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GENETIC AND TECHNOLOGICAL CHARACTERIZATION OF INDIGENOUS *SACCHAROMYCES CEREVISIAE* STRAINS ISOLATED FROM 'MERWAH' WINE (Lebanon)

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INTRODUCTION

1. Spontaneous fermentation

Fermented foods and beverages represent a worldwide category of edible products with a prominent significance for the human economy, nutrition and health for millennia (De Roos and De Vuyst, 2019).

The consumption of fermented foods containing live microorganisms present as natural microbiota has emerged to serve the needs and desires of humans, i.e., to preserve milk, fruits, and vegetables, and to enhance the quality of life with the resultant beverages, cheese, bread, pickled foods, and vinegar (Rezac *et al*., 2018). Moreover, several beneficial effects of food fermentation have been reported including reduced loss of raw materials, reduced cooking time, prolonged shelf-life, enhanced bio-availability of micronutrients, and probiotic effects (Marco *et al,.* 2017; Rezac *et al*., 2018; Motlhanka *et al.,* 2018; Johansen *et al.,* 2019).

The fact that indigenous fermented foods and beverages are easily accessible makes these foods ideal ecosystems for investigating mechanisms of microbial community formation (Wolfe and Dutton, 2015). A huge diversity of microorganisms has been detected in association with spontaneous fermentations all around the world (Tamang *et al*., 2016).

The importance of fermentation is reflected in the amount and variety of foods and beverages traded not only for nutritional benefits and health-promoting effects but also for preservation, safety, and their peculiar appreciated sensory attributes (Bell *et al*., 2017).

Traditional food fermentation represents an extremely valuable cultural heritage in most regions. Indigenous spontaneously fermented food and beverages were produced according to knowledge passed down from one generation to the other without understanding the potential role of the microorganisms involved in the process, in which yeasts are of significant importance (Marco *et al*., 2017; Johansen *et al*., 2019).

Lebanon has a rich fermented food culinary heritage, including but not limited to fermented milk products (i.e., yogurt: Aryan, labneh, cheese, kishk), fermented vegetables (cucumber pickles, sauerkraut and olives), baking bread, and alcoholic fermented beverages (i.e., arak, wine...) (EL-Gendy, 1982; Saleh, 2014; Salemeh and Hosri, 2016).

In the last few years, climate change phenomena associated to global warming had both direct and indirect effects on agricultural productivity which favors a greater use of pesticides that affect the microbial diversity and therefore can have an influence on spontaneous fermented food (Berbegal *et al*., 2019).

For this reason it is important to safeguard the microbial diversity associated to food fermentation. Various scientific evidence on the comparison of spontaneous versus inoculated fermentation processes testified the crucial importance of the starter culture technology to assure food safety worldwide, at all levels of fermented food production (Capozzi *et al.,* 2017). The potential of

microbial activities as mitigating strategies in the wine sector renews interest in the continuous exploration of microbial diversity-associated to specific *terroirs,* autochthonous grapevines, and natural wines (Berbegal *et al.,* 2019).

2. Lebanese oenology

Through history, phoenician spread their knowledge of wine making and viticulture to several regions that today have significant wine making industries, like Lebanon, Algeria, Tunisia, Egypt, Greece, Italy, Spain, France and Portugal (Johnson, 2015).

In addition to the diversity of the climate and the availability of favorable soil structure sandy in the coastal plain, sandstone and clay-sandstone in central Lebanon, and clayey-limestone in Bekaa (Gauthier *et al.,* 2011), there is a low parasitic pressure which makes possible to produce high-quality grapes for winemaking, thereby reducing the cost of phytosanitary treatment and promoting the production of organic grapes (ENITA, 2003).

