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Wine microbiome: impact of vitivinicultural practices

Faculty of Sciences and Technology

2020

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Master in Molecular and Microbial Biology

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Authorship Statement

I hereby declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in the text and are listed in the included references.

Agradecimentos

Começo por agradecer às minhas orientadoras, Doutora Margaret Soares e Doutora Leonor Faleiro, pela oportunidade que me deram ao realizar este trabalho, por toda a disponibilidade e compreensão, e principalmente pela confiança que depositaram em mim.

Agradeço aos meus colegas de laboratório Pedro, Bruno e Isabel. Um agradecimento especial ao Zé por toda a ajuda, e à Liseta por toda a companhia e boa disposição.

Agradeço ao Tiago e à Sara pelo apoio e por todos os momentos de descontração. Agradeço ao Cabé pelo amigo incansável que é, pelo apoio e incentivo constantes e por compreender as minhas ausências.

Agradeço aos meus tios Valdemar e Lídia, à Rita e à Martita, por todo o carinho e apoio.

Agradeço aos meus irmãos, cunhadas e sobrinhos por sempre me apoiarem e impulsionarem a ser melhor.

Agradeço ao Bruno por todo o apoio e compreensão, por toda a paciência que teve durante este processo e por acreditar sempre nas minhas capacidades.

Para finalizar quero agradecer aos meus pais. Obrigada por todo o amor e carinho e por acreditarem sempre em mim. Obrigada por todo o esforço que fizeram para me dar as ferramentas que me permitiram chegar aqui. E por fim, obrigada por me deixarem voar e por desculparem as minhas ausências.

Abstract

Portugal is worldwide known for the quality and distinction of its wines and has been sharing a strong bond with the vitivinicultural sector since early days, with great economic impact. Several current problems, such as climate change and the scarcity of natural resources, have been affecting the agricultural sector, with winemaking exemplifying this. Aiming the overcome of those problems, several studies have been carried out in order to develop sustainable vitivinicultural practices, while preserving wine's regionality and quality. One of the most researched subjects has been the *terroir* impact on wine's organoleptic properties, and several studies have been reporting the influence of vine and wine's microbiome, thus arising the importance of the microbial *terroir*. Therefore, there is a need to clarify the microbial *terroir* role on winemaking processes, identifying the microbial consortium and its evolution along the process, to understand their effect on the final product. Thus, a comparative study was developed, in which two grape varieties (Touriga Nacional and Aragonez) were produced simultaneously under two production modes (Organic and Integrated) and fermented by two different methods (spontaneous fermentation *vs.* commercial inoculation). Samples of Initial Must, Medium Fermentation and Final Fermentation were analysed by culture-dependent methods, molecular biology techniques and metagenomic tools, to evaluate the influence of vinicultural processes on microbiome and organoleptic properties of the 8 wines produced.

This study demonstrated that the mode of production affects the diversity of initial must microbiome in both eukaryotes and prokaryotes. However, differences in the beginning of fermentation tend to decrease throughout the fermentation process. Regarding the fermentation method, in this study musts inoculated with the commercial yeast presented a lower biodiversity and a larger number of pathogenic microorganisms, unlike the musts resulting from spontaneous fermentation, which not only showed a higher biodiversity, but also exhibited a higher number of phytoprotective microorganisms.

Keywords: Wine microbiome, Integrated production mode, Organic production mode, Autochthonous yeasts, Commercial yeasts, Spontaneous fermentation, Inoculated fermentation, Microbial *terroir*.

Resumo

Portugal é mundialmente conhecido pela qualidade e distinção dos vinhos que produz e comercializa, sendo que este forte vínculo com o setor vitivinícola perdura desde os primeiros dias, com grande impacto económico. Dados referentes ao ano de 2018 mostram que, a nível mundial, Portugal é o 12º maior produtor, o 11º maior consumidor e o 9º maior exportador. Diversos problemas atuais, tais como as mudanças climáticas e a escassez de recursos naturais, têm vindo a afetar o sector agrícola, sendo que a produção de vinho exemplo disso mesmo. De forma a contornar este problema é importante estudar, desenvolver e aplicar soluções sustentáveis, que não só permitam ultrapassar os problemas assinalados, como também possibilitem preservar as propriedades e consequente qualidade do produto final, o vinho. Ao longo dos últimos anos têm sido realizados vários estudos com o objetivo de desenvolver práticas vitivinícolas sustentáveis, diminuindo a utilização de produtos nocivos para o ambiente, aumentando a utilização consciente dos recursos naturais e preservando a regionalidade do local, com o objetivo que esta se espelhe nas características do vinho. Portugal, a par com países um pouco por todo o mundo, tem acompanhado esta tendência ao nível do desenvolvimento e adoção de processos inovadores e sustentáveis para a vinificação, através da realização de estudos no seu território.

Um dos temas mais estudados nos dias de hoje envolve o conceito de *terroir*: que engloba o conjunto de interações entre o solo, a topografia, o clima, as características do terreno, a biodiversidade e as práticas de vitivinicultura que ocorrem em determinada área (bem delimitada). A este conceito está associada a regionalidade, ou seja, a singularidade de cada vinho. Por outras palavras: se uma casta for produzida em *terroir* diferentes (por muito ligeiras que sejam essas diferenças), os vinhos produzidos traduzirão as características inerentes de cada local e por isso terão características diferentes. Estas diferenças podem ser reveladas com mais ou menos impacto a nível da análise sensorial, no entanto, quando são aplicadas ferramentas moleculares analíticas é possível observar as diferenças entre a composição de cada vinho. Um dos constituintes do *terroir* responsável por um grande efeito sobre as propriedades organoléticas do vinho é *terroir* microbiano. Este *terroir* é resultado dos diversos microbiomas presentes ao longo dos processos de produção e fermentação do vinho, ou seja, o microbioma da vinha (como é o exemplo do microbioma do solo, do tronco da videira, das folhas, dos bagos, da pele das uvas e das grainhas) e o microbioma do mosto (durante o processo de fermentação). Embora o efeito deste *terroir* nos processos de vitivinificação esteja já comprovado em diferentes castas e diferentes locais do

mundo, as interações que conduzem a esse efeito e a forma como afetam as propriedades do produto final ainda não se encontra clarificada. É por isso importante continuar o estudo desta temática, através da identificação do consórcio microbiano e do estudo da sua evolução ao longo dos processos de produção e fermentação, de forma a compreender o seu papel no produto final. Para além disso, e porque o conceito de *terroir* traduz isso mesmo, seria importante aliar o estudo acima referido a outras variáveis, como o modo de produção e o método de fermentação.

Tendo em vista esse mesmo objetivo, foi desenvolvido um estudo comparativo, no qual duas castas tintas (Touriga Nacional e Aragonez) foram produzidas no Alentejo (Beja, Portugal). Cada casta foi simultaneamente produzida em modo biológico e integrado. Ambos os modos de produção são considerados sustentáveis, uma vez que combinam a utilização ponderada de recursos naturais com praticas agrícolas de incentivo à biodiversidade. A grande diferença entre estes dois modos de produção é o apertado controlo da aplicação de produtos fitofarmacêuticos, sendo que no modo de produção biológico apenas um pequeno número destes produtos se encontra aprovado para aplicação. No modo de produção integrada, embora sejam autorizados alguns tipos de tratamento, o seu emprego é também evitado altamente controlado.

De forma a avaliar o poder do processo de fermentação nas propriedades do vinho, os mostos obtidos foram fermentados através de dois métodos diferentes: fermentação comercial e fermentação espontânea. Na fermentação comercial o mosto foi inoculado com uma levedura comercial cujo efeito nas propriedades finais do vinho já era esperado. Na fermentação espontânea não ocorreu qualquer inoculação, o que significa que nesses mostos apenas estavam presentes leveduras autóctones – provenientes da vinha (solo, videira, folhas, bagos, pele da uva, grainhas, etc.). Assim, as propriedades de um vinho resultante de uma fermentação espontânea vão depender exclusivamente do *terroir*, ou seja, é esperado que estas leveduras traduzam uma maior regionalidade no produto final.

De maneira a clarificar a forma como o *terroir* microbiano se comporta ao longo do processo de fermentação foram recolhidas amostras de Mosto Inicial, mosto em Meio de Fermentação e em Fim de Fermentação. Todas as amostras foram analisadas por métodos dependentes de cultura, técnicas de biologia molecular e ferramentas metagenómicas, para avaliar a influência dos processos vitiviniculturais no microbioma e nas propriedades organoléticas dos 8 vinhos produzidos. Através do método de espalhamento em meio YEPD (Extrato de Levedura, Peptona e Dextrose), procedeu-se à contagem e isolamento das leveduras cultiváveis, que foram depois

identificadas por sequenciação da região ITS. Foi também realizada a análise metagenómica de cada um dos oito mostos, de forma a obter uma caracterização da composição da comunidade microbiana ao longo dos diferentes processos de produção e fermentação em estudo. Para completar o estudo da influência do modo de fermentação, foram amplificadas as sequências interdelta dos mostos em final de fermentação.

Este estudo veio demonstrar que o modo de produção afeta a diversidade do microbioma do mosto inicial tanto em eucariotas como em procariotas. No entanto, as diferenças registadas no início da fermentação tendem a diminuir ao longo do processo de fermentação. Em relação ao método de fermentação, neste estudo os mostos inoculados com a levedura comercial apresentaram uma menor biodiversidade e um maior número de microrganismos patogénicos, ao contrário dos mostos resultantes da fermentação espontânea, que não só apresentaram uma maior biodiversidade, como também exibiram um maior número de microrganismos fitoprotetores.

Palavras chave: Microbioma do vinho, Produção integrada, Produção biológica, Leveduras autóctones, leveduras comerciais, Fermentação espontânea, Fermentação inoculada, *terroir* microbiano.

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Abbreviations

AG	Aragonez
CFU	Colony-Forming Units
CO_2	Carbon Dioxide
EDTA	Ethylenediaminetetraacetic acid
EF	End of Fermentation
EU	European Commission
EUR	Euro
FT	Fermentation
FT13	Integrated Production/Inoculated fermentation
FT14	Organic Production/Inoculated fermentation
FT15	Integrated Production/Spontaneous fermentation
FT16	Organic Production/Spontaneous fermentation
FT33	Integrated Production/Inoculated fermentation
FT34	Organic Production/Inoculated fermentation
FT35	Integrated Production/Spontaneous fermentation
FT36	Organic Production/Spontaneous fermentation
HMN	Herdade da Malhadinha Nova
IM	Initial Must
IP	Integrated Production
ITS	Internal Transcribed Spacer
kg	Kilogram
kha	Thousands of Hectares
khl	Thousands of Hectolitres
km	Kilometre
MF	Middle of Fermentation
ml	Millilitre
mha	Millions of Hectares
mhl	Millions of Hectolitres

NaCl	Sodium Chloride
N_2O	Nitrous Oxide
OP	Organic Production
PPP	Plant Protection Products
TN	Touriga Nacional
YEPD	Yeast Extract Peptone Dextrose

1. Introduction

1. Introduction

1.1 World Vitiviniculture

Wine is one of the best known and most appreciated beverages in the world, being the earliest records of its existence date back to the seventh millennium before Christ (B.C.) (McGovern *et al*. 2004). Nowadays, grape and wine production (vitiviniculture) takes place all over the world in an estimated area of 7.4 mha (3.3 mha in EU). In 2018, about 292.3 mhl of wine were produced worldwide (181.9 mhl in the EU), followed by a slight growth in the wine market compared to 2017 (31.2 billion EUR) (OIV 2019).

The increasing wine consumption and economy impact has driven not only the production but also the pursuit of strategies that can result in a unique product whose quality reflects the inherent and autochthonous regional characteristics (Bokulich *et al*. 2016; Knight *et al*. 2015).

1.1.1 Portuguese vitiviniculture

Portugal has been associated with wine production and commercialization since the early days: it is thought that the first vineyard grown on the Iberian Peninsula (Tejo and Sado valley) dates from 2000 years B.C., being the first book to share Portuguese winemaking techniques published in 1900 (IVV 2019; Costa 1900). Most recent data (referring to 2018) show that the 192 kha of Portuguese territory used for wine production have made Portugal the 12th world's largest producer (with 6.1 mhl), the 11th world's main consumer (with 5.5 mhl), and the 9th world's biggest exporter (worth 804 million of EUR) (OIV 2019).

Although its small area (92212 km^2), Portugal confines various types of soil, climate, topography, landscape characteristics, biodiversity features and winemaking practices– characteristics that constitute the concept of "*terroir*". The region's *terroir* is the responsible for wine organoleptic properties and flavour, i.e., wines produced in different regions (*terroirs*) will never have the same characteristics. According to the Portuguese Wine and Vine Institute (IVV, I.P.), the richness of Portuguese *terroirs* have resulted in 14 distinct appellations (**Figure 1.1**) (OIV 2010; Knight *et al*. 2015).

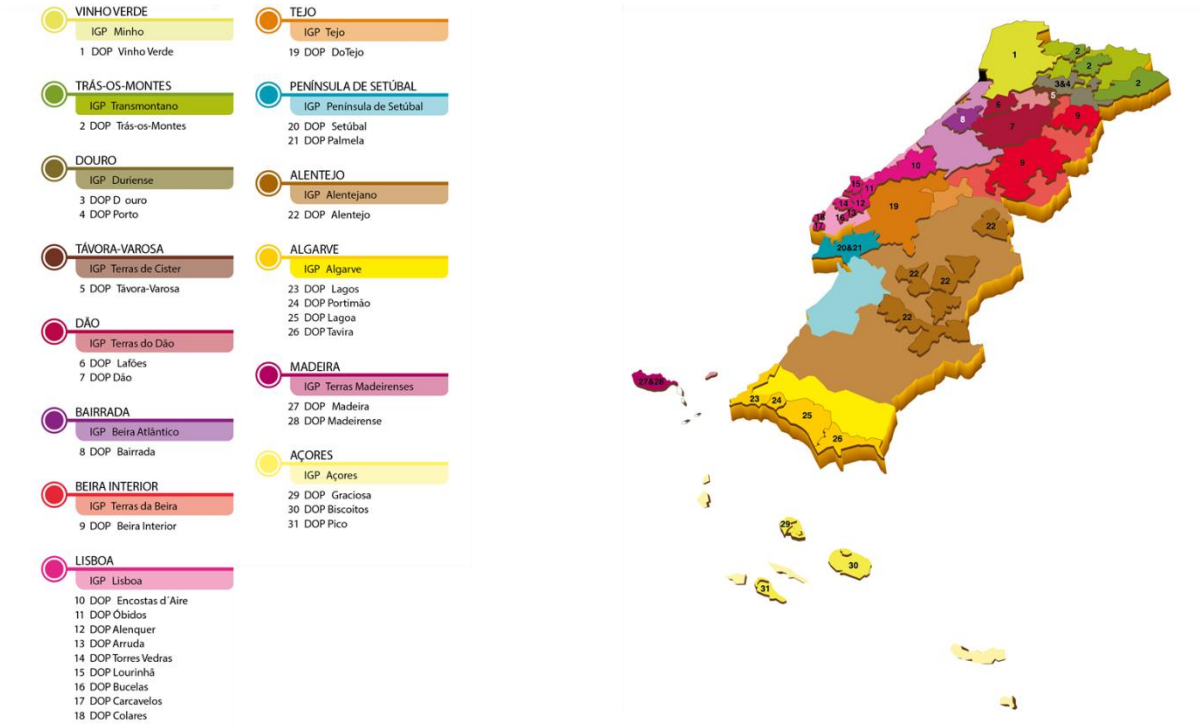

Figure 1.1 – Portugal Wine Regions. (IGP – Protected Geographical Indication; DOP – Protected Designation of Origin) (Adapted from: https://www.ivv.gov.pt/np4/regioes/)

Over the last years Portugal has been following the worldwide trend on development and adoption of innovative and sustainable processes for winemaking (Alves *et al*. 2015; Franco-Duarte *et al*. 2016; Pinto *et al*. 2015a).

1.2 Wine *terroir*

The "*terroir*" concept is used in the winemaking field to express the interaction between soil, topography, climate, landscape characteristics, biodiversity features and vitivinicultural practices in a delimited area (OIV 2010; Alves *et al*. 2015; Knight *et al*. 2015). Collectively, wine *terroir* features are responsible for defining its organoleptic properties and flavour, i.e., its quality and uniqueness (**Figure 1.2**) (Koundouras 2018). Many of the reported studies have demonstrated the impact that each *terroir* feature has on the final product (**Figure 1.3**), and the knowledge of these interactions has been very helpful, allowing winemakers to adapt the grape variety and/or winemaking practices to the *terroir* characteristics in order to maintain or improve the wine quality (Karaoğlan *et al*. 2015; Belda *et al*. 2017; Canfora *et al*. 2018).

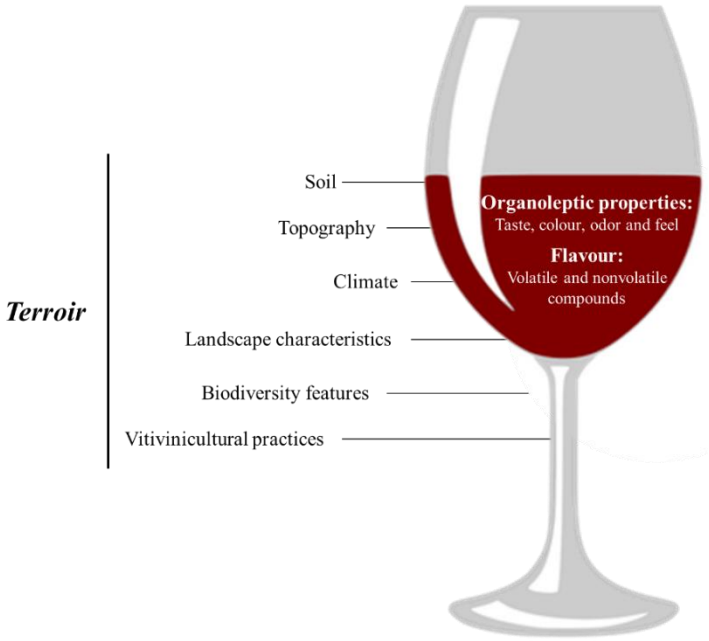

Figure 1.2 – Schematic representation of the *terroir* features and their effects on wine organoleptic properties and flavor (OIV 2010; Koundouras 2018). Wine glass source: Openclipart – https://publicdomainvectors.org/en/free-clipart/Half-wine-glass/67030.html).

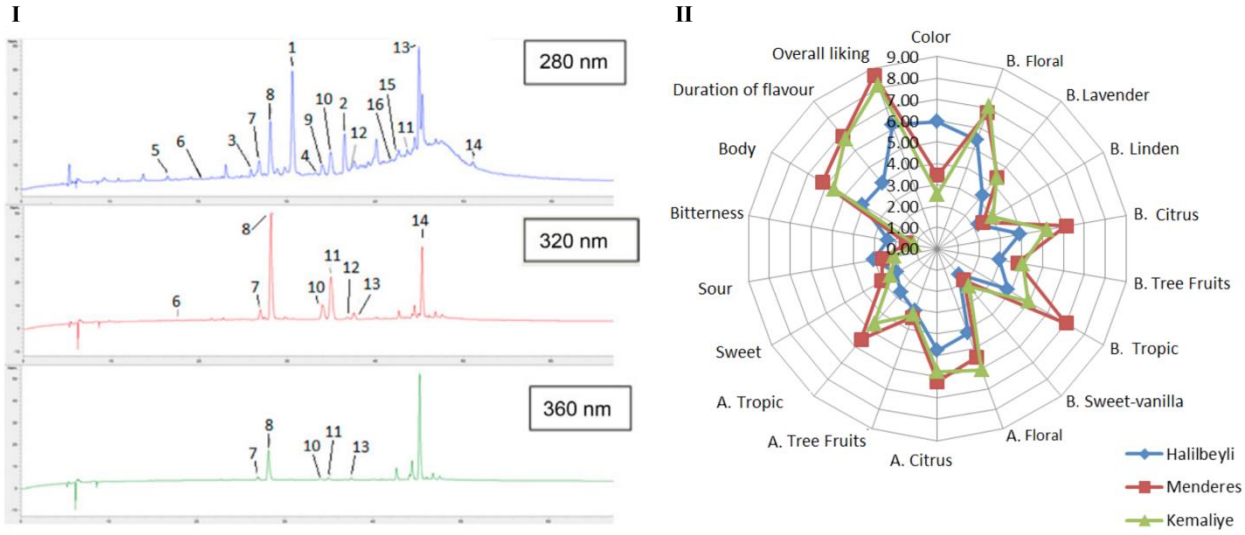

Figure 1.3 – *Terroir* effect on phenolic compounds (I) and flavour profile of Muscat of Bornova wines from three different *terroirs*: Halilbeyli, Menderes and Kemaliye (II). (A – Chromatograms in 280, 320 and 360 nm of the identified phenolic compounds; B – Spider web diagram of flavour profile analysis of Muscat of Bornova wines: (A) data shows palate descriptors, (B) data shows odour descriptors.) Source: Karaoğlan *et al*. 2015.

1.2.1 Microbial *terroir*

Although it is currently accepted, the microbial influence in the wine production has only been acknowledged in the two last decades, but, since then, the number of studies and data generated on this topic has been increasing worldwide (Carreto *et al*. 2008; Schuller *et al*. 2012; Pinto *et al*. 2015b; Bokulich *et al*. 2016; Wei *et al*. 2018; Bagheri *et al*. 2019).

Over the years, the development and improvement of available tools has culminated in a library of information on the soil, grapevine, leaves, grape, must and wine microbiome (Burns *et al*. 2016; Vitulo *et a*l. 2019; Mezzasalma *et a*l. 2017; Wei *et al*. 2018; Karaoğlan *et al*. 2015).

Studies have been revealing the impact of microbial *terroir* on wine properties, and it is now known that the grape and wine microbiome can improve viticulture yields and benefit the production of valuable and unique wines (Pinto *et al*. 2015; Setati 2015; Castañeda and Barbosa 2017; Mezzasalma *et al*. 2017).

