullulans* (M438DRAFT_264767) and *E. nigrum* (B5807_00488) and aldehyde dehydrogenase in *A. pullulans* (M438DRAFT_282832) and *E. nigrum* (B5807_0481), respectively. Citrate and malolactic fermentation-related enzymes are linked to diacetyl and diacetyl derivatives, which are important aroma compounds, contributing to the 'buttery' aroma of wine (Bartowsky and Henschke, 2004). In addition to contributing to the overall aroma/flavour profile of wines, the metabolism of organic acids and their carboxylic acid derivatives have an influence on the resulting total acid-base chemistry and pH (Vicente *et al*., 2022), alcohol content, biological colour and stability of wine which influences the organoleptic and overall quality of the wine. Furthermore, organic and carboxylic acids also have antioxidant properties, which have beneficial effects on human health and help protect the aromatic wine compounds from oxidation (Kamzolova and Morgunov, 2021) and subsequent loss of flavour and aroma (Papadopoulou and Roussis, 2008). Along with the previously mentioned enzymes involved in organic acid metabolism, an isocitrate dehydrogenase (CC77DRAFT_1056855) and isocitrate lyase (H) (M438DRAFT_343020) were identified in *A. alternata* (CC77DRAFT_1056855) and *A. pullulans* (M438DRAFT_343020), respectively, and both have reported antioxidant activity (Benhar *et al*., 2009) protecting wine aromatic compounds against oxidation (Kamzolova and Morgunov, 2021).

With regards to antioxidant properties glutamate cysteine lyase (H) from (M438DRAFT_342907) and a hydroxyacylglutathione hydrolase/glyoxylase II (M438DRAFT_273852), gamma-glutamylcyclotransferase (M438DRAFT_323211) pertaining to glutathione metabolism were identified from *A. pullulans*. Glutamate cysteine ligase is a rate-limiting enzyme in glutathione biosynthesis, hydroxyacylglutathione hydrolase/glyoxylase II catalyses the hydrolysis of S-D-lactoyl-glutathione from glutathione, and d-lactic-acid (Junior *et al*., 2021) and gamma-glutamylcyclotransferases are involved in the catabolism of many compounds that are conjugated to glutathione. Complementary to its strong antioxidant capabilities, glutathione has been shown to inhibit the decline of several volatiles such as isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and linalool in Debina wine during storage (Papadopoulou and Roussis, 2008). Furthermore, many distinctive flavour and aroma compounds present in Sauvignon Blanc wines originate from C5/C6-glutathione conjugates or their catabolised derivatives.

The alcohol concentration of wines is of crucial importance. In the current study, a galactose oxidase (M438DRAFT_276942) was identified from *A. pullulans* which catalyses the oxidation of primary alcohols to multiple compounds, including aldehydes. In addition to having an important effect on the alcohol concentration, the latter process has important implications for wine aroma and flavour formation. Similarly, a glycerol kinase and a 6-phosphogluconolactonase (B5807_08165) were found in *A. alternata* (CC77DRAFT_76448) and *E. nigrum* (B5807_01031), respectively, which is responsible for the metabolism/degradation of glycerol (Wang *et al*., 2020).

In addition to several enzymes having functional roles other than contributing to aroma and antioxidant activity, some enzymes were identified which play a role in the detoxification of potentially toxic compounds in wine and flavour preservation. The S-formylglutathione hydrolase found in *A. alternata* (CC77DRAFT_1003962) and *E. nigrum* (B5807_08147) contributes to the formation of formate-derived compounds that have an apple-like wine aroma (Kelebek and Selli, 2011) but also play a role in wine detoxification, converting formaldehyde to formate (Schug, 2018). Furthermore, the kynureinase from *A. pullulans* (M438DRAFT_265743) is responsible for the degradation of kynurenine to anthranilic acid and provides the wine with an untypical 'ageing-off' flavour (Hoenicke *et al*., 2002).

CONCLUSION

Even though *Botrytis cinerea* is the predominant fungus associated with NR and its functional role in contributing to the physicochemical changes that take place during this process have been documented, this paper provides insight into how other NR-associated filamentous and yeast fungi contribute to specifically the precursors and intermediate compounds of wines made from NR grape berries. We hypothesised that in addition to *B. cinerea,* other filamentous fungi such as *A. alternata* and *E. nigrum,* as well as yeasts *A. pullulans* and *R. graminis* commonly associated with NR berries, also contribute to the aroma profile of NR wines during botrytisation which has not really been studied to date. To identify the most significant impacts of the berry microbiome on the wine aroma during NR, the most relevant biochemical pathways and gene functions were evaluated and analysed, all of which provide insight into the noble rot process through further investigation.

In the current study, several enzymes pertaining to the formation of wine aroma and flavour, mostly precursor molecules pertaining to an amino acid, lipid and carbohydrate metabolism, were identified from filamentous fungi *Alternaria*, *Epicoccum* and the yeast *Aureobasidium.* Amongst these, enzymes were identified that directly impact wine aroma, like the cynnamyl alcohol dehydrogenase found in *A. alternata* (CC77DRAFT_404246), responsible for a cherry-type aroma (Burdock, 2016). In addition to enzymes that have a functional role pertaining to wine flavour and aroma, enzymes were identified which have other functional roles in wine, including aroma preservation, such as the carboxylic and organic acids (Kamzolova and Morgunov, 2021) found in all species, including the citrate synthase in *A. alternata* (CC77DRAFT_250650), the malate dehydrogenase in *E. nigrum* (B5807_02946) and the acetolactate synthase in *A. pullulans* (M438DRAFT_264767). Furthermore, the S-formylglutathione hydrolase found in *A. alternata* (CC77DRAFT_1003962) and *E. nigrum* (B5807_08147) is responsible for the detoxification of formate-related compounds (Schug, 2018). This study has provided insight that even though the main fermentative processes resulting in the development of flavour and aroma compounds in NR wines occur during fermentation by *Saccharomyces* species during vinification (Goddard, 2008), the microbial community on the grape berry skin during NR are responsible for the synthesis of several enzymes pertaining to the aroma, aroma preservation and other functional roles including wine stability and detoxification.

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