Grape vine has been cultivated in Lebanon for at least 5,000 years. Currently, the cultivated area of wine grapes is estimated at 3000 hectares using over 25 different international and local grape varieties (ENITA, 2003) of which 80% are red grape varieties (such as the Cinsault, Cabernet-Sauvignon, Cabernet Franc, Merlot, Syrah, Carignan...) and 20% of white grape varieties (the white Ugni, the white Sauvignon, Chardonnay, Clairette, Obeideh and Merwah (Chalak *et al*., 2016).

However, Lebanon has a rich heritage of native grape varieties such as ‘Merwah’ and ‘Obeideh’ which is very used in Arak and winemaking production (Chalak *et al.,* 2016). Such varieties thrive at altitudes of 600 meters and above. ‘Merwah’ grape (Figure 1) is known by its resistance to frost, to Oïdium and Mildiou attack and is usually cultivated in a mountainous region (Musallem, 2018).

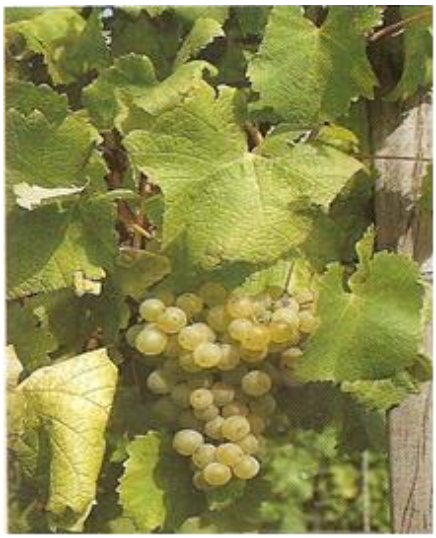

Figure 1: Merwah is characterized by long-sized, scattered clusters; round to oval berries, golden yellow color; cultivated as goblet size mode.

Despite the many conflicts of the region, the country has developed this sector, where a growing wine production is observed in last 10 years with an increasing number of wineries with over 50 domains across the country and produced approximately 28.7 million tonnes (Chbeir and Mikhael, 2019). According to the *Union Viticole du Liban* (UVL) website, Lebanon produce around 8.5 million bottles annually where approximately 50% of these are exported to worldwide destinations and the other 50% go into local market consumption. 'UVL' was founded in 1997, one year after Lebanon joined the *Organisation International de la Vigne et du Vin* (OIV), as the official association of Lebanese wine producers. It has developed legitimacy for Lebanon's export ambitions within the EU and other international markets like the US and Canada. The majority of Lebanon's wine producers export a hefty percentage of their bottles, ranging from 20 to 60 percent according to the wineries interviewed (Rahhal, 2019).

Wine quality depends on the environmental characteristics of where the grapes are grown. Many factors are involved such as climatic conditions, soil composition (geology, soil type and soil depth) and topography. All these factors act simultaneously, and they interact.

If each *terroir* factor is studied separately, studies remain highly descriptive and fail to explain why wine shows such extraordinary sensory diversity (Van Leeuwen *et al.,* 2018).

Nowadays, there are more demands for wines that express their *terroir*, and which can be distinguished from each other (Marlowe and Baumen, 2019).

In the last few decades, the climate change and uncontrolled environment may have affected the ecology of the microbial community present naturally in food products and consequently the quality of fermented products (Tamang *et al.,* 2016; Berbegal *et al*., 2019).

Therefore, the use of indigenous selected starters represents a useful tool to control alcoholic grape must fermentation, safeguarding the typical sensory characteristics of wine produced from specific regions (Capece *et al*., 2019).

In this perspective, studying the diversity of the microbial community associated with this native grape variety, isolating and using indigenous strains in winemaking could contribute to Lebanese native wines and help traditional wineries to preserve their local heritage.

3. Approach of wine appellation: natural, organic, biodynamic wines

There have been many changes in winemaking several thousand years ago. Currently, winemakers have realized about the importance of preserving the biodiversity during wine process by changing from conventional winemaking to create something truly special.