All *terroir* elements interact with each other and, from this complex network, result single microbiomes of soil, vine, grapes, leaves and consequently, wine. Knowing that, several studies have demonstrated that:

- climate, grape variety and vintage weather conditions leads to soil and grape microbiome changes (Pinto *et al*. 2015b);
- climate and vitivinicultural practices affect soil composition (Burns *et al*. 2016; Blotevogel *et al*. 2019);
- different winemaking processes – e.g. fermentations inoculated with *Saccharomyces* and native non-*Saccharomyces* yeasts *vs* fermentations inoculated with only *Saccharomyces* yeasts – lead to modifications on microbial populations of wine fermentations, as well as different wines (Padilla *et al*. 2017).

Although the scientific community has already achieved the foregoing results, some questions remained unclarified:

i. Will the implementation of a more sustainable production mode lead to major changes on wine microbiome?
ii. Will these changes have a positive influence on the final product?
iii. Will they contribute for the wine's regionality?
iv. Will a winemaking process with indigenous yeasts lead to a more improved and differentiated wine, in comparison to others inoculated with only commercial yeasts?

It is therefore important to continue the study on this subject, not only to produce new quality wines but also in pursuance to adopt sustainable winemaking techniques, that allow the conscious and thoughtful use of natural resources to obtain unique and healthy wines, enhancing its quality and regionality.

On **Figure 1.4** a summarized schematic representation of factors known to influence the composition of microbial populations involved in wine fermentations is shown.

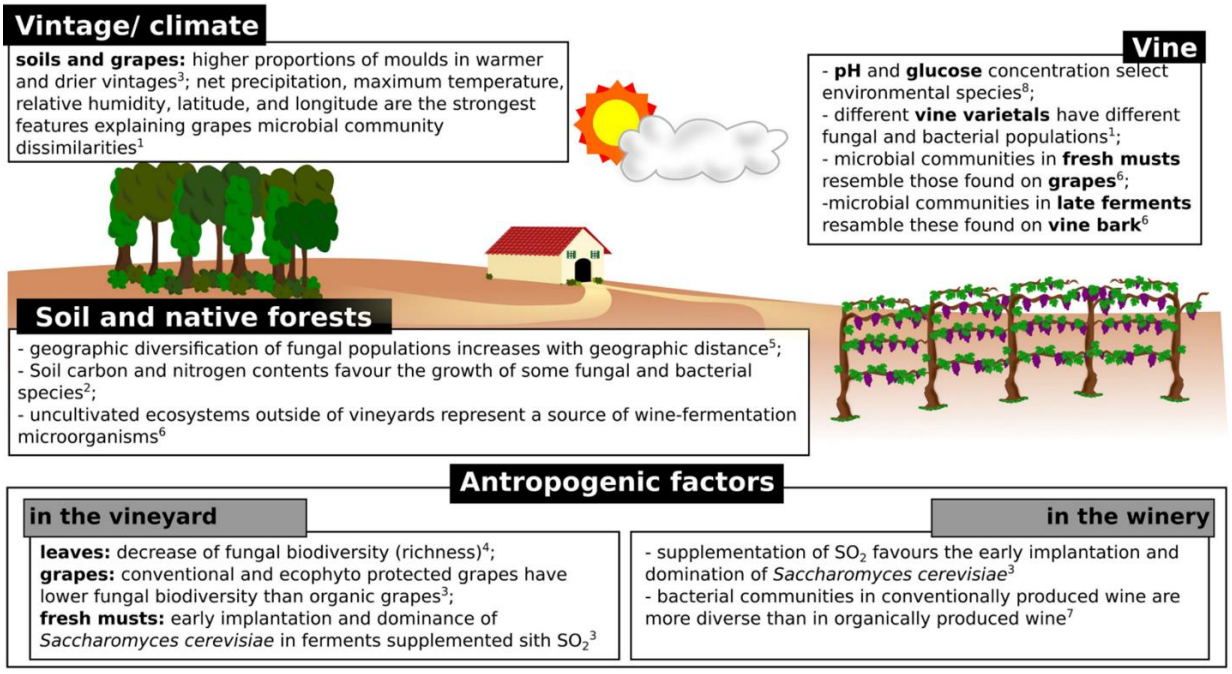

Figure 1.4 – Schematic representation of the factors known to influence the composition of microbial populations involved in wine fermentations ([1](Bokulich *et al*. 2014); [2](Burns *et al*. 2016); [3](Grangeteau, Roullier-Gall, et al. 2017); [4](Pinto *et al*. 2014); [5](Miura *et al*. 2017); [6](Morrison-Whittle and Goddard 2018); [7](Piao *et al*. 2015); [8](Stefanini *et al*. 2016). Image source: Stefanini and Cavalieri 2018.

1.3 Production Modes

Current issues, such as global warming and climate change have been triggering the adoption of pro-environmental positions. Despite the fact that the agricultural sector is one of the most affected by this problem, it was also one of the most responsible for its occurrence, namely in 2017 this sector was the second largest emitter of CO_2 and the first main emitter of N_2O in European Union (EEA, 2019). The adoption of unsustainable practices for the environment and human health has led to an alarming increase in the number and intensity of outbreaks of pests and diseases of animals and plants REFS. This has consequences on the food system, which has been facing food safety threats due to emergencies linked to the global warming and climate change (FAO 2017). One of

the strategies that has been implemented to combat climate change and its effects, is the implementation of sustainable and environmentally friendly production systems (PMEET 2019). The integrated and organic production are two agricultural systems based on sustainable processes, in order to obtain quality products through rational management of natural resources and the adoption of sustainable measures designed to protect and preserve the nature, the environment and the human species (Decreto-Lei n.º 256/2009 de 24 de Setembro 2009).

1.3.1 Integrated Production

Integrated Production (IP) "is an agricultural system for the production of food and other high-quality food products, with rational management of natural resources and giving priority to the use of natural regulation mechanisms in place of inputs, thus contributing to sustainable agriculture" (Decreto-Lei n.º 256/2009 de 24 de Setembro 2009). The exercise of IP is governed by a set of rules based on the Decree of Law n.º 256/2009 de 24 de Setembro 2009, of which it is important to reiterate the phytosanitary protection and the fertilizers use:

- The phytosanitary protection is ruled by the principles of integrated protection, which means that the use of Plant Protection Products (PPPs) and similar forms of intervention is only endorsed at economically and ecologically justifiable levels, reducing its risks to human health and the environment (Decreto-Lei n.º 256/2009 de 24 de Setembro 2009). Therefore, PPPs may only be applied when the Economic Level of Attack is reached – it corresponds to the time when the intensity of crop attack justifies the application of restrictive or fight measures to prevent the damage from exceeding the cost of the control measures to be taken, and undesirable effects. When the Economic Level of Attack is not established at national level, the PPPs can be applied when the importance and extent of damage caused by the enemy to fight is justifiable (Decreto-Lei n.º 256/2009 de 24 de Setembro 2009).
- The use of fertilizers must comply with current legal standards and must be exempt or have very low levels of heavy metals or other environmentally hazardous substances. Micronutrient fertilizers should only be applied when their need is technically recognized.

1.3.2 Organic Production

The Organic Production (OP) "is an overall system of farm management and food production that combines best environmental practices, a high level of biodiversity, the preservation of natural

resources, the application of high animal welfare standards and a production method in line with the preference of certain consumers for products produced using natural substances and processes" (Council Regulation (EC) N.º 834/2007 of 28 June 2007). On OP only PPPs approved by law may be used (Decreto-Lei n.º 94/98 de 15 de Abril 1998).

In OP, the European Commission shall authorize for use and include a restricted list of products and substances which may be used as PPPs, fertilizers, soil conditioners and products for cleaning and disinfection.

Both IP and OP may have an impact on the microbial terroir and ultimately in the wine quality. Several omics approaches can be used to examine the influence of these production mode on the wine microbiome.

1.4 Wine and multi-omics

The wine microbiome is composed of a complex interaction network between each microbial member of the community. During winemaking, the existing yeasts and bacteria act together as a consortium, and all the resulting metabolites end up influencing the wine aroma and taste. The fact that these effects may result in a positive or negative impact on wine quality makes this issue one of the objectives of the wine microbiome study: once these interactions are known, it will be possible to manage the fermentation elements in order to control wine quality (Morrison-Whittle and Goddard 2018).

Over the years, culture-independent molecular methods have been developed to assemble more detailed and complete information, in order to better understand the ongoing interactions and their meanings in winemaking processes and wine properties.

Metagenomics, Metatranscriptomics, Metaproteomics and Metabolomics are four molecular and informatic tools widely used in this field (Setati, Jacobson, and Bauer 2015; Carreto *et al*. 2011; Zhao *et al*. 2015; Franco-Duarte *et al*. 2016).

As shown in **Figure 1.5**, each of these tools can be used at any stage of winemaking, however it needs a specific sample type (DNA, total RNA and mRNA, proteins or volatile and nonvolatile compounds).

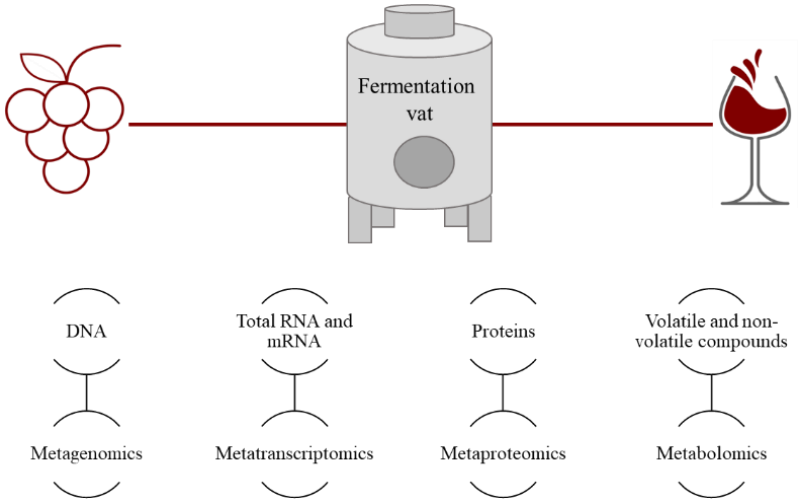

Figure 1.5 – **Multi-omics applications in wine production.** Images source: Grapes – Openclipart, https://publicdomainvectors.org/en/free-clipart/Grapes-symbol/67038.html; Wine glass – https://publicdomainvectors.org/en/free-clipart/Wine-only/64422.html. (Source: Sirén *et al*. 2019).

1.4.1 Wine and metagenomics

Metagenomics is one of the most used tools to describe and compare the compositions of microbial populations of wine, since it is a culture-independent method that allows for in-depth analysis providing additional information. With this tool microbial communities from any environmental sample (soil, leaves, grapes, vine bark, fermentations, wine, winery surfaces) can be established as long as we can extract its DNA. (Mathabatha Evodia Setati *et al*. 2012; Zarraonaindia *et al*. 2015; Pinto *et al*. 2015b; Canfora *et al*. 2018; Mezzasalma *et al*. 2018; Vitulo *et al*. 2019; Bokulich *et al*. 2013).

The application of metagenomics in the study of wine has already opened many doors regarding the knowledge of the composition of the microbial *terroir* not only of the products (grapes, must, wine) but also the wine processing environment (vineyard, cellar). However, it is necessary to perform more studies that work simultaneously with different variables (in terms of viniculture and viticulture) to decode more key points of this complex process. The more obtained information, the easier it will be to apply sustainable and appropriate techniques for each *terroir*.

1.5 Objectives

The main objective of this study was to evaluate the impact of different winemaking practices on wine microbiome during the winemaking process. For this purpose, two Portuguese red wine grape

varieties (Touriga Nacional and Aragonez) were produced under two production modes (organic and integrated), which fermentation occured by two different approaches (Table 1):

i. spontaneous fermentation (autochthonous yeasts);
ii. inoculated fermentation (commercial yeasts).

Table 1.1 – Innovation strategy at vitivinicultural processes.

		Viticultural Processes	
		Integrated Production (IP)	Organic Production (OP)
Oenological processes	Inoculated Fermentation: Commercial + wild yeasts	Undifferentiated conventional wine	Undifferentiated organic wine
	Spontaneous Fermentation: Only wild yeasts	Differentiated conventional wine	Differentiated organic wine

In order to achieve the study aim, the following tasks were established:

1. Characterization of the impact triggered by the shift from integrated to organic production, concerning wine fermentations' microbiome.

To allow comparative and statistical analysis of the viticultural changes impact on grape must and wine, an experimental field on the studied vineyards was implemented. In this field, Touriga Nacional and Aragonez grapevine varieties were simultaneously produced under Organic and Integrated Production. Grapes, must and wine samples were collected, preserved and used for microbiological, molecular e metagenomic approaches.

2. Evaluation of the indigenous microbial consortium ability to impose against commercial yeasts, being responsible for wine fermentation.

The fermentation process had occurred in a pilot unit installed in an isolated area of the cellar. This unit were constituted by eight regulated temperature fermentation vats, allowing the comparison between inoculated and uninoculated fermentations.

In order to evaluate the indigenous microbial consortium ability to impose against the commercial yeast used, the interdelta profile from each one of the eight produced wines was determined. Each profile was compared with the interdelta pattern of the commercial yeas in order to find out the yeast type responsible for the fermentation (indigenous or commercial).

3. Yeast identification.

Through the application of culture-dependent methods and sequencing, the cultivable yeasts present on initial must, middle fermentation and end fermentation were isolated and identified. The obtained results were analyzed in combination with the ones obtained through metagenomic analysis.

The current study was performed in the Herdade da Malhadinha Nova (located in Beja, Portugal) and in UALG – CBMR in the frame of the project CRESC I&DT, Nº 17987 – "MicroBioWines", which received funding through the European Regional Development Fund (FEDER) through the Operational Program CRESC Algarve2020, of Portugal.

2. Methods

2 Methods

2.1 Equipment

- AE200 Analytical Balance – Mettler Toledo©
- Analog Incubator – Raypa
- Bio48 Biological Laminar Flow Cabinet – Faster, S.r.l.
- BTG Shaking Thermostatic Bath – Bunsen, S.A.
- Bullet Blender Storm 24 – Next Advance, Inc.
- Centrifuge 5810R – Eppendorf
- Electrophoresis Power Supply EPS 301 – Amersham Biosciences Corp.
- Grant Bio Combi-spin PCV-2400 – Grant Instruments™
- Kodak DC290 Digital Camera – Kodak
- Kodak EDAS 290 Analysis System – Kodak
- Masticator Homogenizator Classic, IULmicro
- Mini-V/PCR Compact Vertical Laminar Flow Bench – Telstar
- pH-Meter GLP 21 – Crison Instruments
- T-Personal Thermal Cycler – Biometra
- Ultraviolet Transilluminators – Uvitec Ltd.
- XS-410 Analytical Balance – Fisher Scientific, Inc.

2.2 Material and Solutions

- 1 kb DNA Ladder – SIGMA-ALDRICH
- 4you4 dNTP Mix, 10 mM each – Bioron
- AccuGENE Molecular Biology Water – Lonza
- Acetic Acid Glacial – PanReac AppliChem
- Bacteriological Agar Type E – Biokar Diagnostics
- δ12 primer (5´-TCAACAATGGAATCCCAAC-3´) – Sigma-Aldrich
- δ2 primer (5´-GTGGATTTTTATTCCAAC-3´) – Sigma-Aldrich
- Dextrose – Difco

- Ethanol, absolute – Fisher Scientific, Inc.
- Ethylenediaminetetraacetic acid disodium salt dihydrate, 99% – Sigma-Aldrich
- General Purpose Agarose – Lonza
- Glass Beads 0.50 to 0.75 mm – Retsch
- Glycerine ≥99%, Electran Molecular biology grade – VWR Chemicals
- GoTaq G2 Flexi DNA Polymerase – PROMEGA
- GreenSafe Premium – NZYTECH
- ITS1 primer (5´-TCCGTAGGTGAACCTGCGG-3´) – Integrated DNA Technologies©
- ITS4 primer (5´-TCCTCCGCTTATTGATATGC-3´) – Integrated DNA Technologies©
- Loading Buffer 10X – Bioron
- Lyticase 10 U/µl – A&A Biotecnology©
- Peptone water (buffered) – Merck
- Polyvinylpyrrolidone, Molecular Biology Grade – Calbiochem
- Propan-2-ol – VWR Chemicals
- Sodium Chloride – PanReac AppliChem
- Sterile gauze swabs 10 cm x 10 cm – Bastos Viegas, S.A.
- TrackIT™ 100 bp DNA Ladder – Invitrogen™
- Trizma base – Sigma-Aldrich
- Yeast Extract – Biokar Diagnostics.

2.3 Commercial Kits

- DNeasy Plant Mini Kit – Qiagen
- Wizard Genomic DNA Purification Kit – Promega
- Wizard SV Gel and PCR Clean-Up System – Promega

2.4 Sampling

2.4.1 Grape varieties

For this study two red grape varieties were selected: Aragonez (AG) and Touriga Nacional (TN). Aragonez is an Iberian red grape variety, which prefers a soil with low water availability and a dry hot climate, characteristics that lead to complex and aromatically intense wines (Instituto da Vinha e do Vinho 2018a).

Touriga Nacional is an autochthonous grape variety with a high adaptive capacity to most soil types, requiring high insolation and heat. Touriga Nacional wines are known as complex and of very high quality (Instituto da Vinha e do Vinho 2018b).

Regarding the constitution of its bunches and leaves, each grape variety exhibit different features (**Figure 2.1**). Aragonez grapes usually have a large leaf with five-lobe pentagonal limb, a medium cylindrical-conical cluster and a strong skin small berry (Infovini 2019b). Touriga Nacional grapes can present a medium to small very heterogeneous leaf, a small and compact cluster, and a medium berry with thick skin (Infovini 2019a).

Figure 2.1 – Aragonez and Touriga Nacional grapes and leaves (Image source: © 2019 Infovini).

2.4.2 Grape production

Both studied grapevine varieties were planted in 2006 and produced since then under integrated production in a 2.04 hectares vineyard at Herdade da Malhadinha Nova, located in Beja (Portugal) (**Figure 2.2**).

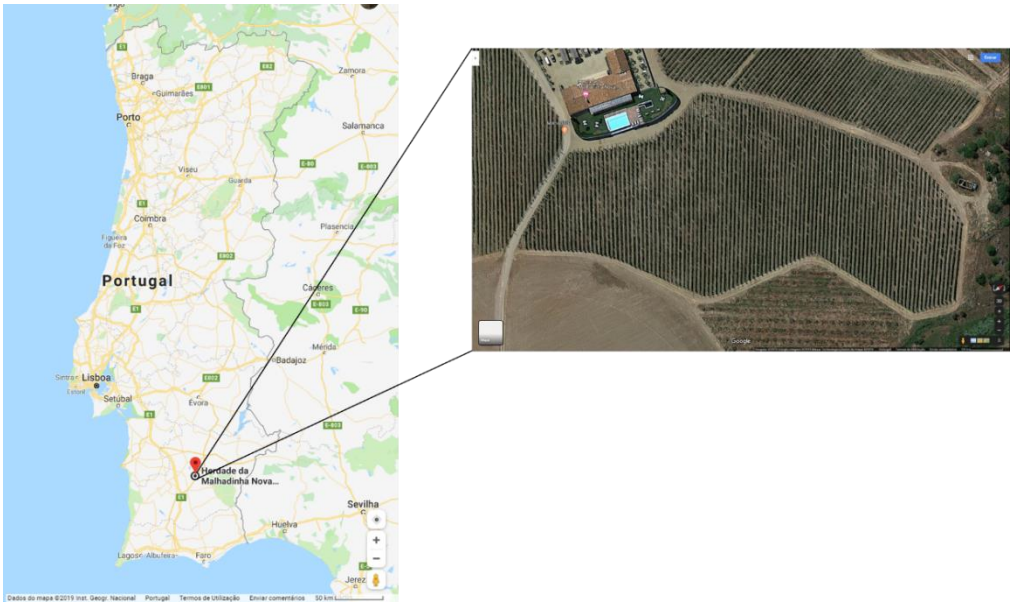

Figure 2.2 – Map of mainland Portugal with a highlight for Herdade da Malhadinha Nova (Beja, Portugal). Satellite photo of the 2.04 hectares studied vineyard. Image Source: ©2019 Google Images. Screenshot by author.

Following 2016 vintage, an experimental field was established on this vineyard with the objective of producing both grapevine varieties through integrated and organic production, simultaneously. Therefore, the two grapevine varieties located in the periphery of the experimental field started to be produced under organic mode, in compliance with all the standards set forth in Regulation (CE) No. 834/2007 of the Council of June 28, 2007 (**Figure 2.3**).

The experimental field was equally divided relatively to grapevine varieties and production modes, and, in order to prevent potential contaminations from other production areas, it was bounded to its full length by a dirt road.

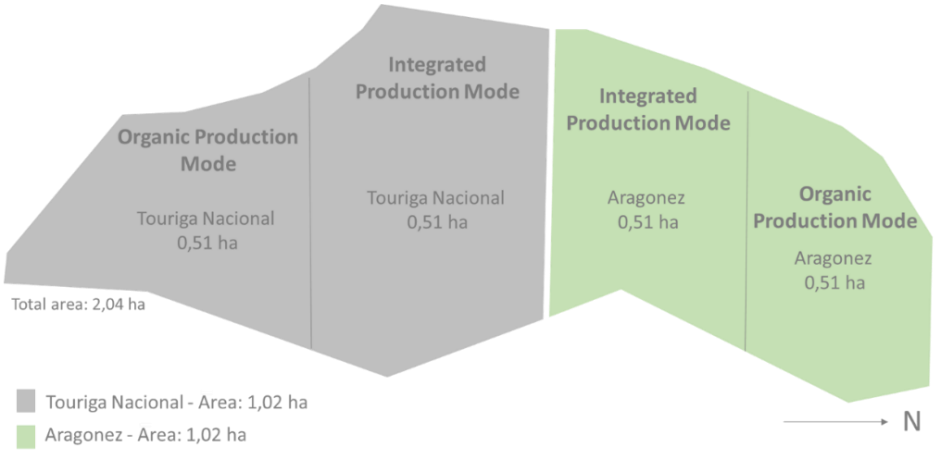

Figure 2.3 – Scheme of the experimental field established in Herdade da Malhadinha Nova (Beja, Portugal). ha – hectare; N – North.

2.4.3 Must fermentation

The studied grapes were harvested at 2017 vintage and fermented under aseptic and controlled conditions in the pilot unit, placed in an isolated area of Herdade da Malhadinha Nova cellar (**Figure 2.4**).

Figure 2.4 – Pilot unit placed in Herdade da Malhadinha Nova cellar, composed by eight fermentation vats (four in the front and four in the back).

In order to achieve the objectives of this study, each grape variety was fermented in four fermentation vats with 1000 kg capacity and controlled temperature: one with integrated production grape must, inoculated with the commercial yeast VQ51 (Enartis); another with integrated production grape must not inoculated; other with organic production grape must inoculated with the same commercial yeast (VQ51); and another one with organic production grape must not inoculated (**Figure 2.5**).

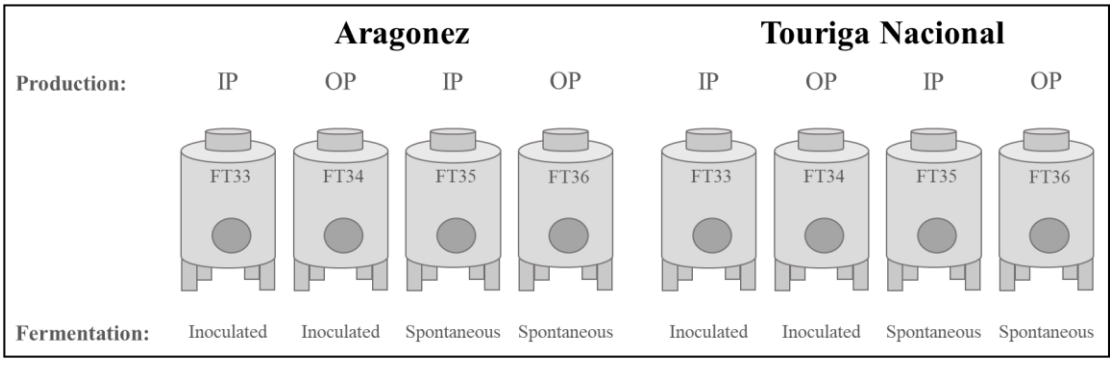

Figure 2.5 – Scheme of the eight fermentation vats placed at Herdade da Malhadinha Nova pilot unit. FT – Fermentation; FT13 – Integrated Production/Inoculated fermentation; FT14 – Organic Production/Inoculated fermentation; FT15 – Integrated Production/Spontaneous fermentation; FT16 – Organic Production/Spontaneous fermentation; FT33 – Integrated Production/Inoculated fermentation; FT34 – Organic Production/Inoculated fermentation; FT35 – Integrated Production/Spontaneous fermentation; FT36 – Organic Production/Spontaneous fermentation.

All the performed procedures until the samples reception on the laboratory were conducted by the Herdade da Malhadinha Nova enology technical team. The characterization of the four fermentations of each grape variety is shown in **Tables 2.1** and **2.2**.

Table 2.1 Characterization of Aragonez fermentations for the year 2017, according to production mode (integrated/organic) and fermentation process (inoculated/spontaneous).

Grape variety	Fermentation identification	Production mode	Fermentation process
Aragonez	FT13	Integrated	Inoculated (VQ51)
	FT14	Organic	Inoculated (VQ51)
	FT15	Integrated	Spontaneous
	FT16	Organic	Spontaneous

FT – Fermentation; FT13 – Integrated Production/Inoculated fermentation; FT14 – Organic Production/Inoculated fermentation; FT15 – Integrated Production/Spontaneous fermentation; FT16 – Organic Production/Spontaneous fermentation; VQ51 – Commercial yeast used on inoculated fermentations.

Table 2.2 Characterization of Touriga Nacional fermentations for the year 2017, according to production mode (integrated/organic) and fermentation process (inoculated/spontaneous).

Grape variety	Fermentation identification	Production mode	Fermentation process
Touriga Nacional	FT33	Integrated	Inoculated (VQ51)
	FT34	Organic	Inoculated (VQ51)
	FT35	Integrated	Spontaneous
	FT36	Organic	Spontaneous

FT – Fermentation; FT33 – Integrated Production/Inoculated fermentation; FT34 – Organic Production/Inoculated fermentation; FT35 – Integrated Production/Spontaneous fermentation; FT36 – Organic Production/Spontaneous fermentation; VQ51 – Commercial yeast used on inoculated fermentations.

Aragonez and Touriga Nacional musts pH and total acidity values are indicated in **Table 2.3** and **2.4**.

Table 2.3 Characterization of Aragonez musts for the year 2017, according to pH and total acidity values.

Grape variety	Fermentation identification	pH	Total acidity
Aragonez	FT13	3.84	3.75
	FT14	3.86	3.97
	FT15	3.83	4.70
	FT16	3.86	4.35

FT – Fermentation; FT13 – Integrated Production/Inoculated fermentation; FT14 – Organic Production/Inoculated fermentation; FT15 – Integrated Production/Spontaneous fermentation; FT16 – Organic Production/Spontaneous fermentation; VQ51 – Commercial yeast used on inoculated fermentations.

Table 2.4 Characterization of Touriga Nacional musts for the year 2017, according to pH and total acidity values.

Grape variety	Fermentation identification	pH	Total acidity
Touriga Nacional	FT33	3,90	6.22
	FT34	3.98	5.47
	FT35	3.87	7.28
	FT36	3.99	5.02

FT – Fermentation; FT33 – Integrated Production/Inoculated fermentation; FT34 – Organic Production/Inoculated fermentation; FT35 – Integrated Production/Spontaneous fermentation; FT36 – Organic Production/Spontaneous fermentation; VQ51 – Commercial yeast used on inoculated fermentations.

The fermentation progress (density determination) of each must was daily monitored by Herdade da Malhadinha Nova technical team of oenology (**Figure 2.6** and **2.7**).

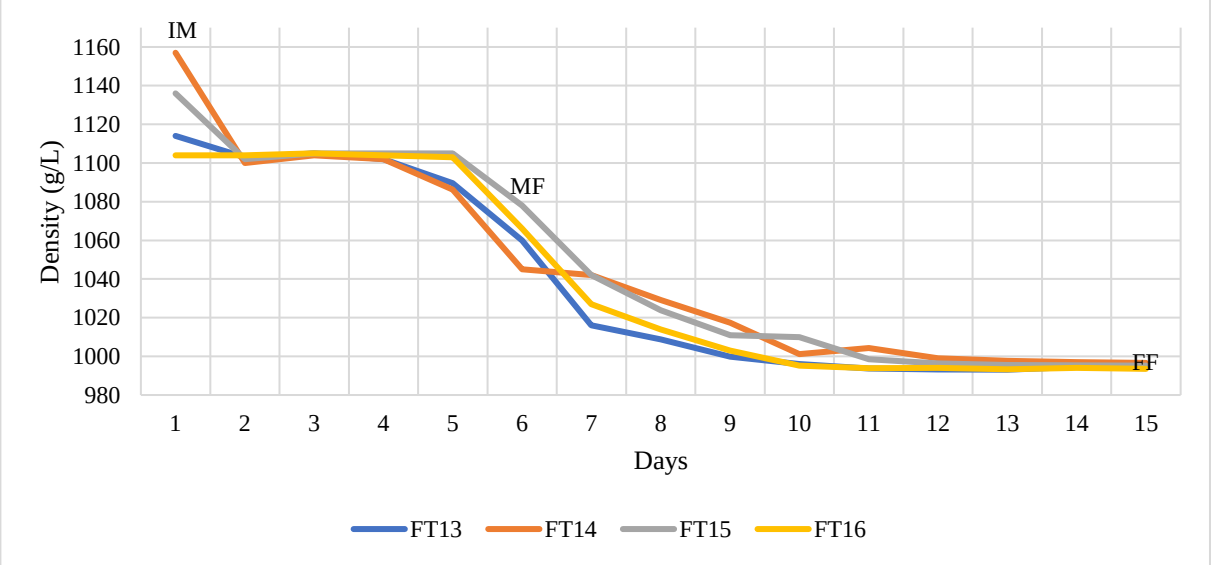

Figure 2.6 – **Evolution of the density (g/L) of Aragonez fermentations for the year 2017, from Initial Must to End of Fermentation.** IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; FT – Fermentation; FT13 – Integrated Production/Inoculated fermentation; FT14 – Organic Production/Inoculated fermentation; FT15 – Integrated Production/Spontaneous fermentation; FT16 – Organic Production/Spontaneous fermentation.

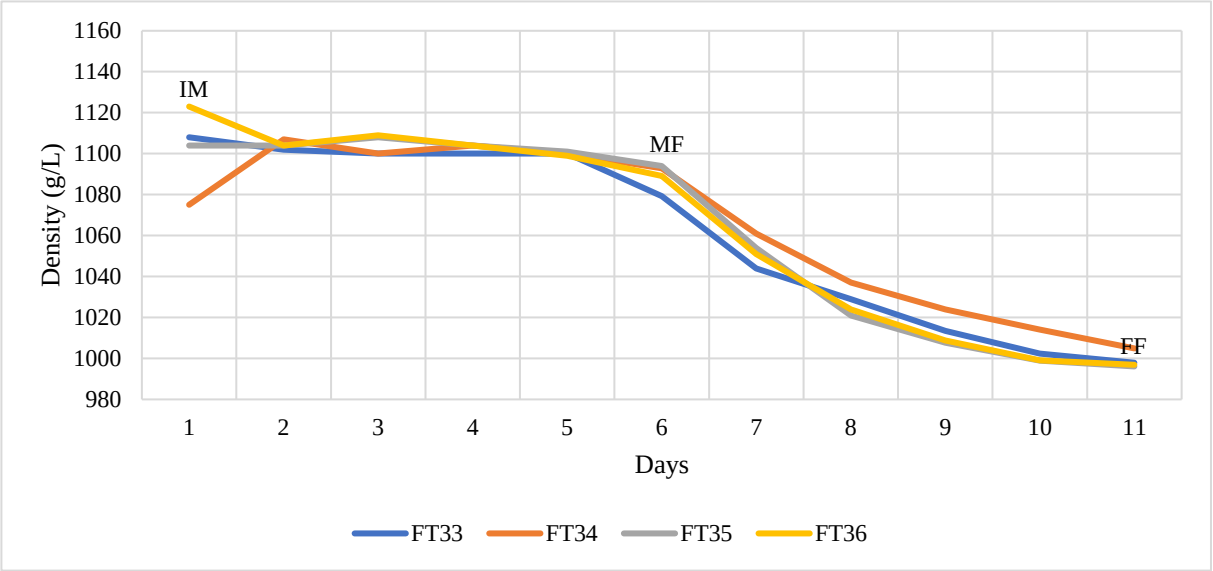

Figure 2.7 – Evolution of the density (g/L) of Touriga Nacional fermentations for the year 2017, from Initial Must to End of Fermentation. FT – Fermentation; FT33 – Integrated Production/Inoculated fermentation; FT34 – Organic Production/Inoculated fermentation; FT35 – Integrated Production/Spontaneous fermentation; FT36 – Organic Production/Spontaneous fermentation.

2.4.4 2017 vintage

The studied samples OF WHAT? were produced and collected during the 2017 vintage, which have occurred later in comparison to previous years, due to the meteorologically atypical year of production.

For the mainland Portugal, 2016 fall was classified as hot and dry, and even though the 2017 winter had registered normal temperatures for the season, it was also dry (Instituto Português do Mar e da Atmosfera 2016, 2017h). During the very hot and very dry spring of 2017, Beja district was affected by a period of mild to moderate drought, during which two heat waves have occurred (Instituto Português do Mar e da Atmosfera 2017i, 2017f, 2017b, 2017e, 2017d). The 2017 summer was considered as hot and extremely dry, especially in Beja, where the period of extreme to moderate drought lasted up until october (Instituto Português do Mar e da Atmosfera 2017j, 2017a, 2017c, 2017k, 2017g).

2.4.5 Sample collection

The sampling process has occurred at three stages of alcoholic fermentation: initial must (IM), middle of alcoholic fermentation (MF) and end of alcoholic fermentation (EF).

Initial must samples correspond to the harvested grapes immediately sent to the laboratory, and the Middle and End of Fermentation samples correspond to the must and wine obtained directly from the fermentation vats.

Grape sampling was composed by 2 kg of Aragonez and Touriga Nacional grapes, aseptically collected into sterile plastic sampling bags, from different branches of different vines, randomly distributed throughout the experimental field, in triplicate. Only healthy undamaged grapes were picked and sent to laboratory, under controlled cooling conditions (± 4°C). At laboratory, all the samples were processed (as resumed in section 2.4.6), resulting in 6 Falcons of 50 mL for yeast counts and isolation and 6 Falcons of 50 mL for metagenomic analysis, for each grape variety.

Middle and End of Fermentation samples were directly transferred from the fermentation vats to two sterile 50 mL falcons (50 mL for metagenomic analysis and 50 mL for yeast counts and isolation), in triplicate. All the samples were immediately transported to laboratory under controlled cooling conditions (± 4°C).

In total, 120 samples were collected: 24 Initial Must samples, 48 Middle of Fermentation samples and 48 End of Fermentation samples.

2.4.6 Sample processing

At the laboratory, the harvested grapes were manually and mechanically crushed (Masticator Homogenizator Classic, IULmicro) and incubated during 20 min at room temperature. The resulting liquid – initial must – was filtered through a sterile gauze swab into two Falcons of 50 mL (50 mL for yeast counts and isolation and 50 mL for metagenomic analysis).

All collected tubes from the three sampling points for yeast counts and isolation were centrifuged (3200 x *g* for 10 min at 4°C) and the supernatant was discarded. The pellet was resuspended in liquid Yeast Extract Peptone Dextrose (YEPD – Yeast Extract, 1% w/v; Peptone, 2% w/v; Dextrose, 2% w/v) and glycerol (80%, v/v) and stored at –80°C until further processing.

All the tubes collected from the three sampling points for metagenomic analysis were centrifuged (3200 x *g* for 10 min at 4°C), the supernatant was discarded, and the pellet was washed with 50 mL of NaCl (0.9%, v/v). The tubes were re-centrifuged (3200 x *g* for 10 min at 4°C) and the supernatant was discarded. The resulting pellet was resuspended in Tris-EDTA 1X (TE) and glycerol (80%, v/v) and stored at –80°C until further processing.

2.5 Metagenomic analysis

2.5.1 DNA Extraction of Grape Musts and Wine

The DNA used for metagenomic analysis was extracted directly from must and wine samples (corresponding to Initial Must, Middle and End of Fermentation samples previously processed and stored at –80°C). This procedure was performed using the DNeasy Plant Mini Kit (Qiagen), through an optimization of the manufacturer's instructions. Firstly, 200 µL of sample were added to a tube with 200 µL of glass beads, 400 µL of preheated Buffer AP1 and 40 µL of a 10% polyvinylpyrrolidone solution. Then, 3 lysis cycles of 1 min were performed in the Bullet Blender Storm (Next Advance) at maximum speed. The tubes sit on ice between each cycle, for 2 min. The tubes were centrifuged (5,000 x *g* for 10 min at 4°C), the supernatant was carefully transferred to a clean tube and the tubes were washed with 300 µL of Buffer AP1. Afterwards, 2 more lysis cycles were completed (at maximum speed for 1 min) and a final cycle were performed at maximum speed for 2 min. The tubes sit on ice between each cycle, for 2 min. The tubes were centrifuged (5,000 x *g* for 10 min at 4°C), the supernatant was carefully added to the previously collected. Therein, 4 µL of 100 mg/mL RNase A were added. The tubes were incubated at 65 °C for 10 min. Hereafter, 130 µL of Buffer P3 were added and mixed by vortex, and incubated on ice for 5 min. The lysates were centrifuged (20,000 x *g* for 5 min at 4°C) and the supernatant was collected. After being centrifuged (5,000 x *g* for 5 min at 4°C), the supernatant was transferred into a QIAshredder spin column, that were centrifuged (5,000 x *g* for 2 min at 4°C). The flow through were transferred to 2 mL tubes with 1.5 volumes of Buffer AW1. Fractions of 650 µL of the resulting mixture were transferred into DNeasy Mini spin column, that were centrifuged (6,000 x *g* for 1 min at 4°C). When all the mixture had passed through the column, this one was placed in a new collection tube a washed with 500 µL of Buffer AW2. After being centrifuged (6,000 x *g* for 1 min at 4°C) the column was re-washed with 500 µL of Buffer AW2 and re-centrifuged (20,000 x *g* for 2 min at 4°C). The flow through was discarded. The column was re-centrifuged (20,000 x *g* for 1 min at 4°C) and transferred to a new 1.5 mL tube. Then, 50 µL of Buffer AE were added directly to the column membrane, which were incubated (for 5 min at room temperature) and centrifuged (10,000 x *g* for 1 min at 4°C). The column was discarded, and the DNA was stored at – 20°C.

2.5.2 rDNA Library Construction and sequencing

Samples were prepared for Illumina Sequencing using the *16S rRNA* gene and the Internal Transcribed Spacer 2 region amplification of the microbial communities. The DNA was amplified for the hypervariable regions with specific primers and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes. First PCR reactions were performed for each sample using KAPA HiFi HotStart PCR Kit according to manufacturer suggestions, 0.3 μM of each PCR primer: forward primer Bakt_341F 5'– CCTACGGGNGGCWGCAG-3' and reverse primer Bakt_805R 5'– GACTACHVGGGTATCTAATCC-3' for bacteria and a pool of forward primers: ITS3NGS1_F 5'-CATCGATGAAGAACGCAG-3', ITS3NGS2_F 5'-CAACGATGAAGAACGCAG-3', ITS3NGS3_F 5'CACCGATGAAGAACGCAG-3', ITS3NGS4_F 5'-CATCGATGAAGAACGTAG-3', ITS3NGS5_F 5'-CATCGATGAAGAACGTGG-3', and ITS3NGS10_F 5'CATCGATGAAGAACGCTG-3' and reverse primer ITS4NGS001_R 5'TCCTSCGCTTATTGATATGC-3' for fungi and 12.5 ng of template DNA in a total volume of 25 μL (Herlemann et al. 2011)Klindworth *et al*. 2013; Tedersoo *et al*. 2014). The PCR conditions involved a 3 min denaturation at 95 ºC, followed by 30 cycles (bacterial region)/25 cycles (fungal region) of 98 ºC for 20 s, 55 ºC (bacterial region)/60 ºC (fungal region) for 30 s and 72 ºC for 30 s and a final extension at 72 ºC for 5 min. Second PCR reactions added indexes and sequencing adapters to both ends of the amplified target region according to the manufacturer's recommendations (Illumina, 2003). Negative PCR controls were included for all amplification procedures. PCR products were then one-step purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific), pooled and pair-end sequenced in the Illumina MiSeq sequencer with the V3 chemistry, according to manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal) (Comeau, Douglas, and Langille 2017). Sequence data was processed at Genoinseq (Cantanhede, Portugal). Raw reads were extracted from Illumina MiSeq System in fastq format and quality-filtered with PRINSEQ version 0.20.4 to remove sequencing adapters, reads with less than 100 bases for samples targeting ITS region and 150 for samples targeting 16S rRNA gene and trim bases with an average quality lower than Q25 in a window of 5 bases (Schmieder and Edwards 2011). The forward and reverse reads were merged by overlapping paired-end reads with AdapterRemoval version 2.1.5 using default parameters (Schubert, Lindgreen, and Orlando 2016). The QIIME package version 1.8.0 was used for Operational Taxonomic Unit (OTU) generation, taxonomic identification, sample

diversity and richness indices calculation(Caporaso *et al*. 2010). Sample IDs were assigned to the merged reads and converted to fasta format. Chimeric merged reads were detected and removed using UCHIME against Greengenes database version 13.8 for samples targeting 16S rRNA gene and UNITE/QIIME ITS database version 12.11 for samples targeting ITS region. ITSx version 1.0.11 was used on samples targeting the ITS region to extract the highly variable fungal ITS2 subregion from the merged reads (Edgar et al. 2011) DeSantis *et al*. 2006; Abarenkov *et al*. 2010; Bengtsson-Palme *et al*. 2013). OTUs were selected at 97% similarity threshold using the open reference strategy. Merged reads were pre-filtered by removing sequences with a similarity lower than 60% against Greengenes database version 13.8 for samples targeting *16S rRNA* gene and UNITE version 7.1 for samples targeting *16S rRNA* gene and the remaining merged reads were then clustered at 97% similarity against the same database (DeSantis et al. 2006) Kõljalg *et al*. 2013). Merged reads that did not cluster in the previous step were *de novo* clustered into OTUs at 97% similarity. OTUs with less than two reads were removed from the OTU table. A representative sequence of each OTU was then selected for taxonomy assignment.

2.6 Yeast identification

2.6.1 Isolation of Yeasts

Initial Must, Middle and End of Fermentation samples previously processed and stored for yeast isolation, were subjected to successive decimal dilutions (from 10^{-1} to 10^{-6}) with NaCl (0.9%, v/v). On duplicate, 100 µL of each dilution was spread on YEPD plates (Yeast Extract, 1% w/v; Peptone, 2% w/v; Dextrose, 2% w/v; Agar, 2% w/v) and incubated at 30°C for 48 h. Colony-forming units (CFU/mL) were calculated from plates with 30-300 colonies.

In order to achieve its molecular identification, up to six colonies sharing the same morphologic characteristics were randomly selected and isolated by streaking on YEPD plates.

A total of 263 isolates were cryopreserved in liquid YEPD and glycerol (80%, v/v) at –80°C until further processing.

2.6.2 DNA Extraction of Yeasts

To achieve the molecular identification of the isolated yeasts, its DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's instructions. Initially, each isolate has grown for 20 h in liquid YEPD (at 30°C with 185 rpm agitation). Then, 1 mL of the grown culture was centrifuged (16000 x *g* for 2 min) and the supernatant was discarded. The cell pellet was resuspended in 293 µL of EDTA (50 mM pH 8.0) and 7.5 µL lyticase (5 units/µL).