Generally, three styles of wines (organic, biodynamic and natural wine) use traditional winemaking methods that predate modern technology to make wine that gets the most out of nature. These wine-styles are based on sustainable agriculture and the respect of land and nature. In addition, these wines

are made with minimal intervention and truly reflect their *terroir*, which includes climate, soil and vintage (Merlot, 2018).

Organic wine is made from organically grown grapes that were grown without any herbicides, pesticides or other non-organic soil treatments and shouldn't have any genetically modified ingredients, such as yeast. Organic certification in the vineyard means the farmer only uses naturally occurring non-synthetic inputs (Mann *et al.,* 2012). There is no global certifying body for organic wine and each country has developed its own set of rules for organic farming. These rules will take into consideration factors such as the geographical location, the climate, the existing legal regulations as well as the farming tradition and philosophy of the local grape growing farmers (Sanders *et al.,* 2013).

However, the U.S. has different standards for organic wine than Europe and Canada. The U.S. does not allow added sulfites in organic wine, while Europe and Canada do allow the addition of sulfites in small amounts (McEvoy, 2017). In Europe, The International Organisation of Vine & Wine (OIV) is an intergovernmental science and technical agency which has made standards for organic wine. The OIV states that organic viticulture must look to maintain and protect ecosystems, soil fertility, natural resources, increase biodiversity and promote ecological cycles. Sulfite may be added to these wines up to a maximum of 100 parts per million. To be considered an organic wine, the limits of sulfite are reduced of 30 to 50 mg/L, according to EU Regulation No. 203/2012 (Lisanti *et al.,* 2019). Organically fermented wines are also based on wild ferments or organically certified commercial yeasts which permit to quickly start the fermentation.

While, the biodynamic is a holistic approach that believes to find the natural rhythm of the farm and prevent problems before they occur. A biodynamic farm is inherently organic, but biodynamic certification goes much further by addressing the health of the soil, encouraging vigorous populations of microbes and embracing the natural structure of the earth. Biodynamic certification is closely regulated by Demeter USA (Sinskey, 2011). In organic and biodynamic wine process, the use of sulfite is much lower levels than conventional winemaking and without any chemical inputs during spontaneous fermentation (Goode and Harrop 2011). The allowable levels of sulfur are much less in organic wine (less than 100 ppm) than conventionally farmed wine (Sinskey, 2011). Noting that sulfites are present in small quantities in all wines because of natural production of SO_2 by some yeast strains such *S. cerevisiae* during fermentation process (Pezley, 2015).

The term "natural wine" is often used. But there are no legal standards for this designation, and it is even forbidden in some EU member states. A 'true' natural wine could be considered one produced using organic (or biodynamic) principles with a minimum of technological intervention. Private standards (e.g., Demeter International in the EU; Bioland, Germany; Naturland, Germany; AIAB, Italy; Delinat, Switzerland; Bio Suisse, Switzerland; Bio Coherence, France) are appreciated by many winemakers and are recognised by many consumers as indications of quality wine which authentically express *terroir* (Szeremeta *et al.,* 2013). Producing organic or natural wines is a sustainable way to

preserve yeast diversity present on the grapes and during winemaking process in order to preserve the *terroir.*

4. Yeast associated to spontaneous wine fermentation

Several studies showed the positive effects of spontaneous fermentations on the organoleptic complexity of wine, providing wine with unique regional characters; because of the growth of natural microbial community (Francesca *et al.,* 2016; Tempère *et al.,* 2018).

Among oenological yeasts, the following categories can be found: (i) Yeast belonging to the *Saccharomyces* genus, and particularly to the *Saccharomyces cerevisiae* species, which are mainly responsible for alcoholic fermentation in wine and (ii) the heterogeneous category of the so-called non-*Saccharomyces* yeasts (Berbegal *et al.,* 2017).