After 60 min of incubation at 37°C, the tubes were cooled to room temperature. Subsequently, the tubes were centrifuged (16000 x *g* for 2 min), the supernatant was removed and 300 µL of Nuclei Lysis Solution were gently added. Then, 100 µL of Protein Precipitation Solution were added, followed by a vigorous vortex at maximum speed for 20 s.

After 5 min on ice, the tubes were centrifuged (16000 x *g* for 3 min) and the supernatant was carefully transferred to a clean 1.5 mL tube containing 300 µL of room temperature isopropanol. The tubes were gently mixed by inversion until the thread-like strands of DNA were visible. The tubes were centrifuged (16000 x *g* for 2 min), the supernatant was carefully decanted, and the tubes were drained on absorbent paper.

Therefore, 300 µL of room temperature 70% ethanol were added, followed by a gentle DNA wash by tube inversion. After another centrifugation (16000 x *g* for 2 min) all the ethanol was carefully aspirated, the tubes were drained, and the pellet was air-dried during 15 min. To finalize, 50 µL of DNA Rehydration Solution and 1.5 µL of RNase Solution were added. The tubes were vortexed for 1 s, briefly centrifuged for 5 s and incubated at 37°C for 15 min.

The DNA was rehydrated overnight at 4°C and its concentration and purity were quantified by measuring its absorbance at 260 nm and 280 nm, with NanoDrop 2000/c (Thermo Fisher Scientific). The DNA was stored –20ºC.

2.6.3 Amplification of ITS region

The amplification of ITS region was performed using a pair of ITS primers: ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) (Integrated DNA Technologies)(Mathabatha E. Setati, Jacobson, and Bauer 2015). For each 100 ng of DNA, a 50 µL reaction mixture was prepared with 5 µL of 5x Colourless GoTaq Flexi Buffer

(Promega), 4 μL of 25 mM $MgCl_2$, 0,5 μL 10 mM dNTPs, 1 μL of primer ITS1 at 10 pmol/μL, 1 μL of primer ITS4 at 10 pmol/μL, 0.125 μL of GoTaq G2 Flexi DNA polymerase (Promega) and Molecular Biology Water (Lonza) up to 50 μL. The PCR amplification conditions consisted of a denaturation cycle of 95°C for 6 min, 35 cycles of annealing (94°C for 40 s, 53°C for 40 s, 72°C for 1 min) and an extension cycle of 72°C for 5 min. A total of 2 μL of each PCR product were separated by electrophoresis on a 1% w/v agarose gel stained with Greensafe Premium (NZYtech) and visualized using the Kodak DC290 camera (Kodak) and the Kodak 1D software (Kodak).

2.6.4 Purification of PCR products and sequencing of ITS region

The remaining 48 μL of each PCR product already amplified for ITS region, were purified using Wizard SV Gel and PCR Clean-Up System kit (Promega), according to the manufacturer's instructions.

Briefly, 48 μL of Membrane Binding Solution were added to the PCR product. The mixture was transferred into the Minicolumn assembly and incubated at room temperature for 1 min. After being centrifuged (16,000 x *g* for 1 min at 4°C) the Minicolumn flowthrough was discarded. The Minicolumn was placed into a new collection tube and 700 μL of Membrane Wash Solution were added. The tubes were centrifuged, the flowthrough discarded and the membrane was washed one more time with 500 μL of Membrane Wash Solution. The Minicolumn were centrifuged (16,000 x *g* for 5 min at 4°C), the flowthrough discarded and the centrifugation repeated for 1 min (with the microcentrifuge lid off). The Minicolumn was prudently transferred to a new 1.5 mL tube and 30 μL of Nuclease-Free Water were added. After 1 min of incubation at room temperature, the tubes were centrifuged (16,000 x *g* for 1 min at 4°C) and the Minicolumn discarded.

The purified PCR products DNA concentration were quantified by measurement of its absorbance at 260 nm and 280 nm, with NanoDrop 2000/c (Thermo Fisher Scientific) and stored at –20°C.

2.6.5 Sequencing of ITS region

The sequencing process was performed at CCMAR Technologies and Services Platform (Centro de Ciências do Mar, Faro, Portugal). The sequencing process occurred in a 3130xl Genetic Analyzer (Applied Biosystems) using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), the POP7™ Polymer (Applied Biosystems) and the pair of ITS primers: ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3')

(Integrated DNA Technologies)(Mathabatha E. Setati, Jacobson, and Bauer 2015). The DNA sequences were assessed through BioEdit Sequence Alignment Editor V7.0.5.3 (Tom Hall) and the isolates identification was achieved using the NCBI/GenBank nucleotide blast (BLASTn), by finding the closest match (based on the maximum identity percentage and query cover and along with the lower e-value).

2.7 Interdelta sequences amplification and analysis

The amplification of the interdelta sequences was carried out in the eight fermentations under study (at End of Fermentation), as well as in the commercial yeast used (VQ51), using a pair of δ primers: δ12 (5´-TCAACAATGGAATCCCAAC-3´) and δ2 (5´-GTGGATTTTTATTCCAAC-3´) (Franco-Duarte *et al*. 2011). The DNA extraction of the fermentations was carried out through DNeasy® Plant Mini Kit (Qiagen) and the DNA of the commercial yeast was isolated using Wizard® Genomic DNA Purification Kit (Promega) – through the procedures already reported above (sections 2.5.1 and 2.6.2). For each 100 ng of DNA, a 25 µL reaction mixture was prepared with 5 µL of 5x Colourless GoTaq Flexi Buffer (Promega), 4 µL of 25 mM $MgCl_2$, 0.5 µL 10 mM dNTPs, 4.2 µL of primer δ12 at 10 pmol/µL (Sigma-Aldrich), 4.2 µL of primer δ2 at 10 pmol/µL (Sigma-Aldrich), 0.125 µL of GoTaq G2 Flexi DNA polymerase (Promega) and Molecular Biology Water (Lonza) up to 25 µL. The positive control was the commercial yeast used on the inoculation of the fermentations on study (VQ51 – ENARTIS). PCR amplification conditions consisted of a denaturation cycle of 95°C for 2 min, 35 cycles of annealing (95°C for 30s, 43.2°C for 1min, 72°C for 1min) and an extension cycle of 72°C for 10 min. PCR products were separated by electrophoresis on a 2.3% w/v agarose gel stained with Greensafe Premium (NZYTECH) and visualized using the Kodak DC290 camera (KODAK) and the Kodak 1D software (KODAK).

3. Results

3 Results

3.1 Metagenomic Analysis

Aragonez and Touriga Nacional Initial Must, Middle and End of Fermentation samples for each production mode (integrated and organic) and fermentation process (commercial and spontaneous) were prepared for Illumina Sequencing of Internal Transcribed Spacer 2 region and 16S rRNA (V3V4 region), for fungal and bacterial targets, respectively. For Aragonez samples, the sequencing generated a total of 4.675.544 sequences for ITS2 and V3V4 region collected at the three sampling points, from which 84.6% passed the quality control parameters (**Table 3.1**).

Table 3.1 – Total sequences obtained for eukaryotic (ITS2) and prokaryotic (V3V4) microbial community for Initial Must, Middle and End of Fermentation of Aragonez vintage 2017. IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; AGIP – Aragonez Integrated Production; AGOP – Aragonez Organic Production; FT – Fermentation; FT13 – Integrated Production/Inoculated fermentation; FT14 – Organic Production/Inoculated fermentation; FT15 – Integrated Production/Spontaneous fermentation; FT16 – Organic Production/Spontaneous fermentation.

Sampling point	Sample	Target region	Number of Reads	
			Total	High quality
IM	AGPI	ITS	278567	270224
		V3V4	261821	211390
	AGPB	ITS	248850	236766
		V3V4	254629	189147
MF	FT13	ITS	172001	165668
		V3V4	208649	159522
	FT14	ITS	205092	198127
		V3V4	224563	174012
	FT15	ITS	235225	215064
		V3V4	314682	249565
	FT16	ITS	255605	226044
		V3V4	185341	143763
EF	FT13	ITS	236840	211890
		V3V4	289225	213462
	FT14	ITS	158471	147672
		V3V4	199082	143478
	FT15	ITS	270544	245079
		V3V4	189898	146101
	FT16	ITS	218807	195249
		V3V4	267652	210988
		Eukaryotic	2280002	2111783
		Prokaryotic	2395542	1841428
		Total	4675544	3953211

For Touriga Nacional samples, the sequencing generated a total of 5.310.153 sequences of ITS2 and V3V4 regions collected at the three sampling points, from which 83.1% passed the quality control parameters (**Table 3.2**).

Table 3.2 – Total sequences obtained for eukaryotic (ITS2) and prokaryotic (V3V4) microbial community for Initial Must, Middle and End of Fermentation of Touriga Nacional vintage 2017. IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; TNIP – Touriga Nacional Integrated Production; TNOP – Touriga Nacional Organic Production; FT – Fermentation; FT33 – Integrated Production/Inoculated fermentation; FT34 – Organic Production/Inoculated fermentation; FT35 – Integrated Production/Spontaneous fermentation; FT36 – Organic Production/Spontaneous fermentation.

Sampling point	Sample	Target region	Number of Reads	
			Total	High quality
IM	TNPI	ITS	331346	284886
		V3V4	148816	112820
	TNPB	ITS	299244	267082
		V3V4	136142	102682
MF	FT33	ITS	178345	158433
		V3V4	136781	109334
	FT34	ITS	277164	255396
		V3V4	147604	108488
	FT35	ITS	243063	216261
		V3V4	141465	102912
	FT36	ITS	713689	647178
		V3V4	270214	197462
EF	FT33	ITS	223265	195989
		V3V4	273964	210764
	FT34	ITS	276885	229208
		V3V4	676444	537815
	FT35	ITS	256092	212072
		V3V4	162209	126071
	FT36	ITS	256286	217951
		V3V4	161135	119620
		Eukaryotic	3055379	2684456
		Prokaryotic	2254774	1727968
		Total	5310153	4412424

3.1.1 General Characterization of Eukaryotic Population

3.1.1.1 Aragonez Eukaryotic Population

To evaluate the Aragonez eukaryotic population dynamics, the relative abundance at genus level was analysed (**Figure 3.1**). About 14.2% of the sequenced population has not been identified. The entire Aragonez eukaryotic microbial population was mostly characterized by *Saccharomyces* (69.7%), *Aureobasidium* (7.7%), *Lachancea* (6%), *Alternaria* (5.5%), *Hanseniaspora* (5.2%), *Sporobolomyces* (2.5%) and *Torulaspora* (1%) genera.

At Initial Must sampling point, the entire Aragonez eukaryotic population was mostly characterized by *Aureobasidium* (38.5%), *Alternaria* (27.6%), *Sporobolomyces* (12.4%), *Saccharomyces* (10.8%), *Filobasidium* (2.4%), *Rhodotorula* (1.3%), and *Aspergillus* (1.2%) genera. Regarding the Initial Must under integrated production (AGIP), the most abundant eukaryotic genera were *Alternaria* (28.2%), *Sporobolomyces* (24%), *Saccharomyces* (16.9%), *Aureobasidium* (15.2%) *Filobasidium* (4.5%), *Rhodotorula* (2.6%), and *Aspergillus* (2.1%). For the Initial Must organically produce (AGOP), the most abundant eukaryotic genera were *Aureobasidium* (61.8%), *Alternaria* (27%) and *Saccharomyces* (4.7%).

At the Middle of Fermentation, the entire Aragonez eukaryotic population was mostly characterized by *Saccharomyces* (74.8%), *Lachancea* (11.6%), *Hanseniaspora* (10.7%) and *Torulaspora* (2.1%) genera. Concerning to the most abundant eukaryotic genera in each fermentation: FT13 presented mainly *Saccharomyces* (92.8%), *Lachancea* (4.2%) and *Hanseniaspora* (1.5%); FT14 showed predominantly *Saccharomyces* (90.4%), *Hanseniaspora* (5.3%), *Lachancea* (2.5%) and *Aspergillus* (1.2%); FT15 revealed mostly *Saccharomyces* (61.3%), *Lachancea* (27.6%), *Hanseniaspora* (7.3%) and *Torulaspora* (3.6%); FT16 displayed a population predominantly composed by *Saccharomyces* (54.9%), *Hanseniaspora* (28.7%), *Lachancea* (12.1%), and *Torulaspora* (4%).

Regarding the End of Fermentation, the Aragonez eukaryotic population was mainly characterized by *Saccharomyces* (94%), *Lachancea* (3.1%) and *Hanseniaspora* (2.1%). Concerning the most abundant eukaryotic genera in each fermentation: FT13 presented mainly *Saccharomyces* (97.8%) and *Lachancea* (1.4%); FT14 showed predominantly *Saccharomyces* (98%); FT15 revealed mostly *Saccharomyces* (91.9%) and *Hanseniaspora* (6.3%); FT16 displayed a population predominantly composed by *Saccharomyces* (88.5%) and *Lachancea* (9.6%).

Concerning the number of different eukaryotic genera identified in each production mode and fermentation process over the three sampling points: at the Initial Must yield under integrated production (AGIP) were identified 26 different eukaryotic genera; at the Initial Must organically produce (AGOP) were identified 22 different eukaryotic genera; during the Middle of Fermentation were identified, 8, 7, 6 and 5 different eukaryotic genera from FT13, FT14, FT15 and FT16, respectively; at the End of Fermentation were identified 6 different eukaryotic genera for FT13 and FT15, and 5 different eukaryotic genera for FT14 and FT16 (**Figure 3.2**).

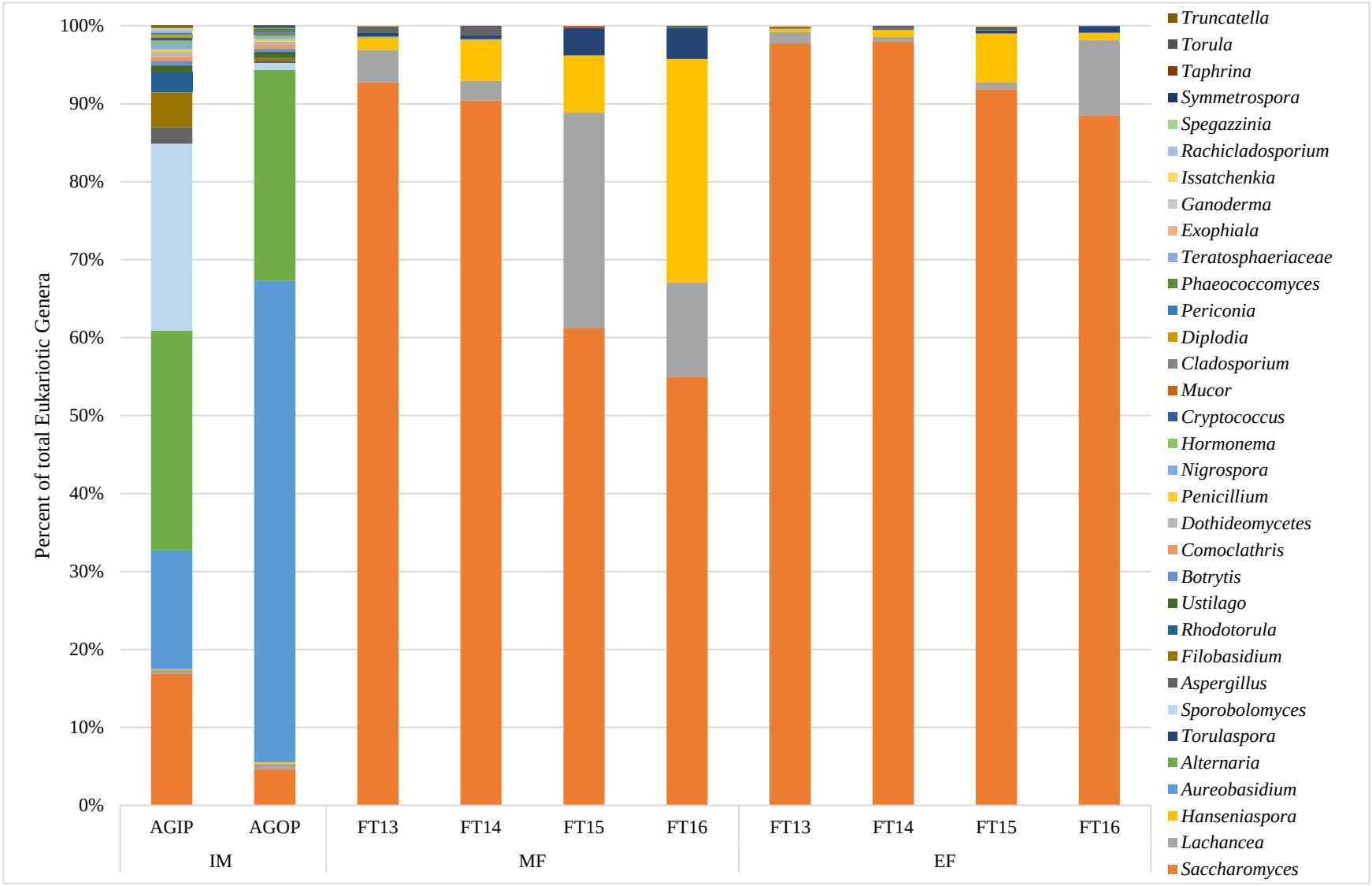

Figure 3.1 – Eukaryotic community distribution over Initial Must, Middle and End of Fermentation of Aragonez vintage 2017 at genus level. Relative abundance of the eukaryotic community through genus analysis. About 14.2% of the sequenced population has not been identified. IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; AGIP – Aragonez Integrated Production; AGOP – Aragonez Organic Production; FT – Fermentation; FT13 – Integrated Production/Inoculated fermentation; FT14 – Organic Production/Inoculated fermentation; FT15 – Integrated Production/Spontaneous fermentation; FT16 – Organic

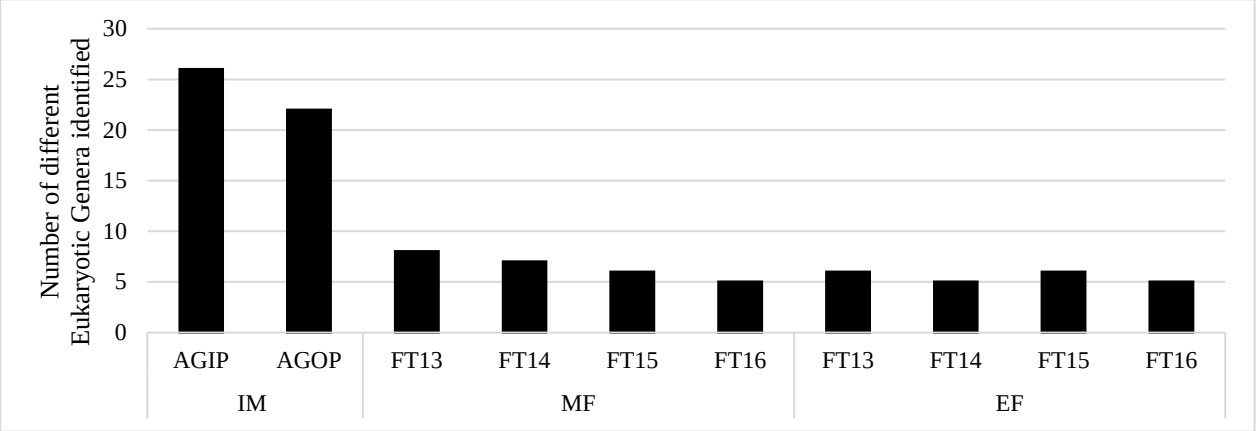

Figure 3.2 – Number of different eukaryotic genera identified over Initial Must, Middle and End of Fermentation of Aragonez vintage 2017. IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; AGIP – Aragonez Integrated Production; AGOP – Aragonez Organic Production; FT – Fermentation; FT13 – Integrated Production/Inoculated fermentation; FT14 – Organic Production/Inoculated fermentation; FT15 – Integrated Production/Spontaneous fermentation; FT16 – Organic Production/Spontaneous fermentation.

The dynamics of Aragonez eukaryotic communities present at the three sampling points (Initial Must, Middle and End of Fermentation) were explored by principal component analysis (PCA) (**Figure 3.3**).

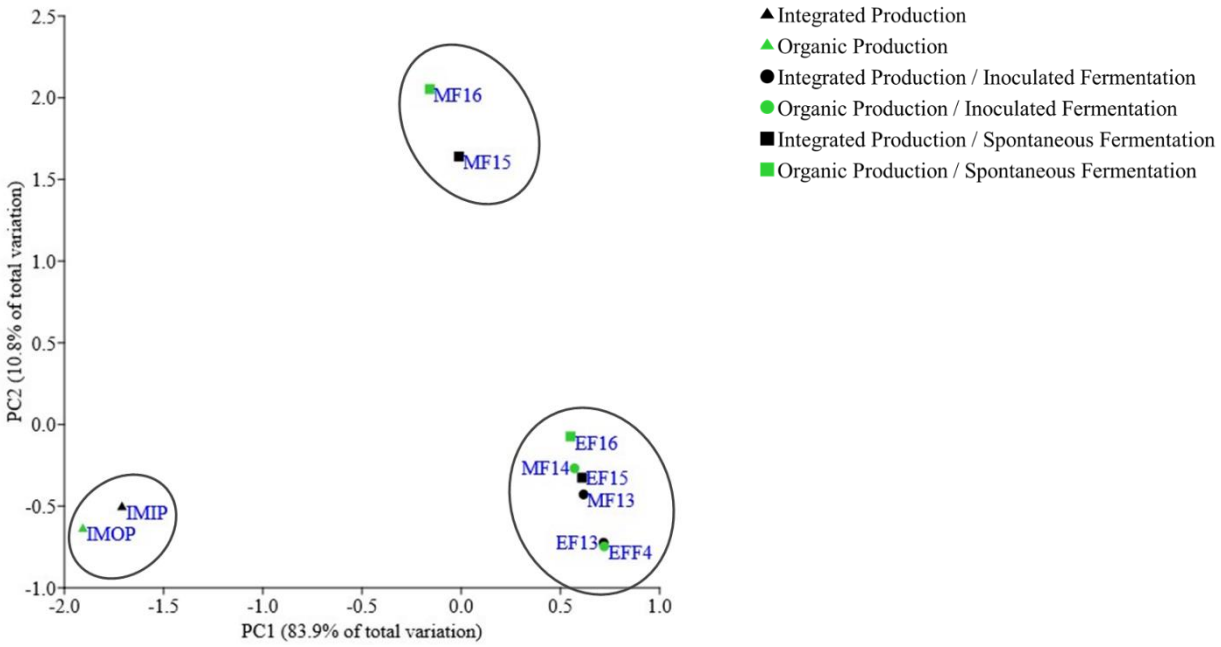

Figure 3.3 – Principal Component Analysis (PCA) biplot diagram of Aragonez eukaryotic community during the fermentation process, based on sequence abundance of eukaryotic genera. Principal component analysis (showing the first and second components) of Aragonez eukaryotic genera. IMIP – Initial Must/Integrated Production; IMOP – Initial Must/Organic Production; MF13 – Middle of Fermentation/FT13; MF14 – Middle of Fermentation/FT14; MF15 – Middle of Fermentation/FT15; MF16 – Middle of Fermentation/FT16; EF13 – End of Fermentation/FT13; EF14 – End of Fermentation/FT14; EF15 – End of Fermentation/FT15; ED16 – End of Fermentation/FT16; FT13 – Integrated Production/Inoculated fermentation; FT14 – Organic Production/Inoculated fermentation; FT15 – Integrated Production/Spontaneous fermentation; FT16 – Organic Production/Spontaneous fermentation.

Samples were grouped according to their fermentative stage (Initial Must, Middle and End of Fermentation), production mode (Integrated or Organic) and fermentation process (inoculated or spontaneous), where the x axis explains 83.9% of the total variation, and the y axis explains 10.8% of the total variation. The results show the formation of 3 distinct clusters: one composed by the initial must samples (IMIP and IMOP); another cluster constituted by the middle fermentation FT13 and FT15; the bigger cluster is formed by middle fermentation FT14 and FT16 – and by all end of fermentation samples – FT13, FT14, FT15, FT16.

3.1.1.2 Touriga Nacional Eukaryotic Population

Touriga Nacional eukaryotic population dynamics, the relative abundance at genus level was analysed (**Figure 3.4**). About 16.1% of the sequenced population has not been identified. The entire Touriga Nacional eukaryotic population was mostly characterized by *Saccharomyces* (55.3%), followed by *Hanseniaspora* (25.8%), *Aspergillus* (8.1%), *Aureobasidium* (3.7%), *Alternaria* (2.8%), *Rhizopus* (1.5%) and *Lachancea* (1.1%) genera.

At Initial Must sampling point, the Touriga Nacional eukaryotic population was mostly characterized by *Saccharomyces* (28%), *Aspergillus* (22%), *Aureobasidium* (18.4%) *Alternaria* (14.1%), *Rhizopus* (6.9%), *Hanseniaspora* (3.9%) and *Diplodia* (2.4%) genera. Regarding the Initial Must yield under integrated production (TNIP), the most abundant eukaryotic genera were *Aspergillus* (38.5%), *Aureobasidium* (20.8%), *Rhizopus* (13.7%), *Alternaria* (11.7%), *Diplodia* (4.7%), *Hanseniaspora* (4.6%) and *Saccharomyces* (3%). For the Initial Must organically produce (TNOP), the most abundant eukaryotic genera were *Saccharomyces* (53%), *Alternaria* (16.4%) *Aureobasidium* (16.1%), *Aspergillus* (5.5%), *Hanseniaspora* (3.2%) and *Sporobolomyces* (1.2%).

At Middle of Fermentation sampling point, the Touriga Nacional eukaryotic population was mostly characterized by *Saccharomyces* (54.4%), *Hanseniaspora* (36.8%), *Aspergillus* (6.4%) and *Lachancea* (1.4%) genera. Concerning the most abundant eukaryotic genera in each fermentation: FT33 showed mainly *Saccharomyces* (62.8%), *Hanseniaspora* (31.9%), *Aspergillus* (3.7%) and *Lachancea* (1.1%); FT34 showed essentially *Saccharomyces* (61.2%), *Hanseniaspora* (34.8%) and *Aspergillus* (3.3%); FT35 revealed mostly *Saccharomyces* (52.6%), *Hanseniaspora* (37.9%),

Aspergillus (6%), and *Lachancea* (2%); FT36 displayed a population predominantly composed by *Hanseniaspora* (42.7%), *Saccharomyces* (40.8%), *Aspergillus* (12.6%), and *Lachancea* (2%).

At End of Fermentation sampling point, the entire Touriga Nacional eukaryotic population was mostly characterized by *Saccharomyces* (69.8%), *Hanseniaspora* (25.7%), *Aspergillus* (2.8%) and *Lachancea* (1%) genera. Concerning the most abundant eukaryotic genera in each fermentation: FT33 showed mainly *Saccharomyces* (75%), *Hanseniaspora* (20.3%), *Lachancea* (1.8%), *Aspergillus* (1.7%) and *Torulaspora* (1%); FT34 exhibited mostly *Saccharomyces* (70.4%), *Hanseniaspora* (25.5%) and *Aspergillus* (3.4%); FT35 revealed mainly *Saccharomyces* (70.8%), *Hanseniaspora* (24%), *Aspergillus* (3%) and *Lachancea* (1.4%); FT36 displayed a population predominantly composed by *Saccharomyces* (63%), *Hanseniaspora* (32.9%) and *Aspergillus* (3%).

Concerning the number of different eukaryotic genera identified in each production mode and fermentation process over the three sampling points: at the Initial Must produce under integrated production (TNIP) were identified 20 different eukaryotic genera; at the Initial Must produce organically (TNOP) were identified 27 different eukaryotic genera; during the Middle of Fermentation, at FT33, FT34, FT35 and FT36 were identified, respectively, 10, 8, 13 and 15 different eukaryotic genera; at the End of Fermentation were identified 7 different eukaryotic genera for FT33 and FT34, and 9 different eukaryotic genera for FT35 and FT36 (**Figure 3.5**).

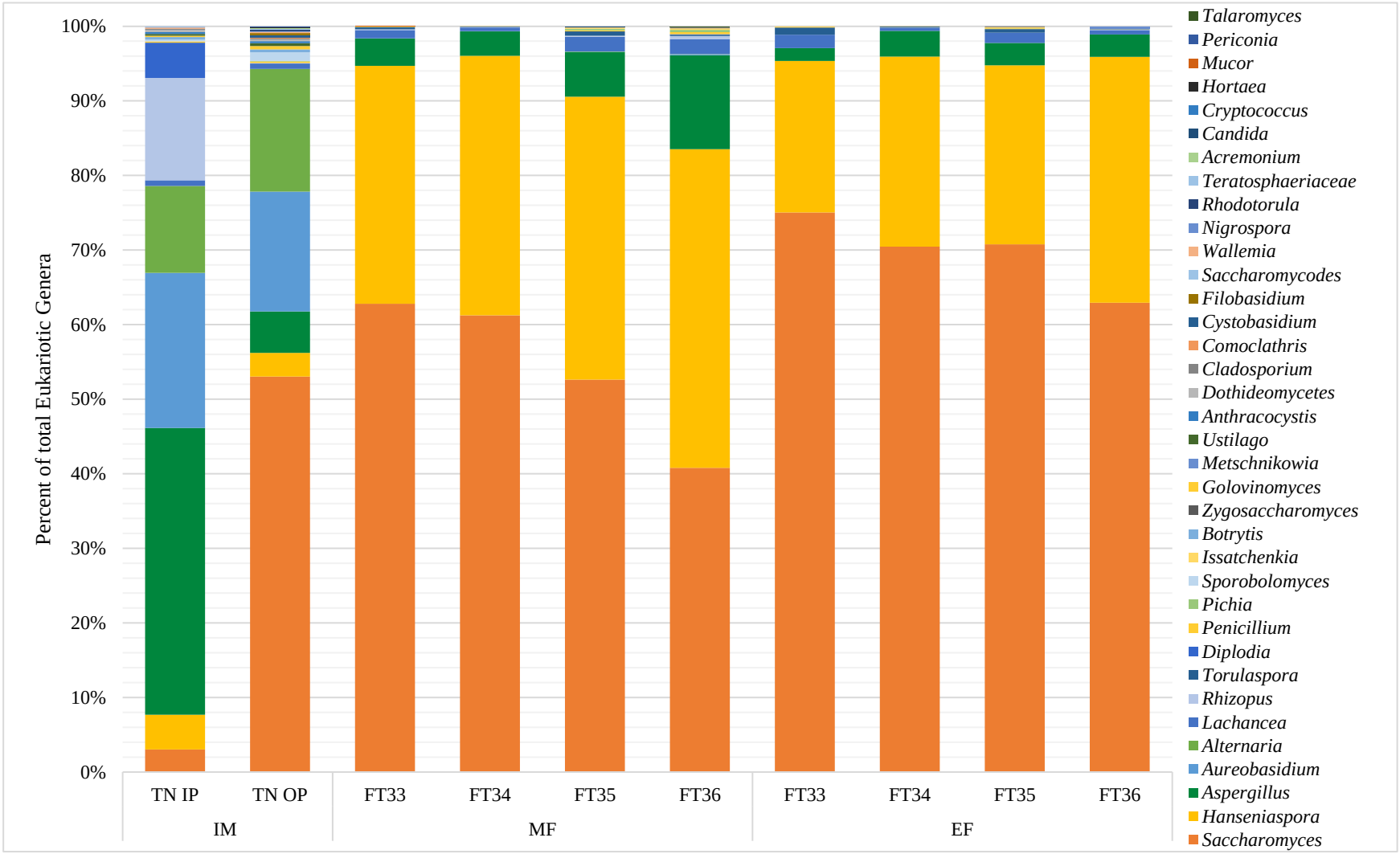

Figure 3.4 – Eukaryotic community distribution over Initial Must, Middle and End of Fermentation of Touriga Nacional vintage 2017 at the genus level. Relative abundance of the eukaryotic community through genus analysis. About 16.1% of the sequenced population has not been identified. IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; TNIP – Touriga Nacional Integrated Production; TNOP – Touriga Nacional Organic Production; FT – Fermentation; FT33 – Integrated Production/Inoculated fermentation; FT34 – Organic Production/Inoculated fermentation; FT35 – Integrated Production/Spontaneous fermentation; FT36 – Organic Production/Spontaneous fermentation.

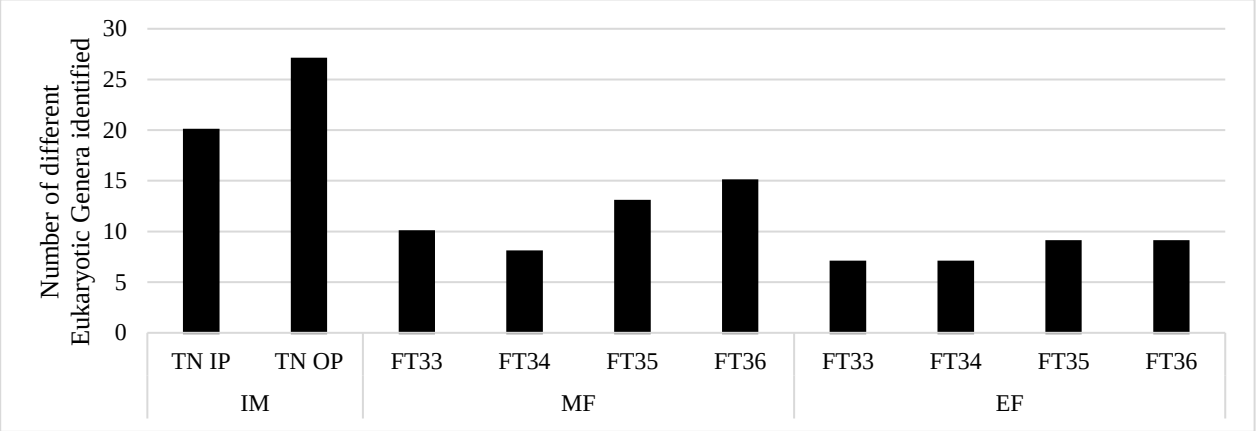

Figure 3.5 – Number of different eukaryotic genera identified over Initial Must, Middle and End of Fermentation of Touriga Nacional vintage 2017. IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; TNIP – Touriga Nacional Integrated Production; TNOP – Touriga Nacional Organic Production; FT – Fermentation; FT33 – Integrated Production/Inoculated fermentation; FT34 – Organic Production/Inoculated fermentation; FT35 – Integrated Production/Spontaneous fermentation; FT36 – Organic Production/Spontaneous fermentation.

The dynamics of Touriga Nacional eukaryotic communities at the three sampling points (Initial Must, Middle and End of Fermentation) were examined by principal component analysis (PCO) (**Figure 3.6**). Samples were grouped according to their fermentative stage (Initial Must, Middle and End of Fermentation), production mode (Integrated or Organic) and fermentation process (inoculated or spontaneous), where the x axis explains 86% of the total variation, and the y axis explains 12.6% of the total variation. The results show the formation of 3 distinct clusters: one composed by the initial must samples (IMIP and IMOP); another constituted by the end of fermentation FT33, FT34 and FT35; the bigger cluster is formed by middle of fermentation samples FT33 and FT34, and by the end fermentation FT36; one last cluster is formed by middle of fermentation FT35 and FT36.

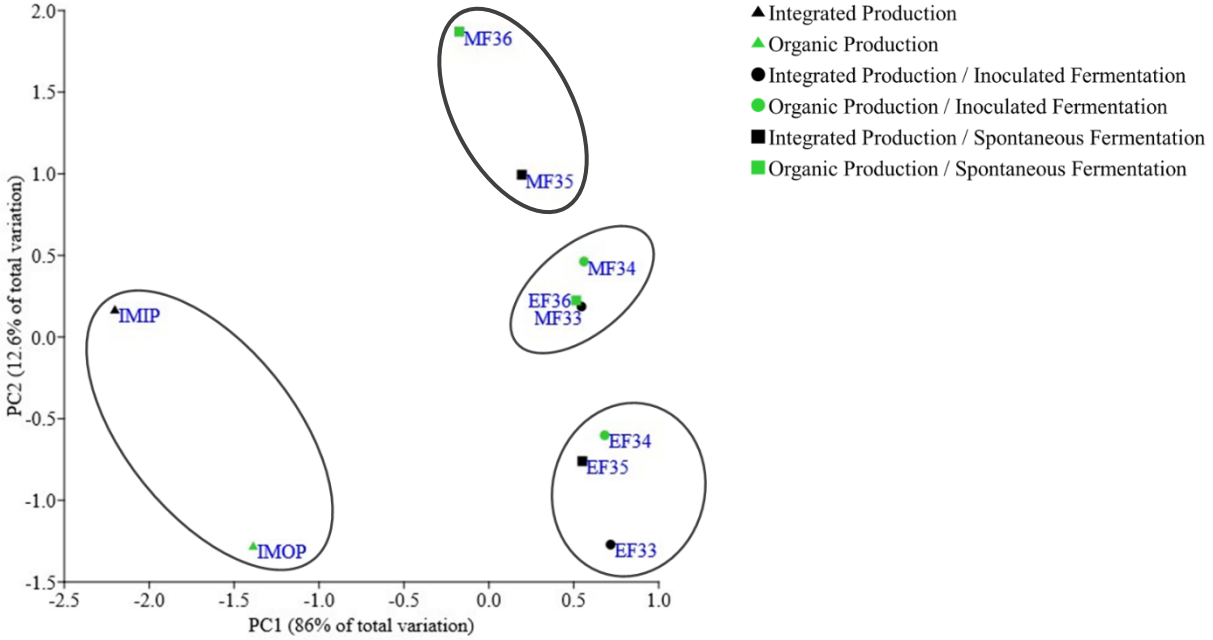

Figure 3.6 – Principal Component Analysis (PCA) biplot diagram of Touriga Nacional eukaryotic community during the fermentation process, based on sequence abundance of eukaryotic genera. Principal component analysis (showing the first and second components) of Touriga Nacional eukaryotic genera. IMIP – Initial Must/Integrated Production; IMOP – Initial Must/Organic Production; MF33 – Middle of Fermentation/FT33; MF34 – Middle of Fermentation/FT34; MF35 – Middle of Fermentation/FT35; MF36 – Middle of Fermentation/FT36; EF33 – End of Fermentation/FT33; EF34 – End of Fermentation/FT34; EF35 – End of Fermentation/FT35; ED36 – End of Fermentation/FT36; FT33 – Integrated Production/Inoculated fermentation; FT34 – Organic Production/Inoculated fermentation; FT35 – Integrated Production/Spontaneous fermentation; FT36 – Organic Production/Spontaneous fermentation.

3.1.2 General Characterization of Prokaryotic Population

3.1.2.1 Aragonez Prokaryotic Population

To better understand Aragonez prokaryotic population dynamics, the relative abundance at class level was analysed (**Figure 3.7**). About 1% of the sequenced population has not been identified. Moreover, chloroplast and Miscellaneous *Crenarchaeota* Group identifications were ignored. The dominant class across the entire Aragonez prokaryotic population was *Alphaproteobacteria* (77.7%) followed by *Gammaproteobacteria* (15.7%), *Bacilli* (2%), *Betaproteobacteria* (1.5%), *Bacteroidia* (1.4%) and *Clostridia* (1%).

At Initial Must sampling point, the Aragonez prokaryotic population was mostly characterized by *Alphaproteobacteria* (85.2%), *Bacteroidia* (6.1%), *Clostridia* (4.6%) and *Bacilli* (3.2%) classes.

Regarding the Initial Must yield under integrated production (AGIP), the most abundant prokaryotic class was *Alphaproteobacteria* (99%). For the Initial Must organically produce (AGOP), the most abundant prokaryotic classes were *Alphaproteobacteria* (71.3%), *Bacteroidia* (12.2%), *Clostridia* (9.3%) and *Bacilli* (5.8%).

At Middle of Fermentation sampling point, the Aragonez prokaryotic population was mostly characterized by *Alphaproteobacteria* (72%) and *Gammaproteobacteria* (27%) classes. Regarding the most abundant prokaryotic classes in each fermentation: FT13 presented mainly *Alphaproteobacteria* (78.3%) and *Gammaproteobacteria* (20.6%). FT14 showed principally *Alphaproteobacteria* (90.2%) and *Gammaproteobacteria* (9.2%); FT15 revealed mostly *Alphaproteobacteria* (62%) and *Gammaproteobacteria* (37.2%); FT16 displayed a population predominantly composed by *Alphaproteobacteria* (57.6%) and *Gammaproteobacteria* (41.2%).

At the End of Fermentation sampling point, the entire Aragonez prokaryotic population was mostly characterized by *Alphaproteobacteria* (79.7%), *Gammaproteobacteria* (12%), *Betaproteobacteria* (3.3%) and *Bacilli* (3%) classes. Regarding to the most abundant prokaryotic classes in each fermentation: FT13 presented mainly *Alphaproteobacteria* (79.8%), *Gammaproteobacteria* (14.5%), Betaproteobacteria (2.4%) and Bacilli (1.3%). FT14 showed principally *Alphaproteobacteria* (86.1%), *Gammaproteobacteria* (7.6%) and *Betaproteobacteria* (2.5%); FT15 revealed mostly *Alphaproteobacteria* (71.5%), Gammaproteobacteria (13.8%), Bacilli (8.5%) and *Betaproteobacteria* (4.8%); FT16 displayed a population predominantly composed by *Alphaproteobacteria* (81.4%), *Gammaproteobacteria* (12.3%), *Betaproteobacteria* (3.5%) and *Bacilli* (1.3%).

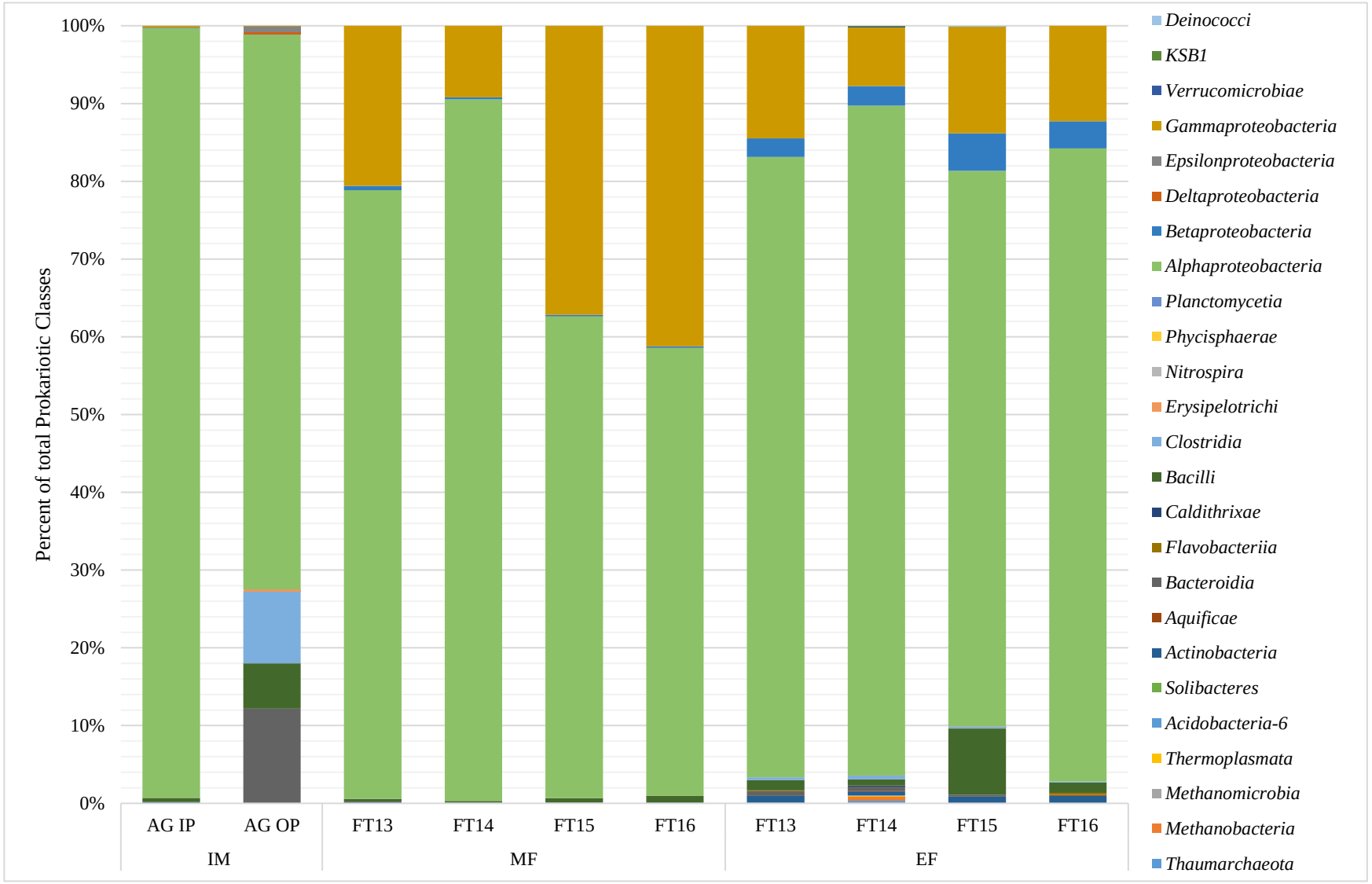

Figure 3.7 – Prokaryotic community distribution over Initial Must, Middle and End of Fermentation of Aragonez vintage 2017 at class level. Relative abundance of the prokaryotic community through class analysis. About 1% of the sequenced population has not been identified. Chloroplast and Miscellaneous Crenarchaeota Group identifications were ignored. IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; AGIP – Aragonez Integrated Production; AGOP – Aragonez Organic Production; FT – Fermentation; FT13 – Integrated Production/Inoculated fermentation; FT14 – Organic Production/Inoculated fermentation; FT15 – Integrated Production/Spontaneous fermentation; FT16 – Organic Production/Spontaneous fermentation.