The complexity of indigenous non-*Saccharomyces* and *Saccharomyces* yeast microbiota present during spontaneous grape must fermentation has a major impact on the organoleptic and sensory properties of the final wines (Jolly *et al*., 2013; Padilla *et al.,* 2016; Varela and Borneman, 2016).

Traditional winemaking is characterized by spontaneous fermentations of grape must with naturally occurring microflora or what is known as wild yeast. As revealed by several studies, the main yeast genera associated with grapes are: *Hanseniaspora*, with lesser representations of *Candida*, *Metchnikowia*, *Cryptococcus*, *Pichia* and *Lachancea* which were found to decline rapidly at the initial or middle stages of fermentation; while others exist until the end of fermentation, including *Saccharomyces cerevisiae, Torulaspora delbrueckii, Klyveromyces marxianus* and *Streptomyces bacillaris* (Bagheri *et al.,* 2015; Grangeteau *et al.,* 2016; Hranilovic *et al.,* 2018; Anagnostopoulos *et al.,* 2019). Their growth is important and can influence the chemical composition of the wine (Walker, 2014).

The species *S. cerevisiae* is the best adapted to carry out alcoholic fermentation, whereas some species intervene before (*Rhodotorula sp* ...), and others (*B. bruxellensis, ZygoSaccharomyces bailii)* can cause microbial alterations of wine (Escott *et al.,* 2018). Despite the presence of a large number of non-*Saccharomyces* yeast species at the beginning of the fermentation process, *Saccharomyces cerevisiae* will remain the predominant species in wine alcoholic fermentations (Walker and Stewart, 2016).

5. Genetic diversity and origin of wine *Saccharomyces cerevisiae* strains

The genetic diversity within the population of *Saccharomyces cerevisiae* has been well documented (Schuller *et al*., 2012; Peter and Schacherer, 2016; Viel *et al*., 2017; Tra Bi *et al.,* 2019).

Currently, industrial yeasts can be divided into five sub-lineages (beer, wine, bread, spirits, sake), that are genetically and phenotypically separated from wild strains and originate from only a few ancestors

through complex patterns of domestication and local divergence (Gallone *et al.,* 2016). *Saccharomyces cerevisiae* has traditionally been the main yeast responsible for most fermentation processes. However, the market is changing due to climate change and consumer demands. The solutions were the application of yeast hybrids (Peris *et al.,* 2018).

Some studies have suggested that *Saccharomyces cerevisiae* diverged from the common ancestor of *Saccharomyces paradoxus* and *Saccharomyces cariocanus* approximately 5–10 million years ago (Mya), whereas *Saccharomyces kudriavzevii*, *Saccharomyces bayanus,* and *Saccharomyces mikatae* diverged 10–15, 15–20, and 20 Mya, respectively (Tofalo *et al.,* 2013; Nguyen and Boekhout, 2017). Recently, a new species similar to *Saccharomyces bayanus* (Libkind *et al.,* 2011) and called *Saccharomyces eubayanus* existing in apparent sympathy in Patagonia forests has been identified (Gayevskiy and Goddard, 2016). The widely used lager yeast is a *S. cerevisiae* x *S. eubayaneus* hybrid, and many *S. cerevisiae* x *S. kudriavzevii* have been isolated from beers as well (Langdon *et al.,* 2019). In addition, *S. bayanus* appears to be a hybrid of three species: *S. cerevisiae, S. eubayanus*, and *S. uvarum*, and may have arisen from a rare mating of *S. pastorianus* and *S. uvarum* (Bisson, 2017). Thus, yeast hybrids arise commonly in nature.

Alternative *Saccharomyces* species, such as *S. uvarum* and *S. kudriavzevii*, and their hybrids with *S. cerevisiae*, exhibit good fermentative capabilities at low temperature, and produce wines with lower alcohol concentration, higher glycerol amounts, excellent aromatic profiles and interesting properties for the wine industry (Peris *et al.,* 2016).