The dynamics of Aragonez prokaryotic families at the three sampling points (Initial Must, Middle and End of Fermentation) were analysed by principal component analysis (PCA) (**Figure 3.8**). Samples were grouped according to their fermentative stage (Initial Must, Middle and End of Fermentation), production mode (Integrated or Organic) and fermentation process (inoculated or spontaneous), where the x axis explains 89.9% of the total variation, and the y axis explains 5.1% of the total variation. The results evidenced the formation of 2 distinct clusters: one composed by the middle of fermentation FT15 and FT16; another constituted by the integrated initial must (IMIP), for the middle of fermentation FT13 and FT14, and also for the end of fermentation FT13, FT14 and FT 16. The end of fermentation FT15 and the organic initial must (IMOP) did not integrated into any of the clusters.

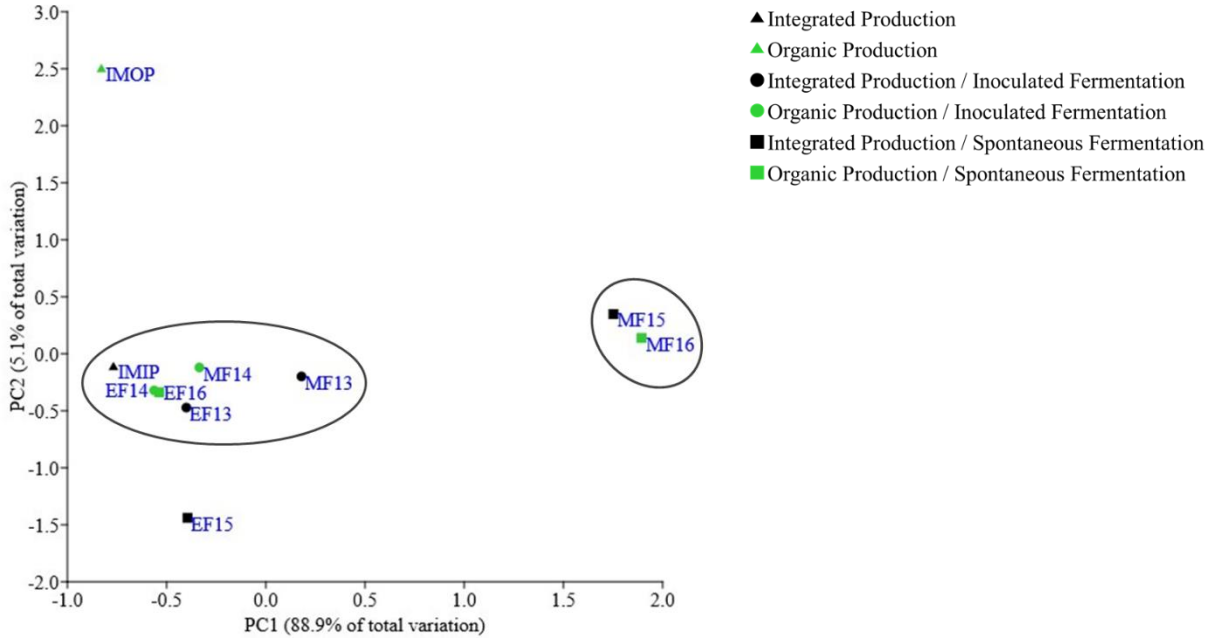

Figure 3.8 – Principal Component Analysis (PCA) biplot diagram of Aragonez prokaryotic community during the fermentation process, based on sequence abundance of prokaryotic families. Principal component analysis (showing the first and second components) of Aragonez prokaryotic families. IMIP – Initial Must/Integrated Production; IMOP – Initial Must/Organic Production; MF13 – Middle of Fermentation/FT13; MF14 – Middle of Fermentation/FT14; MF15 – Middle of Fermentation/FT15; MF16 – Middle of Fermentation/FT16; EF13 – End of Fermentation/FT13; EF14 – End of Fermentation/FT14; EF15 – End of Fermentation/FT15; ED16 – End of Fermentation/FT16; FT13 – Integrated Production/Inoculated fermentation; FT14 – Organic Production/Inoculated fermentation; FT15 – Integrated Production/Spontaneous fermentation; FT16 – Organic Production/Spontaneous fermentation.

3.1.2.2 Touriga Nacional Prokaryotic Population

The relative abundance at class level of the prokaryotic population of Touriga Nacional, was analysed (Figure 3.9). About 0.9% of the sequenced population has not been identified. Moreover, chloroplast identifications were ignored. The dominant class across the Touriga Nacional prokaryotic population was (63.8%) followed by *Gammaproteobacteria* (12.9%) and *Bacilli* (22.3%).

At Initial Must sampling point, the entire Touriga Nacional prokaryotic population was mostly characterized by *Alphaproteobacteria* (98.1%) and *Gammaproteobacteria* (1.2%) classes. Regarding the Initial Must produce under integrated production (TNIP), the most abundant prokaryotic classes were *Alphaproteobacteria* (98.1%) and *Gammaproteobacteria* (1%). For the Initial Must produce by organic production (TNOP), the most abundant prokaryotic class was *Alphaproteobacteria* (98.5%).

At the Middle of the Fermentation sampling point, the Touriga Nacional prokaryotic population was mostly characterized by *Alphaproteobacteria* (49%), *Bacilli* (33.3%) and *Gammaproteobacteria* (17.4%). Concerning the most abundant prokaryotic classes in each fermentation: FT33 presented mainly *Alphaproteobacteria* (44%), *Bacilli* (30.4%) and *Gammaproteobacteria* (25.4%); FT34 showed principally *Alphaproteobacteria* (46.8%), *Bacilli* (37.3%) and *Gammaproteobacteria* (15.8%); FT35 revealed mostly *Bacilli* (42.5%), *Alphaproteobacteria* (40.5%) and *Gammaproteobacteria* (16.7%); FT36 displayed a population predominantly composed by *Alphaproteobacteria* (64.8%), *Bacilli* (23.2%) and *Gammaproteobacteria* (11.7%).

At the End of Fermentation sampling point, the Touriga Nacional prokaryotic population was mostly characterized by *Alphaproteobacteria* (61.3%), *Bacilli* (22.2%), *Gammaproteobacteria* (14.5%) and *Betaproteobacteria* (1.4%). Concerning the most abundant prokaryotic classes in each fermentation: FT33 presented mainly *Alphaproteobacteria* (66.8%), *Gammaproteobacteria* (16.6%) and *Bacilli* (15.9%); FT34 showed principally *Alphaproteobacteria* (66.4%), *Bacilli* (16.7%), *Gammaproteobacteria* (14.4%) and *Betaproteobacteria* (2.1%); FT35 revealed mostly *Alphaproteobacteria* (52.4%), *Bacilli* (35.9%) and *Gammaproteobacteria* (10.6%); FT36 displayed a population predominantly composed by *Alphaproteobacteria* (59.6%), *Bacilli* (20.3%), *Gammaproteobacteria* (16.2%) and *Betaproteobacteria* (2.9%).

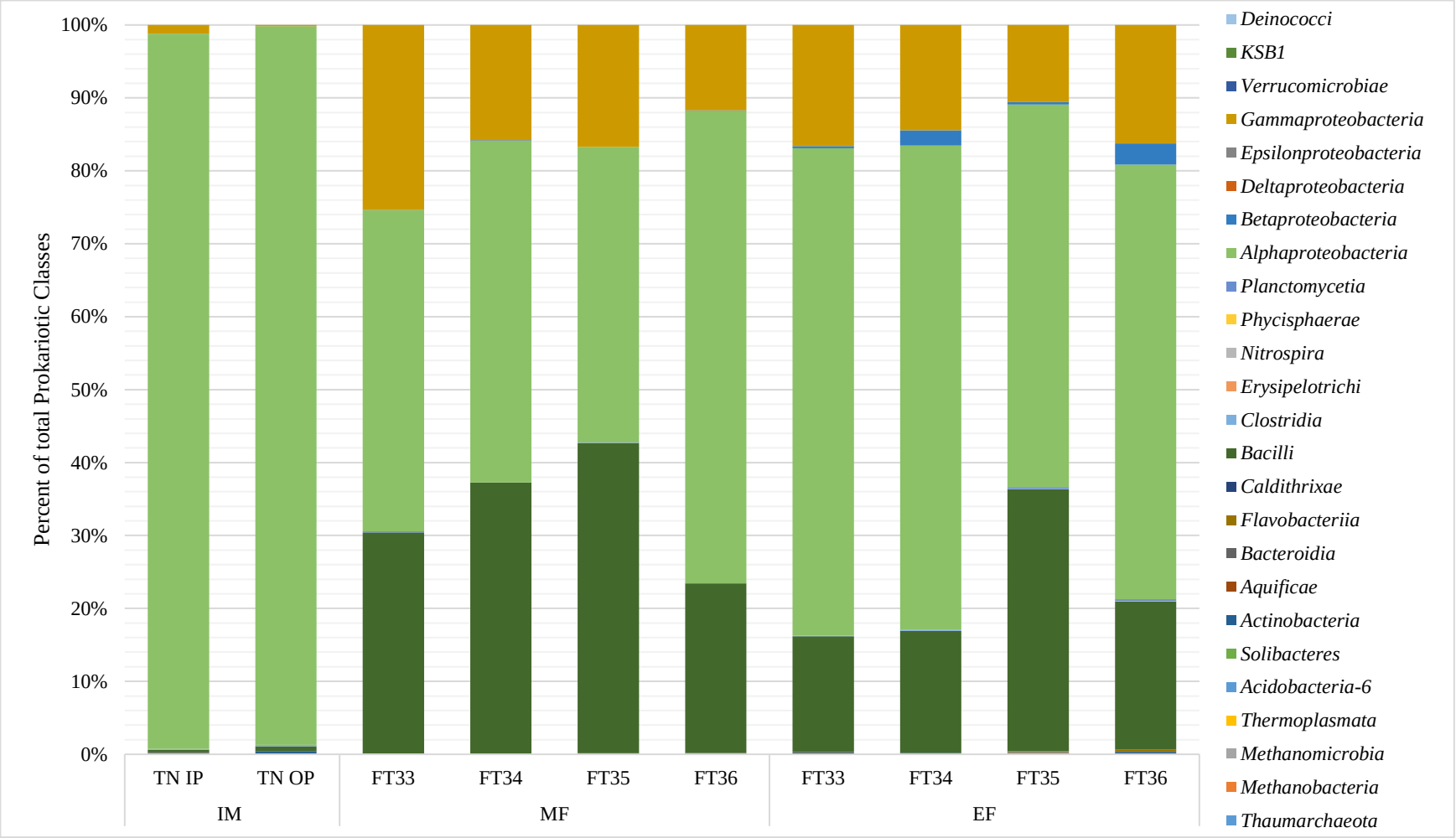

Figure 3.9 – Prokaryotic community distribution over Initial Must, Middle and End of Fermentation of Touriga Nacional vintage 2017 at class level. Relative abundance of the prokaryotic community through class analysis. About 0.9% of the sequenced population has not been identified. Chloroplast identifications were ignored. IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; TNIP – Touriga Nacional Integrated Production; TNOP – Touriga Nacional Organic Production; FT – Fermentation; FT33 – Integrated Production/Inoculated fermentation; FT34 – Organic Production/Inoculated fermentation; FT35 – Integrated Production/Spontaneous fermentation; FT36 – Organic Production/Spontaneous fermentation.

The dynamics of the prokaryotic families present in Touriga Nacional at the three sampling points (Initial Must, Middle and End of Fermentation) were examined by principal component analysis (PCA) (**Figure 3.10**). Samples were grouped according to their fermentative stage (Initial Must, Middle and End of Fermentation), production mode (Integrated or Organic) and fermentation process (inoculated or spontaneous), where the x axis explains 97.4% of the total variation, and the y axis explains 12.6% of the total variation. The results show the formation of 3 distinct clusters: one composed by the integrated and organic initial must (IMIP and IMOP); other formed by all the end of fermentation samples (FT33, FT34, FT35 and FT36) and also by the middle of fermentation FT36; the last cluster was constituted by the middle of fermentation samples FT33, FT34 and FT35.

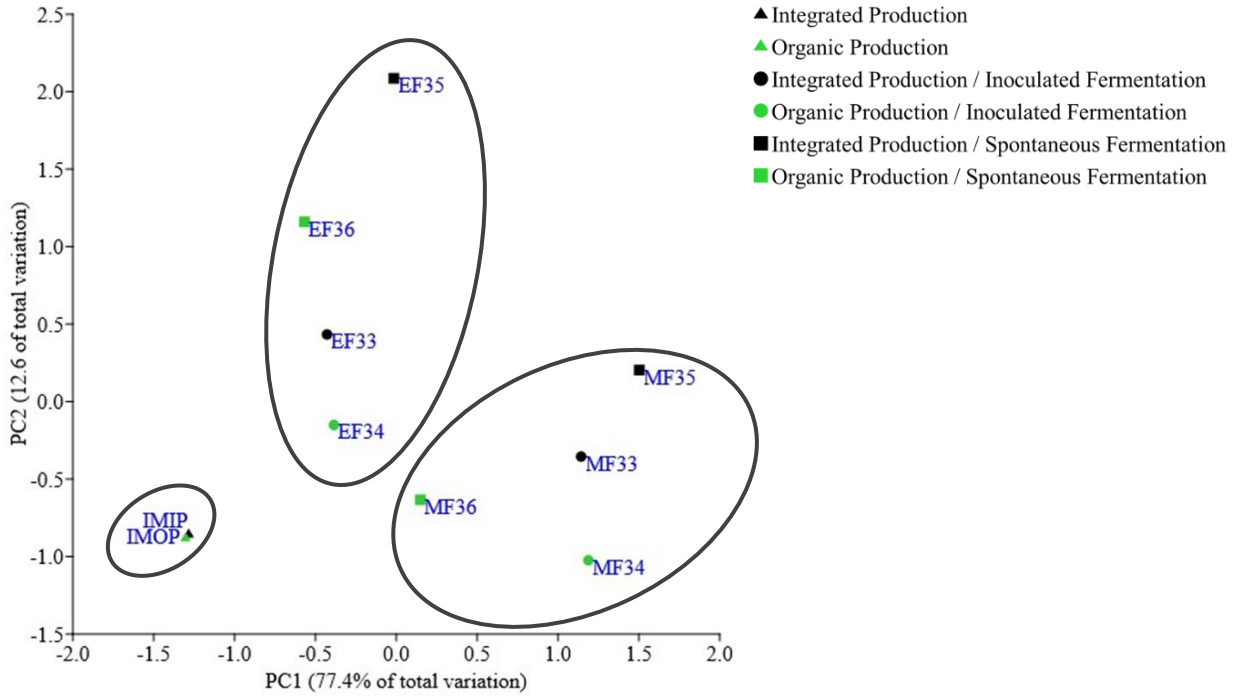

Figure 3.10 – Principal Component Analysis (PCA) biplot diagram of Touriga Nacional prokaryotic community during the fermentation process, based on sequence abundance of prokaryotic families. Principal component analysis (showing the first and second components) of Touriga Nacional prokaryotic families. IMIP – Initial Must/Integrated Production; IMOP – Initial Must/Organic Production; MF33 – Middle of Fermentation/FT33; MF34 – Middle of Fermentation/FT34; MF35 – Middle of Fermentation/FT35; MF36 – Middle of Fermentation/FT36; EF33 – End of Fermentation/FT33; EF34 – End of Fermentation/FT34; EF35 – End of Fermentation/FT35; ED36 – End of Fermentation/FT36; FT33 – Integrated Production/Inoculated fermentation; FT34 – Organic Production/Inoculated fermentation; FT35 – Integrated Production/Spontaneous fermentation; FT36 – Organic Production/Spontaneous fermentation.

3.2 Cultivable Yeasts

3.2.1 Determination of Cultivable Yeasts in Aragonez variety

The number of cultivable yeasts present in the Initial Must, Middle and End of Fermentation of Aragonez fermentations were determined. The results are illustrated in **Figure 3.11**.

Regarding the Initial Must, the number of cultivable yeasts was higher ($p \leq 0.0001$) on samples from grapes organic production, in comparison with the samples from grapes produced under integrated mode.

Concerning the Middle of Fermentation, the number of cultivable yeasts isolated did not show a statistically significant variation ($p > 0.05$) between any of the four fermentations. At the End of Fermentation, the number of cultivable yeasts determined in FT13 and FT16 was not statistically

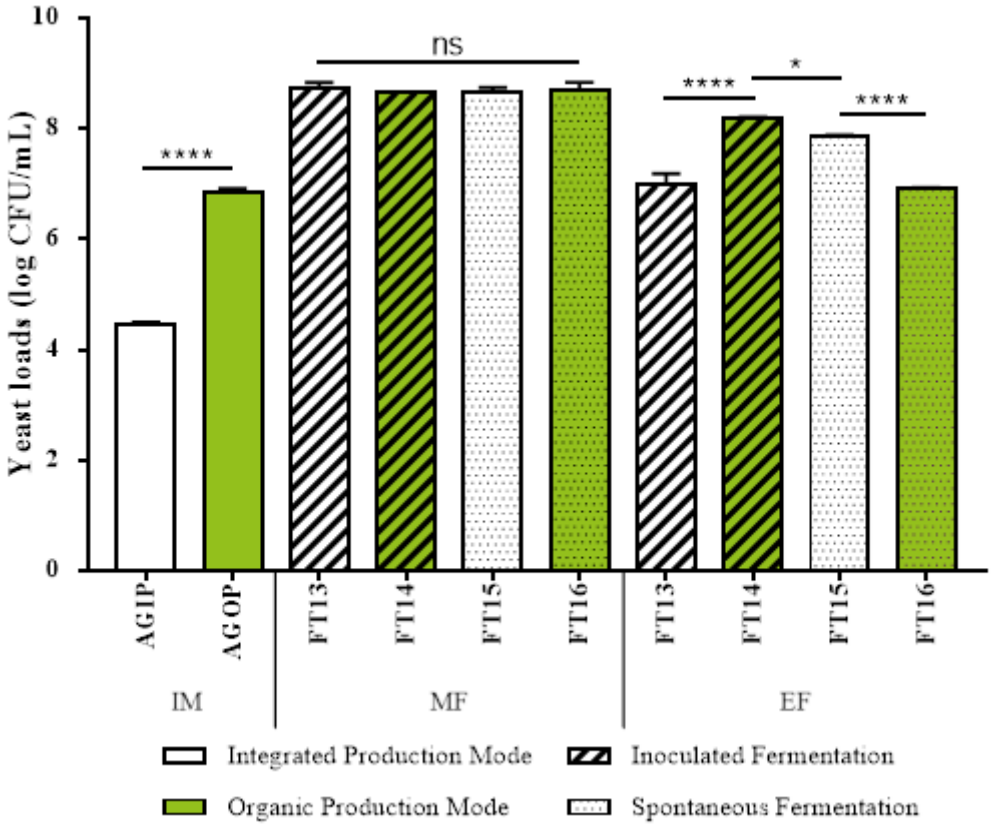

Figure 3.11 – Count of cultivable yeasts on Initial Must, Middle and End of Fermentation of Aragonez for 2017 vintage. The data presented result from the mean of 3 replicates. IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; AGIP – Aragonez Integrated Production; AGOP – Aragonez Organic Production; FT – Fermentation; FT13 – Integrated Production/Inoculated fermentation; FT14 – Organic Production/Inoculated fermentation; FT15 – Integrated Production/Spontaneous fermentation; FT16 – Organic Production/Spontaneous fermentation. ns – No statistical significance ($p > 0.05$); * –Indicate statistical significance at the $p \leq 0.05$ level of confidence; ** – Indicate statistical significance at the $p \leq 0.01$ level of confidence; *** – Indicate statistical significance at the $p \leq 0.001$ level of confidence; **** – Indicate statistical significance at the $p \leq 0.0001$ level of confidence.

significant ($p>0.05$). The comparison of the remaining fermentations, namely FT13 and FT14, FT13 and FT15, FT14 and FT16 and FT 15 and FT16 showed significantly differences $p\leq0.000$, also, the comparison of the number of cultivable yeasts from fermentations FT14 and FT15 was significantly different, but with a less significant level ($p\leq0.05$) ((**Figure 3.11**).

3.2.2 Determination of Cultivable Yeasts in Touriga Nacional variety

The determined number of cultivable yeasts in Touriga Nacional Initial Must and fermentations were evaluated and the results are shown in Figure **3.12**.

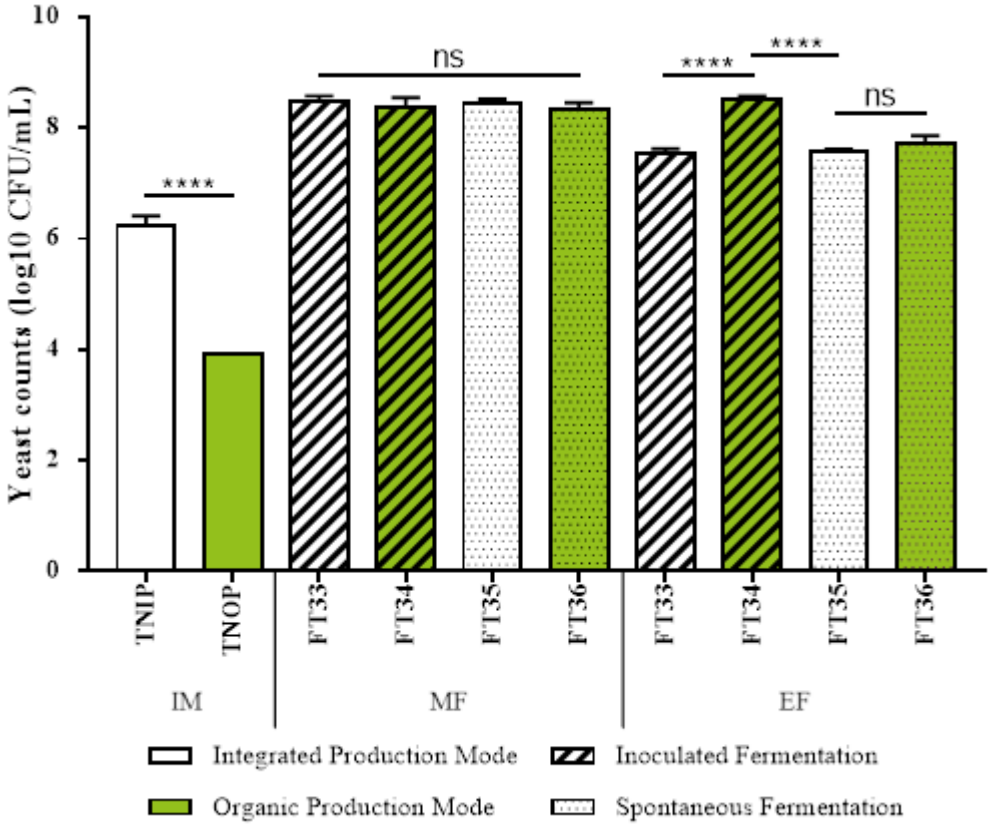

Figure 3.12 – Count of cultivable yeasts on Initial Must, Middle and End of Fermentation of Touriga Nacional for 2017 vintage. The data presented result from the mean of 3 replicates. IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; TNIP – Touriga Nacional Integrated Production; TNOP – Touriga Nacional Organic Production; FT – Fermentation; FT33 – Integrated Production/Inoculated fermentation; FT34 – Organic Production/Inoculated fermentation; FT35 – Integrated Production/Spontaneous fermentation; FT36 – Organic Production/Spontaneous fermentation. ns –No statistical significance ($p>0.05$); * –Indicate statistical significance at the $p\leq0.05$ level of confidence; ** – Indicate statistical significance at the $p\leq0.01$ level of confidence; *** – Indicate statistical significance at the $p\leq0.001$ level of confidence; **** – Indicate statistical significance at the $p\leq0.0001$ level of confidence.

In contrast to the Aragonez variety in the Touriga Nacional the Initial Must under Integrated mode, showed a higher number of cultivable yeasts in comparison with the Initial Must from the organic mode ($p\leq0.0001$) **Figure 3.12**.

Concerning the Middle of Fermentation, the number of cultivable yeasts was similar across the four fermentations ($p>0.05$).

At the End of Fermentation, the comparison of the number of cultivable yeasts isolated in FT33 and FT35, FT33 and FT36, FT35 and FT36 was not statistically significant ($p>0.05$). Regarding the number of cultivable yeasts, it was significantly different ($p\leq0.0001$) in fermentations FT33 and FT34, FT34 and FT35, FT34 and FT36.

3.2.3 Identification of Cultivable Yeasts

3.2.3.1 Identification of Yeasts isolated from Aragonez variety

The sequencing of the ITS regions allowed the identification of 27 of the 138 recovered yeasts isolates collected from three sampling points of Aragonez 2017 vintage (Initial Must, Middle and End of Fermentation). The yeasts identifications and their percentages at each sampling point are shown in **Figure 3.13**.

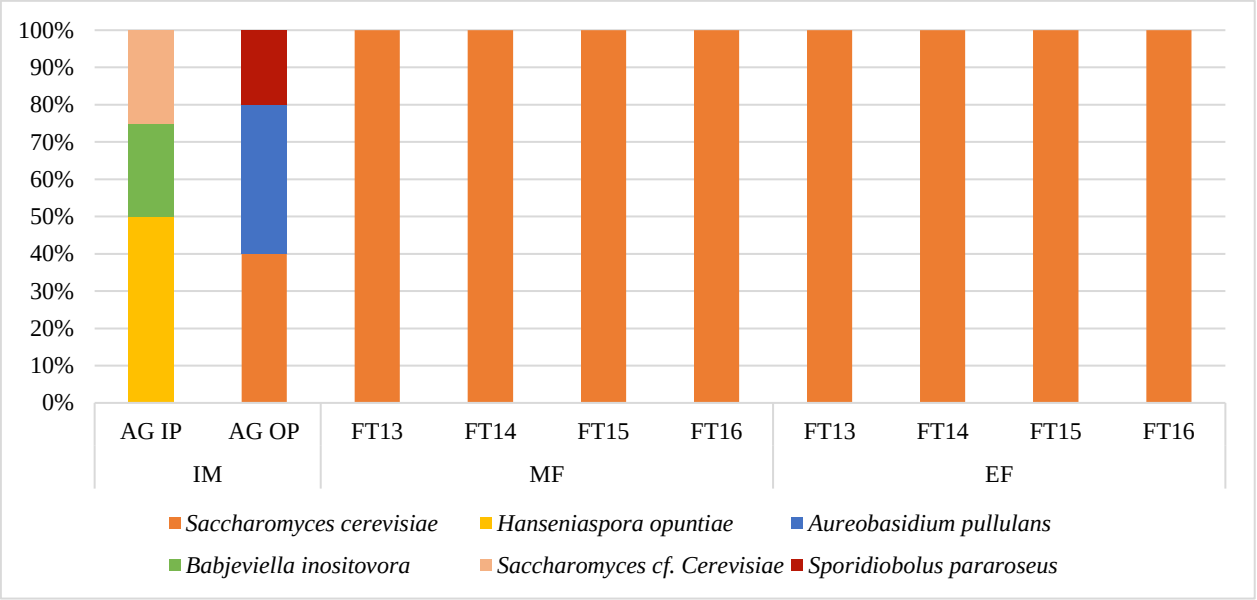

Figure 3.13 – Identification of cultivable yeasts on Initial Must, Middle and End of Fermentation of Aragonez 2017 vintage. IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; AGIP – Aragonez Integrated Production; AGOP – Aragonez Organic Production; FT – Fermentation; FT13 – Integrated Production/Inoculated fermentation; FT14 – Organic Production/Inoculated fermentation; FT15 – Integrated Production/Spontaneous fermentation; FT16 – Organic Production/Spontaneous fermentation.

The cultivable yeasts were collected in the Initial Must from grapes produced under integrated mode was found to belong to *Hanseniaspora opuntiae* (50%), *Saccharomyces cf. cerevisiae* (25%) and *Babjeviella inositovora* (25%). The cultivable yeasts isolated from the Initial Must of grapes produced under organic mode consisted of *Aureobasidium pullulans* (40%), *S. cerevisiae* (40%) and *Sporidiobolus pararoseus* (20%). Concerning the Middle of Fermentation and the End of Fermentation, only one species of yeast (100 %) was identified in all four fermentations, *S. cerevisiae* (**Figure 3.13)**.

3.2.3.2 Identification of Yeasts isolated from Touriga Nacional variety

The sequencing of the ITS regions enable the identification of 32 of the 125 recovered yeasts from three sampling points of Touriga Nacional 2017 vintage (Initial Must, Middle and End of Fermentation). The yeasts identifications and their percentages at each sampling point are shown in **Figure 3.14**.

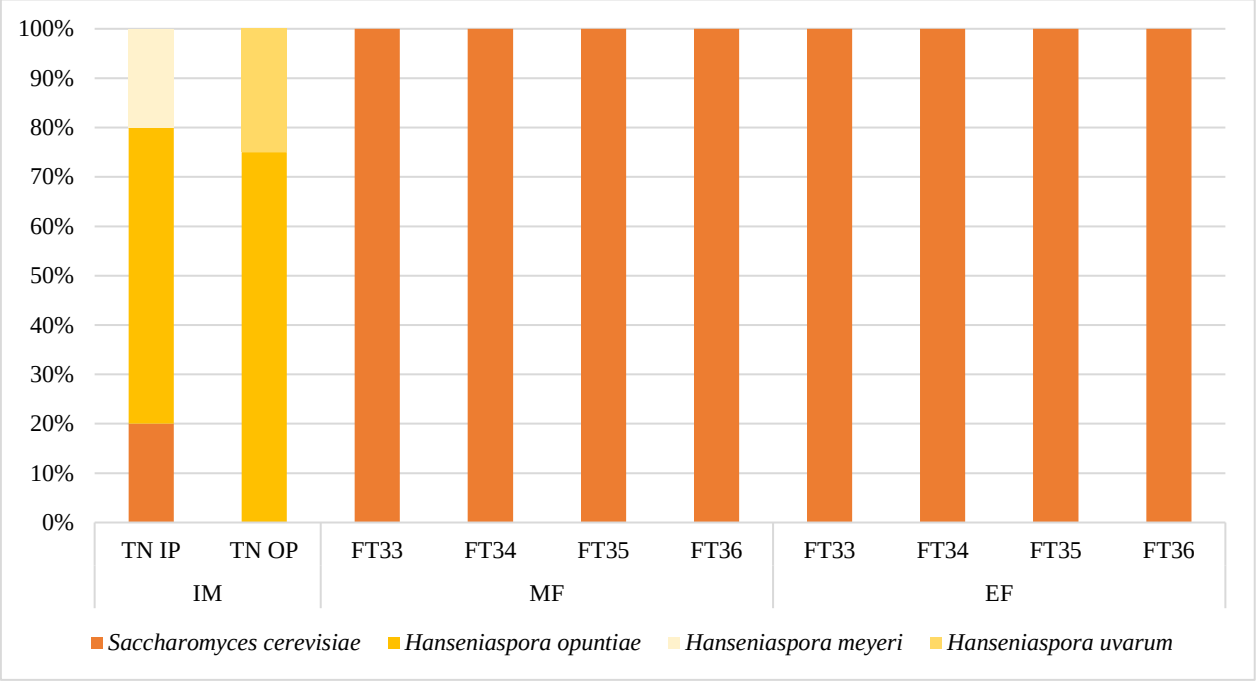

Figure 3.14 – Identification of cultivable yeasts on Initial Must, Middle and End of Fermentation of Touriga Nacional 2017 vintage. IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; TNIP – Touriga Nacional Integrated Production; TNOP – Touriga Nacional Organic Production; FT – Fermentation; FT33 – Integrated Production/Inoculated fermentation; FT34 – Organic Production/Inoculated fermentation; FT35 – Integrated Production/Spontaneous fermentation; FT36 – Organic Production/Spontaneous fermentation.

The isolated yeasts in the Initial Must from grapes produced under integrated mode belong to consist *Hanseniaspora opuntiae* (60%), *H. meyeri* (20%) and *S. cerevisiae* (20%). The population of cultivable yeasts identified in the Initial Must of biologically produced grapes consists of *H. opuntiae* (75%), and *H. uvarum* (25%). Concerning the Middle of Fermentation and the End of Fermentation, only one species, the *S. cerevisiae* (100%) was identified in all four fermentations.

3.2.4 The interdelta patterns of the Aragonez fermentations

In order to evaluate the ability of the indigenous yeasts consortium to impose against commercial yeast the patterns of the interdelta sequence regions using primer pair delta12 and delta2B were evaluated. The results are shown in the **Figure 3.15** for the patterns of the interdelta profile of the commercial yeast VQ51, the commercial fermentations (FT13 and FT14) and the spontaneous fermentations (FT15 and FT16) of Aragonez at the End of Fermentation.

All the autochthonous yeasts profiles share the 1000 bp and 400 bp band with the commercial yeast. The comparison of the interdelta profiles of the commercial fermentations (FT13 and FT14) with the commercial yeast evidence at least four common bands (1000 bp, 600 bp, 500 bp and 400 bp). The interdelta profiles of the spontaneous fermentations (FT15 and FT16) reveal at least four common bands (1000 bp, 70 0bp, 400bp and 300 bp). The Aragonez spontaneous fermentations profiles have, in common with the commercial yeast profiles at least two bands (1000 bp and 400 bp) (**Figure 3.15)**.

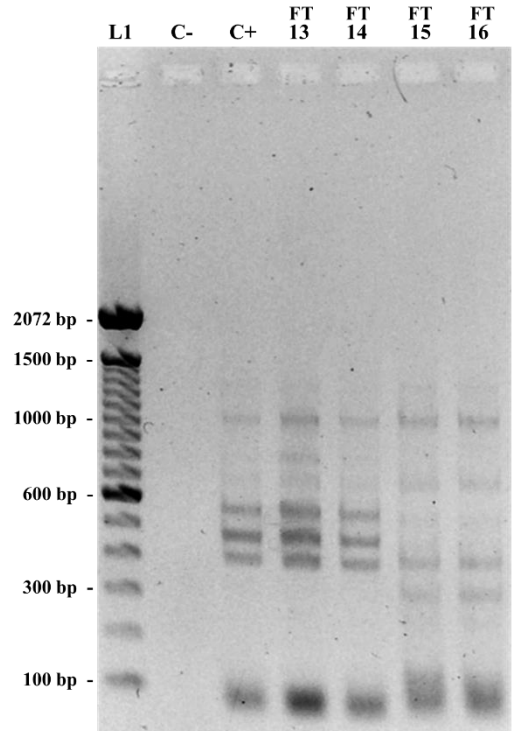

Figure 3.15 – Electrophoretic profiles (C+ to FT16) obtained by PCR-inter-delta using primers delta 12 and 2b on Aragonez (FT13, FT14, FT15, FT16) fermentations. L1 – TrackIT™ 100bp DNA Ladder (Thermo-Scientific); C- – Negative Control; C+ – Positive Control (Commercial yeast VQ51); FT – Fermentation; FT13 – Integrated Production/Inoculated fermentation; FT14 – Organic Production/Inoculated fermentation; FT15 – Integrated Production/Spontaneous fermentation; FT16 – Organic Production/Spontaneous fermentation.

3.2.4 The interdelta patterns of the Touriga Nacional fermentations

The profiles of amplification of the delta sequence regions using primer pair delta12 and delta2B of the commercial yeast VQ51, the commercial fermentations (FT33 and FT34) and the spontaneous fermentations (FT35 and FT36) of Touriga Nacional at the End of Fermentation are shown in **Figure 3.16**.

All profiles shared the 400 bp band with the commercial yeast. The comparison of the interdelta profiles of the commercial fermentations (FT33 and FT34) with the commercial yeast showed that all bands are common between them (1000 bp, 600 bp, 500 bp and 400 bp). The spontaneous fermentations interdelta profiles (FT35 and FT36) evidenced that the two bands are common between them (400 bp and 300 bp) and only the 400 bp band is common with the commercial yeast.

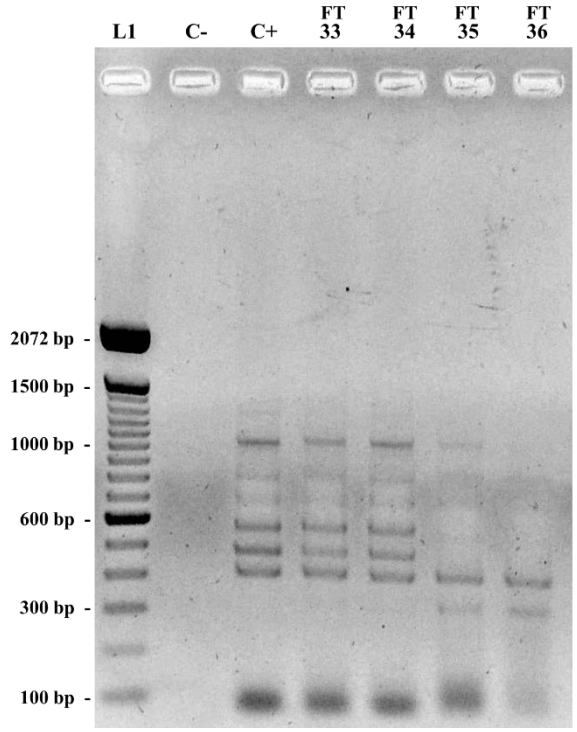

Figure 3.16 – Electrophoretic profiles (C+ to FT36) obtained by PCR-inter-delta using primers delta 12 and 2b on Aragonez (FT13, FT14, FT15, FT16) fermentations. L1 – TrackIT™ 100bp DNA Ladder (Thermo-Scientific).C- – Negative Control; C+ – Positive Control (Commercial yeast VQ51); FT – Fermentation; FT33 – Integrated Production/Inoculated fermentation; FT34 – Organic Production/Inoculated fermentation; FT35 – Integrated Production/Spontaneous fermentation; FT36 – Organic Production/Spontaneous fermentation.

4. Discussion

4 Discussion

The main objective of this study was to evaluate the impact of different winemaking practices on wine microbiome during the winemaking process. For this purpose, two Portuguese red wine grape varieties (Aragonez and Touriga Nacional) were cultivated at Herdade da Malhadinha Nova (Beja, Portugal), under two production modes (organic and integrated) and fermented by inoculation of a commercial yeasts or spontaneously.

Aragonez and Touriga Nacional Initial Must, Middle and End of Fermentation samples (for each production mode and fermentation process), were prepared for Illumina Sequencing for fungal and bacterial analysis (Internal Transcribed Spacer 2 region and 16S rRNA V3V4 region, respectively). The results were assessed, and the eukaryotic and prokaryotic populations of each grape variety were characterized regarding the production method, fermentation process and sampling point.

Aragonez eukaryotic population showed a higher relative abundance of *Aureobasidium* (organic Initial Must), *Alternaria* (integrated Initial Must) and *Saccharomyces* (Middle and End of Fermentation) genera. As expected, the eukaryotic biodiversity of Aragonez decreased during fermentation, along with a sequential substitution of species during the different stages, with a dominance of non-*Saccharomyces* yeasts at the initial stage, and a successive replacement by *Saccharomyces* yeasts at the end (regardless of the production and fermentation method). This is in line with previous studies that have showed that *Saccharomyces* suppresses certain non-*Saccharomyces* yeast species, while also favouring the persistence of other species (Bezerra-Bussoli *et al*. 2013; Bagheri, Bauer, and Setati 2017).

The evaluation of the Aragonez Initial Must eukaryotic populations showed that the integrated Initial Must exhibited a higher relative abundance of *Saccharomyces* yeasts. However, other non-beneficial microbial members were also present, such as *Alternaria* and *Aspergillus* and a lower relative abundance of phytoprotectants. In contrast, organic Initial Must showed a lower number of eukaryotic genera, a lower relative abundance of *Saccharomyces* yeasts. In addition, its population has resulted in a greater relative abundance of phytoprotectants (*Aureobasidium*) and a smaller relative abundance of pathogens (yet it shares the same relative abundance of *Alternaria* with integrated Initial Must). This finding was also reported by Grangeteau *et al*. 2017 and Martins

et al. 2016, where a lower yeast biodiversity in organic vineyards was associated with the sensitivity of yeasts and yeasts-like fungi to copper and sulphur, with the exception of *A. pullulans* that exhibited an higher resistance to both antifungal agents.

At Middle and End of Fermentation the eukaryotic populations of Aragonez originated from integrated and organic mode of production were compared according to the inoculation process, and at the Middle of Fermentation, the integrated and inoculated fermentation (FT13) showed a relevant relative abundance of *Saccharomyces* yeasts. However, the eukaryotic population of this fermentation included at relative abundance a non-beneficial member, the genus *Aspergillus* and a lower relative abundance of phytoprotectors. In contrast, the integrated and spontaneous fermentation (FT15) showed a less relative abundance of *Saccharomyces* yeasts. In addition, the eukaryotic community include at a significant abundance phytoprotectants, such as *Lachancea* and *Torulaspora* and a reduced relative abundance of pathogens. The organic and inoculated fermentation (FT14) showed a significant relative abundance of *Saccharomyces* yeasts. However, the eukaryotic population of this fermentation evidenced a greater relative abundance of pathogens (*Aspergillus*) and a lower relative abundance of phytoprotectors. In contrast, the organic and spontaneous fermentation (FT16) showed less relative abundance of *Saccharomyces* yeasts. In addition, its population showed a marked relative abundance of phytoprotectants (*Lachancea* and *Torulaspora*) and a minor relative abundance of pathogens. Contrary to what was described by Setati *et al.* 2017 and Padilla *et al*. 2017, in the current study, the Aragonez inoculated fermentations (FT13 and FT14) showed a greater relative abundance of *Saccharomyces* yeasts. Given these results it is thought that the commercial *S. cerevisiae* suppressed a lower number or favour a higher number of non-*Saccharomyces* species, than it has been reported.