However, *S. cerevisiae* is not domesticated as a whole, and population genetics analysis reflects different ecological niches; a growing number of wild isolates is continuously offering new insights into the ecological distribution, population structure and biogeography of this species (Marsit and Dequin, 2015).

The dissemination of natural *S. cerevisiae* was associated to human and animal vectors (Borlin *et al*., 2016). The dissemination of yeast by migratory birds and social wasps might contribute to the biodiversity in many geographical locations and to the differences observed in the vineyard ecosystem (Francesca *et al*., 2016; Peter *et al*., 2018).

The results of population genetics analysis of wild Chinese yeast isolates with different ecological and geographical origins indicate that a geographically isolated source is important for *S. cerevisiae* population differentiation in nature (Wang *et al.,* 2012).

A genomic survey of a higher number of strains suggested a model of geographic differentiation, followed by human-associated admixture, primarily occurring between European and Asian populations and more recently between European and North American populations (Cromie *et al*., 2013). Other studies suggested that wine yeast strains could have a Mesopotamian origin (Legras *et al.,* 2007; Sicard and Legras, 2011). According to Legras *et al.,* (2007), there has been a migration of wine yeast strains through the Mediterranean Sea, from Lebanon to Central Europe (Italy, France and Spain), and in France from the Mediterranean coast to Burgundy through the Rhone valley, and

another migration route through the Danube valley. Yeast strains could have also been transferred via co-migration with grape varieties (Sicard and Legras, 2011). The identification of three Chinese wild isolates belonging to the Wine/European lineage by Wang *et al.,* (2012) led these authors to raise the possibility that Wine/European strains have an Asian origin, in line with previous archaeological evidence for fermented beverages in China dated to 9000 years ago (McGovern *et al*., 2004). However, these isolates were sampled from orchard soil and grape and might not be truly natural. The opposite hypothesis stipulating that the wine yeast could have migrated to Asia is also plausible (Marsit and Dequin, 2015).

6. Monitoring and evaluation of yeast diversity during spontaneous wine fermentation

Wild microflora can produce in spontaneous alcoholic fermentation an excellent wine with interesting characteristics by a complexity of flavors, intense aroma persistency, interesting alcoholic degree, vintage variability and a suitable aromatic profile, that can be produced with indigenous *Saccharomyces cerevisiae* strains (Ribéreau-Gayon *et al.,* 2007; Cus *et al.,* 2017). On the contrary, it is difficult to predict spontaneous fermentation with natural microflora, causing multiple complications and uncontrolled fermentation.

However, spontaneous wine fermentation is exposed to a risk of stuck or sluggish fermentations that can occur by spoilage yeast and/or bacterial strains that negatively affect the quality of the final products (Padilla *et al*., 2016).

The monitoring of the different microbial populations allowed us to understand the interactions between the different microorganisms during fermentation.

6.1. Factor that impact yeast diversity

Several key factors have been shown to influence the types of yeasts present and their persistence during fermentation. The biodiversity of yeasts associated with grapes has been studied in different regions of wine producing countries throughout the world. Most of the species associated with the wine environment are similar, while some species are specifically associated with specific regions (Sipiczki, 2011).

The identity and relative abundance of indigenous yeast species in grapes are considered to be dependent on the *terroir* (e.g., soil type, annual mean temperature, and rainfall, etc.), the ripeness and health of the grapes, as well as the production procedures in the vineyards (Bokulich *et al.,* 2014; Capece *et al.,* 2016; Drumonde-Neves *et al.,* 2016; Grangeteau *et al.,* 2016). Additionally, it was demonstrated the effect of vintage on the microbial diversity regarding the geographical site (*terroir*) (Vigentini *et al.,* 2015).