At End of Fermentation the integrated and inoculated fermentation (FT13) showed a similar eukaryotic population as the integrated and spontaneous fermentation (FT15), and a greater relative abundance of *Saccharomyces* yeasts. However, the eukaryotic population of FT13 presented a lower relative abundance of pathogens and a greater relative abundance of phytoprotectors (*Lachancea*). The integrated and spontaneous fermentation (FT15) showed the same eukaryotic population but a lower relative abundance of *Saccharomyces* yeasts. In addition, FT15 population has resulted in a lower relative abundance of phytoprotectants and a higher relative abundance of pathogens (*Aspergillus*). The organic and inoculated fermentation (FT14) showed the same eukaryotic population as the organic and spontaneous fermentation (FT16) and a greater relative

abundance of *Saccharomyces* yeasts. However, the eukaryotic population of FT14 presented the highest relative abundance of pathogens (*Aspergillus*) and the lowest relative abundance of phytoprotectors. The organic and spontaneous fermentation (FT16) showed the same eukaryotic population but a lower relative abundance of *Saccharomyces* yeasts. In addition, FT16 population evidenced a higher relative abundance of phytoprotectants (*Lachancea*) and a lower relative abundance of pathogens. This finding was also reported by Grangeteau *et al*. 2017 and Martins *et al*. 2016, where a lower yeast biodiversity was associated with organic production. Furthermore, Setati *et al.* 2017 and Padilla *et al*. 2017 showed that the commercial *S. cerevisiae* can suppress and favour a number of non-*Saccharomyces* species (phytopathogens and beneficial microorganisms).

Aragonez prokaryotic population showed a greater relative abundance for *Alphaproteobacteria* class (at Initial Must, Middle and End of Fermentation). As expected, the prokaryotic biodiversity of Aragonez increased during fermentation (Pinto *et al*. 2014).

The comparison of Aragonez Initial Must prokaryotic population from integrated and organic mode of production evidenced that the integrated Initial Must the prokaryotic population was less diverse, along with a greater relative abundance of *Alphaproteobacteria* and a lower relative abundance of *Bacilli*. In contrast, organic Initial Must showed a more diverse prokaryotic population, along with less relative abundance of *Alphaproteobacteria* and a significant relative abundance of *Bacteroidia*, *Clostridia* and *Bacilli*.

At Middle and End of Fermentation the prokaryotic populations of Aragonez from integrated and organic mode of production were compared according to the applied inoculation process, and at Middle of Fermentation, the integrated and inoculated fermentation (FT13) showed the same relative abundance of prokaryotic classes as the integrated and spontaneous fermentation (FT15), along with a greater relative abundance of *Alphaproteobacteria* and a lower relative abundance of *Gammaproteobacteria*. The integrated and spontaneous fermentation (FT15) showed the same relative abundance of prokaryotic classes as the integrated and inoculated fermentation (FT13), along with a lower relative abundance of *Alphaproteobacteria* and a higher relative abundance of *Gammaproteobacteria*. The organic and inoculated fermentation (FT14) showed the same relative abundance of prokaryotic classes as the organic and spontaneous fermentation (FT16), together with a greater relative abundance of *Alphaproteobacteria* and a lower relative abundance of

Gammaproteobacteria. The organic and spontaneous fermentation (FT16) show the same relative abundance of prokaryotic classes as the organic and inoculated fermentation (FT13), along with a lower relative abundance of *Alphaproteobacteria* and a greater relative abundance of *Gammaproteobacteria*.

At End of Fermentation the integrated and inoculated fermentation (FT13) showed less biodiversity, along with a greater relative abundance of *Alphaproteobacteria* and *Gammaproteobacteria*, and a lower relative abundance of *Betaproteobacteria* and *Bacilli*. In contrast, integrated and spontaneous fermentation (FT15) showed greater biodiversity, along with a lower relative abundance of *Alphaproteobacteria* and *Gammaproteobacteria* and a greater relative abundance of *Betaproteobacteria* and *Bacilli*. The organic and inoculated fermentation (FT14) showed greater biodiversity, along with a greater relative abundance of *Alphaproteobacteria* and a lower relative abundance of *Gammaproteobacteria* and *Betaproteobacteria*. The organic and spontaneous fermentation (FT16) showed less biodiversity, along with a lower relative abundance of *Alphaproteobacteria* and a greater relative abundance of *Gammaproteobacteria* and *Betaproteobacteria*.

All the identified classes are in line with previous studies (Pinto *et al*. 2015; Salvetti *et al*. 2016).

Touriga eukaryotic population showed a higher relative abundance of *Saccharomyces* genus over the three sampling points. As expected, several members of eukaryotic community of Touriga Nacional decreased during fermentation, in contrast to the increase of the relative abundance of *Saccharomyces* yeasts.

Comparing Touriga National Initial Must eukaryotic populations, the integrated Initial Must showed minor biodiversity, along with a greater relative abundance of non-*Saccharomyces* yeasts. Its population showed a greater relative abundance of pathogens, such as *Aspergillus, Rhizopus* and *Alternaria*, also evidenced the largest relative abundance of phytoprotectants (*Aureobasidium*). In contrast, organic Initial Must showed a greater biodiversity, along with greater relative abundance of *Saccharomyces* yeasts. In addition, its population showed a lower relative abundance of phytoprotectants and pathogens. The loss of integrated Initial Must biodiversity may be due to the atypical, dry and hot vintage year, already known to be a factor that negatively affect yeast biodiversity (Gayevskiy and Goddard, 2012).

At Middle and End of Fermentation the eukaryotic populations of Touriga Nacional integrated and organic fermentations were compared according to the applied inoculation process, and at the Middle of Fermentation, the integrated and inoculated fermentation (FT33) showed less biodiversity, along with a greater relative abundance of *Saccharomyces* yeasts, as mentioned above several studied already reported that *S. cerevisiae* can suppress certain species while favour the persistence of other (Bahareh Bagheri, Bauer, and Setati 2017). The eukaryotic population of this fermentation presented a lower relative abundance of pathogens and phytoprotectors. In contrast, the integrated and spontaneous fermentation (FT35) showed greater number of eukaryotic genera together with less relative abundance of *Saccharomyces* yeasts. In addition, its population has resulted in a greater relative abundance of phytoprotectants (as *Lachancea*) and pathogens (as *Aspergillus*). The organic and inoculated fermentation (FT34) showed less biodiversity, along with a greater relative abundance of *Saccharomyces* yeasts, once again in line with previous studies where it was clarified that *S. cerevisiae* can suppress certain species while favour the persistence of other (Bahareh Bagheri, Bauer, and Setati 2017). However, the eukaryotic population of this fermentation presented a smaller relative abundance of pathogens and phytoprotectors. In contrast, the organic and spontaneous fermentation (FT36) showed greater number of eukaryotic genera, along with less relative abundance of *Saccharomyces* yeasts. In addition, its population showed a greater relative abundance of phytoprotectants (*Lachancea*) and pathogens (*Aspergillus*). These findings are in accordance with those described by Setati *et al.* 2017 and Padilla *et al*. 2017, that showed that that the commercial *S. cerevisiae* can suppress and favour a number of non-*Saccharomyces* species (phytopathogens and beneficial microorganisms), which can lead to an increase of population biodiversity.

At End of Fermentation the integrated and inoculated fermentation (FT33) showed a lower number of eukaryotic genera, together with a greater relative abundance of *Saccharomyces* yeasts. The eukaryotic population of this fermentation presented a lower relative abundance of pathogens and a greater relative abundance of phytoprotectors (as *Lachancea* and *Torulaspora*). In contrast, the integrated and spontaneous fermentation (FT35) showed greater number of eukaryotic genera, along with less relative abundance of *Saccharomyces* yeasts. In addition, its population has resulted in a lower relative abundance of phytoprotectants and a greater relative abundance of pathogens, h as *Aspergillus*. The organic and inoculated fermentation (FT34) showed lower number of eukaryotic genera, along with a greater relative abundance of *Saccharomyces* yeasts. However, the

eukaryotic population of this fermentation presented a greater relative abundance of pathogens (*Aspergillus*) and a lower relative abundance of phytoprotectors. In contrast, the organic and spontaneous fermentation (FT36) showed a greater number of eukaryotic genera, along with less relative abundance of *Saccharomyces* yeasts. In addition, its population has resulted in a greater relative abundance of phytoprotectants (as *Lachancea* and *Torulaspora*) and a lower relative abundance of pathogens. These results are discordant with those reported by Grangeteau *et al*. 2017 and Martins *et al*. 2016, where lower yeast biodiversity was associated with organic production. However, it is important to stress that as mentioned above the studies of Setati *et al.* 2017 and Padilla *et al*. 2017, reported that the commercial *S. cerevisiae* can suppress and favour a number of non-*Saccharomyces* species (phytopathogens and beneficial microorganisms) a finding that is concordant with the reported in this study.

Touriga Nacional prokaryotic population showed a greater relative abundance of *Alphaproteobacteria* class. As expected, the number of Touriga Nacional prokaryotic classes increased during fermentation.

Comparing Touriga Nacional Initial Must prokaryotic population, the integrated Initial Must show greater number of prokaryotic classes, along with a lower relative abundance of *Alphaproteobacteria* and a greater relative abundance of *Gammaproteobacteria*. In contrast, organic Initial Must showed lower number of prokaryotic classes, along with greater relative abundance of *Alphaproteobacteria*.

At Middle and End of fermentation the prokaryotic populations of Touriga Nacional integrated and organic fermentations were compared according to the applied inoculation process and at Middle of Fermentation, the integrated and inoculated fermentation (FT33) showed the same relative abundance of prokaryotic classes as the integrated and spontaneous fermentation (FT35), along with a higher relative abundance of *Alphaproteobacteria* and a lower relative abundance of *Bacilli* and *Gammaproteobacteria*. The integrated and spontaneous fermentation (FT35) showed the same relative abundance of prokaryotic classes as the integrated and inoculated fermentation (FT33), together with a lower relative abundance of *Alphaproteobacteria* and a greater relative abundance of *Bacilli* and *Gammaproteobacteria*. The organic and inoculated fermentation (FT34) showed the lowest relative abundance of prokaryotic classes, along with the lowest relative abundance of *Alphaproteobacteria* and the highest relative abundance of *Bacilli* and *Gammaproteobacteria*. The

organic and spontaneous fermentation (FT36) showed the highest relative abundance of prokaryotic classes, jointly with the highest relative abundance of *Alphaproteobacteria* and the lowest relative abundance of *Bacilli* and *Gammaproteobacteria*.

At End of Fermentation the integrated and inoculated fermentation (FT33) showed lower number of prokaryotic classes, in conjunction with a greater relative abundance of *Alphaproteobacteria* and *Gammaproteobacteria*, and a lower relative abundance of *Bacilli*. The integrated and spontaneous fermentation (FT35) showed greater number of prokaryotic classes, together with a lower relative abundance of *Alphaproteobacteria* and *Gammaproteobacteria*, and a greater relative abundance of *Bacilli*. The organic and inoculated fermentation (FT34) showed lower number of prokaryotic classes, accompanied with the highest relative abundance of *Alphaproteobacteria* and the lowest relative abundance of *Bacilli*, *Gammaproteobacteria* and *Betaproteobacteria*. The organic and spontaneous fermentation (FT36) showed greater number of prokaryotic classes, together with the lowest relative abundance of *Alphaproteobacteria* and the greatest relative abundance of *Bacilli*, *Gammaproteobacteria* and *Betaproteobacteria*.

All the identified classes are in line with previous studies (Pinto *et al*. 2015; Salvetti *et al*. 2016).

Aragonez and Touriga Nacional Initial Must, Middle and End of Fermentation cultivable yeasts were isolated using YEPD medium and by sequencing the ITS region.

In Aragonez, the Initial Must was the sampling point that reflected a greater number of yeasts species identified, with the Middle and End of Fermentation showing the same yeasts members between them. At integrated Initial Must three yeast species were identified: *Hanseniaspora opuntiae* (50%) *Babjeviella inositovora* (25%) and *S. cf. cerevisiae* (25%). At organic Initial Must three yeast species were identified: *S. cerevisiae* (40%), *Aureobasidium pullulans* (40%) *and Sporidiobolus pararoseus* (20%). At Middle and End of Fermentation only one yeast species was recovered: *S. cerevisiae* (100%).

The low diversity of species identified may be due to limiting factors, such as the use of the unique growth conditions applied (such as growth medium and growth temperature) – other studies have reported that the use of more than one growth medium, more different species are isolated and consequently identified (Padilla *et al*. 2016). As it was expected, the Initial Must showed a higher diversity than the Middle and End of Fermentation, that as expected just showed *S. cerevisiae*, a fact also reported by Padilla *et al*. 2016. The identified species by the culture- dependent approach

are in agreement with the metagenomic data, and also with the reported by several studies, except the presence of *B. inositovora* (Bezerra-Bussoli *et al*. 2013; Brysch-Herzberg and Seidel 2015; Padilla *et al*. 2016).

In Touriga Nacional, the Initial Must was the sampling point that reflected a greater biodiversity of the isolated yeasts, with the Middle and End of Fermentation showing the same diversity between them. In the integrated Initial Must three yeast species were identified: *H. opuntiae* (60%), *S. cerevisiae* (20%), and *H. meyeri* (20%). In the organic Initial Must two yeast species were identified: *H. opuntiae* (75%) and *H. uvarum* (25%). At Middle and End of Fermentation only one yeast specie was identified: *S. cerevisiae* (100%).

As reported above the identification of a restricted number of yeasts species may be due to limiting factors, such as the use of single growth (Padilla *et al*. 2016). As it was expected, the Initial Must showed a significant higher number of species identified in comparison with the Middle and End of Fermentation, that also, as expected only *S. cerevisiae* was recovered (Padilla *et al*. 2016). The identified species are in accordance with the metagenomic data and also in agreement with the reported by previous studies (Bezerra-Bussoli *et al*. 2013; Brysch-Herzberg and Seidel 2015; Padilla *et al*. 2016; Martin *et al*. 2018).

In order to evaluate the ability of the indigenous yeasts consortium to impose against commercial yeast the patterns of the interdelta sequence regions using primer pair delta12 and delta2B were evaluated. In addition, these profiles also provided the control of possible contaminations that may occurred during the inoculation and fermentation processes.

The results showed that the inoculated fermentations (FT13, FT14, FT33 and FT34) shared a very similar interdelta profile with the inoculated commercial yeast profile, having in common nearly all bands between them. All the spontaneous fermentations interdelta profiles (FT15, FT16, FT35 and FT36) shared only two bands with the commercial yeast profile, so spontaneous fermentations were not dominated by the commercial yeast, but by a diverse number of strains similar to the commercial one as reported by Martiniuk *et al.* 2016.

According to the interdelta profiles of Aragonez and Touriga Nacional fermentations, the spontaneous fermentations (FT15 and FT16; FT35 and FT36) were not contaminated with the commercial yeast used on the commercial fermentations (FT13 and FT14; FT33 and FT34), during

the inoculation process nor during the procedures carried out throughout the fermentation process at the pilot unit.

5. Conclusion and Future Perspectives

5 Conclusion and Future Perspectives

This study reports the analysis of the wine microbiome of two Portuguese red wine grape varieties ,Aragonez and Touriga Nacional that were simultaneously produced under two production modes, Organic and Integrated, and the fermentations were carried by two different methods: inoculated with a commercial yeast and driven by spontaneous yeast community. The sampling was performed at three sampling points, namely the Initial Must, Middle and End of Fermentation to evaluate the impact of vitivinicultural practices on wine microbiome during the alcoholic fermentation.

This study demonstrated that the production mode (integrated or organic) influences differently the eukaryotic and prokaryotic populations throughout the fermentation, for both Aragonez and Touriga Nacional.

Concerning the eukaryotic population, the organic production mode favoured a *healthier* microbiome for both grape varieties, but in different ways. In Aragonez, the characteristics provided by the organic production mode provided the necessary conditions for a population with a greater abundance of phytoprotectors. However, in Touriga Nacional the characteristics provided by the organic production mode provided the conditions for the development of a population with a lower abundance of pathogens. Regarding the fermentation process (inoculated or spontaneous), it was possible to observe that the addition of commercial yeast leads to a consequent biodiversity decrease and an increase of *Saccharomyces* yeasts abundance. This relationship was observed in both Aragonez and Touriga Nacional, regardless of the production method.

Concerning the prokaryotic population, the organic production mode showed a restriction on the prokaryotic members in the Initial Must, however, with the progress of fermentation this effect disappears. Regarding the fermentation process (inoculated or spontaneous), in both Aragonez and Touriga Nacional, the addition of a commercial yeast does seem to affect the prokaryotic microbiome.

Once this study was carried out during the first harvest since all the vitivinicultural practices were implemented (production mode and fermentation method), it would be important to give continuity, not only to clarify the questions raised by the observed results, but also to monitor and compare the behaviour of the wine microbiome along time, climate and other variables. This should allow a deeper characterization of the terroir, allowing application of the attained knowledge to

develop more sustainable decisions and processes, allowing the increase of the quality of the final product.

Future work should evaluate the contribution of these findings to the chemical composition of wine in order to identify the synthesise metabolites and aroma compounds in the produced wines (metabolome analysis). Furthermore, is required a sensory analysis by panelists, in order to characterize the impact of the different production modes and fermentation types to the final product quality and differentiation.

In this study, the impacts of different winemaking practices (production method and fermentation methods) on the wine microbiome during fermentation were evaluated. The initially proposed objectives were achieved; however, further comprehensive studies are needed to clarify the relationships identified.

This study not only contributed to a better understanding of the behaviour of two Portuguese grape varieties in a Portuguese wine region, but also allowed to reinforce the importance and the role that the terroir has on the microbiome and in the wine's regionality.

6. References

6 References

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7. Appendix

Table 7.1 – **Phytosanitary treatments applied in the experimental field during the year 2017.**

Administration date	Production Mode	Active substance	Cause
March, 2017	Organic	Cuprous oxide	Downy Mildew prevention
		Sulfur	Downy Mildew and Powdery Mildew
		Iron phosphate	Snail invasion
May, 2017	Organic	Cuprous oxide	Downy Mildew prevention
		Sulfur	Downy Mildew and Powdery Mildew
	Integrated	Dimethomorph Fosetil-Al Fluopyram Iprovalicarb Folpet Tebuconazole	Downy Mildew
June, 2017	Organic	Deltamethrin	*Cicadella viridis* invasion
		Spirodiclofen	Acariasis
	Integrated	Dimethomorph Folpet Tebuconazole	Downy Mildew
July, 2017	Integrated	Deltamethrin	*Cicadella viridis* invasion

Kaolin was administered only in the experimental field of the integrated production mode.

Table 7.2 – **Evolution of the density (g/L) of Aragonez fermentations for the year 2017, from Initial Must to End of Fermentation.**

Stages of fermentation	Days of fermentation	Aragonez fermentations density (g/L)			
		FT13	**FT14**	**FT15**	**FT16**
IM	1	1114	1157	1136	1104
	2	1103	1100	1102	1104
	3	1105	1104	1105	1105
	4	1102	1102	1105	1104
	5	1089.5	1086.2	1105	1103
MF	6	1060	1045	1078	1066
	7	1016	1042	1042	1027
	8	1008.7	1029.1	1023.9	1013.9
	9	999.9	1017.3	1010.9	1002.9
	10	996	1001.2	1010	995.2
	11	993.7	1004.3	998.5	993.9
	12	993.2	999	996.3	994.1

	13	993.1	997.7	995.5	993.3
	14	994.5	997	995.4	994
EF	15	993.8	996.6	995.2	993.6

IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; FT – Fermentation.

Table 7.3 – **Evolution of the density (g/L) of Touriga Nacional fermentations for the year 2017, from Initial Must to End of Fermentation.**

Stages of fermentation	**Days of fermentation**	**Touriga Nacional fermentations density (g/L)**			
		FT33	**FT34**	**FT35**	**FT36**
IM	1	1108	1075	1104	1123
	2	1102	1107	1104	1104
	3	1100	1100	1108	1109
	4	1100	1104	1104	1104
	5	1100	1100	1101	1099
MF	6	1079.3	1092.8	1094	1089.1
	7	1044	1061	1054	1051
	8	1029	1037	1021	1024
	9	1013.5	1024	1007.7	1008.7
	10	1002.3	1014.1	998.9	999.1
EF	11	997.9	1004.9	996.1	996.9

IM – Initial Must; MF – Middle of Fermentation; EF – End of Fermentation; FT – Fermentation.

Table 7.4 Number of cultivable yeasts on Initial Must, Middle and End of Fermentation of Aragonez for the year 2017.

Initial Must (log CFU/ml)			
AG IP		AG OP	
4.45±0.06		6.85±0.06	
Middle of Fermentation (log CFU/ml)			
FT13	FT14	FT15	FT16
8.72±0.11	8.65±0.02	8.67±0.07	8.70±0.14
End of Fermentation (log CFU/ml)			
FT13	FT14	FT15	FT16
6.97±0.21	8.18±0.03	7.86±0.03	6.91±0.02

The data presented result from the mean of 3 replicates. AG OP – Aragonez Organic Production; AG IP – Aragonez Integrated Production; FT – Fermentation; FT13 – Integrated Production/Inoculated fermentation; FT14 – Organic Production/Inoculated fermentation; FT15 – Integrated Production/Spontaneous fermentation; FT16 – Organic Production/Spontaneous fermentation

Table 7.5 Number of cultivable yeasts on Initial Must, Middle and End of Fermentation of Touriga Nacional for the year 2017.

Initial Must (log CFU/ml)			
TN IP		TN OP	
6.22±0.19		3.92±0.01	
Middle of Fermentation (log CFU/ml)			
FT33	FT34	FT35	FT36
8.48±0.10	8.37±0.17	8.44±0.08	8.33±0.13
End of Fermentation (log CFU/ml)			
FT33	FT34	FT35	FT36
7.54±0.07	8.51±0.05	7.57±0.04	7.70±0.16

The data presented result from the mean of 3 replicates. TN OP – Touriga Nacional Organic Production; TN IP – Touriga Nacional Integrated Production; FT – Fermentation; FT33 – Integrated Production/Inoculated fermentation; FT34 – Organic Production/Inoculated fermentation; FT35 – Integrated Production/Spontaneous fermentation; FT36 – Organic Production/Spontaneous fermentation.