Furthermore, several studies have shown that chemical composition of the initial must, biotic factors (e.g., microorganisms, killer factors, migratory birds, grape varieties), abiotic factors (e.g., pH, temperature, ethanol, osmotic pressure, nitrogen, molecular sulphur dioxide) and antropogenic factors (eg., use of commercial starters) may affect the diversity of an *S. cerevisiae* population (Setati *et al.,* 2012; Schuller *et al.,* 2012; Ciani *et al.,* 2016; Francesca *et al.,* 2016; Rodríguez-Varela *et al.,* 2017).

Currently, climate change affects grape and wine production and quality; influences the proliferation of certain viticulture pathogens, introducing new insight into pest management in the field (De Orduña *et al.,* 2010). It also entails considerable problems for the sustainability of oenology in several geographical regions, also placing at risk the wine typicity (Berbegal *et al.,* 2019).

There are four main vineyard factors that will affect the fermentation and therefore the management strategies: (i) the nutritional and chemical composition of the fruit, (ii) level and type of berry/cluster damage (a low percentage of damaged clusters can have a negative impact on the microbial flora of fermentation), (iii) the invasion by fruit-visiting insects pre-harvest, and (iv) the use of antimicrobial compounds too close to harvest (Barata *et al.,* 2012). Furthermore, vineyards managed with organic practices showed intermediate to low levels of *S. cerevisiae* strain diversity, whereas conventional practices showed higher levels (De Celis *et al.,* 2019).

Moreover, the use of pesticides have a negative effect on the diversity of the yeast community in grapes and musts (Čus and Raspor, 2008; Setati *et al.,* 2012).

Thus, it has been shown that, for the same efficiency, yeast strains were more sensitive to synthetic treatment than to natural ones. In particular *S. cerevisiae* showed minimum sulfur inhibitory concentrations 200 times lower (> 10 mg/L) than those for penconazole (< 0.05 mg/L) (Cordero-Bueso *et al*., 2014).

In addition, harvesting techniques can also impact the yeasts present in the fermentation, particularly if the berries are damaged during harvest and microbial growth occurs during shipping to the winery (König *et al.,* 2009). Oenological practices such as juice clarification treatment and the type of clarification can influence the interspecific diversity of *S. cerevisiae* and change the frequency of occurrence of dominant strains directly or indirectly by affecting the maceration or oxidation of musts in either of the filter types (Capece *et al.,* 2016). Fermentation conditions and must wine composition could also influence diversity, as well as the interactions between strains and certain factors intrinsic to strains (Dutraive *et al*., 2019). In addition, lowering fermentation temperature can be a better way to control microbial growth and minimize potential spoilage (Jackson, 2014).

The low pH of wine, high sugar content, rapidly generated anaerobic conditions, and presence of phenolic compounds creates the ideal environment to support the growth of yeasts and to enrich these organisms over other microbes (Claus and Mojsov, 2018).

Nowadays, the hygienic practices used in the modern cellars seem to minimize the contamination by the resident cellar flora and, therefore, its diversity (Andorrà *et al.,* 2019).

Other studies suggested the "winery effect", where the winemaking environment can influence the population of cellar habitats, such as wall surfaces, equipment and oak barrels, among others (Andorrà *et al.,* 2019). Some predominant *S. cerevisiae* strains, recovered from spontaneous fermentation in the same winery, could occur over a year, assuming that might have some correlation between strain and winery environment (Capozzi *et al.,* 2015).

6.2. Importance of isolation, preservation and exploitation of local indigenous yeast populations

In order to improve the local heritage, it is important to characterize indigenous yeast as a tool for differentiation, diversification, and quality improvement of wines.

The monitoring of the microbial populations and the control of the spoilage yeasts, focused on the selection and utilization of the starter cultures coming from one's own vineyard, which can enhance the regional character of the wine (Tristezza *et al.,* 2014).

Strains of *Saccharomyces cerevisiae* exert a profound influence on the flavor and aroma characteristics of different fermented beverages (Walker and Stewart, 2016).

Modern large-scale wineries use specially selected starter cultures of *S. cerevisiae* strains available in dried form (e.g., active dry yeast, ADY) which is the most widespread practice in winemaking, because of their very good fermentation and oenological capacities (Ciani *et al*., 2016; Capece *et al*., 2019).

A side-effect of the widespread use of these commercial starter cultures is the elimination of native microbiota, which might result in wines with similar analytical and sensory properties, depriving them from the variability and diversity that define the typicality of a wine (Guillamón and Barrio, 2017).

Recently, winemakers have realized the importance of the indigenous microbial population closely related to the geographical origin which produces a wine of *terroir* (Tristezza *et al.,* 2013; Peter *et al.,* 2018). An increasing demand of organic wine requires winemakers to preserve spontaneous microflora which is essential to obtain the typical flavor and aroma of wines derived from different grape varieties (Capozzi *et al.,* 2015).

The combination of the microbial evolution along with metabolic and sensorial characterization of the produced wines could lead to the suggestion of the microbial *terroir* (Anagnostopoulos *et al.,* 2019).

The potential of microbial activities on the production of organic wines could be a mitigation strategy in the wine sector renovating interest in the continuous exploration of indigenous strains diversity associated to specific *terroirs*. This exploitation has great importance for the characterization and selection of strains with peculiar oenological features (Grieco *et al.,* 2011; Tristezza *et al.,* 2014; Berbegal *et al.,* 2019).

The use of selected indigenous *S. cerevisiae* strains play important roles in winemaking in order to ensure a reproducible product, to preserve biodiversity by employing the most representative strains and to reduce the risk of wine spoilage (Capozzi *et al.,* 2012).

Actually, winemakers are constantly searching for new techniques to modulate wine style and the exploitation of indigenous or "wild" yeasts are perceived to be more complex by showing a greater diversity of flavors (Liu *et al.,* 2016).

6.3. Molecular methods used in the evaluation of *Saccharomyces cerevisiae* yeast diversity

Currently, an important task to understand biodiversity, population structure and evolutionary history of wine yeasts is the study of the molecular mechanisms involved in yeast adaptation to wine fermentation, and on remodeling the genomic features of wine yeast, unconsciously selected since the advent of winemaking (Guillamón and Barrio, 2017).

Genetic diversity of *S. cerevisiae* strains was analysed through several molecular methods such as karyotype determination of strains by pulsed-field gel electrophoresis, mitochondrial DNA restriction analysis (RFLP mtDNA) (Lopez *et al.,* 2001), amplification of the regions between two transposon delta LTR region (Ness *et al.,* 1993; Legras, and Krast, 2003), fingerprinting and genotyping of microsatellite markers (Legras *et al.,* 2007). In addition, MLST (Multi Locus Sequence Typing) has been applied to differentiate between individuals and thus the typing of strains while allowing to deduce phylogenetic relationships in *S. cerevisiae* population, (Fay and Benavides, 2005; Ayoub *et al.,* 2006).

The results of this method have not showed superior yeast differentiation ability in comparison to interdelta PCRs or microsatellite polymorphisms (Ayoub *et al.,* 2006) but allowed to reveal the particular population structure of the yeasts of wine or sake, indicating the domestication of these yeasts (Fay and Benavides, 2005).

Recently, authors determine the sequences of regions between randomly selected restriction sites in the genome (Restriction site Associated DNA SEQuencing or RADSeq). The RAD-seq method allowed the study of the diversity and phylogenetic structure of the S. *cerevisiae* strains from various ecological niches, creating large open databases (Hyma and Fay, 2013).

The use of interdelta PCR and microsatellite are the molecular genotype methods with the higher discriminant power. These methods are the more appropriate for population analysis and have been frequently used to understand *S. cerevisiae* population dynamics (Goddard *et al*., 2010; Borlin *et al*., 2016).

6.3.1. PCR amplification of sequences between two delta LTRs

Interdelta sequencing typing uses the variation of the number and position of the delta element, corresponding to the Long Terminal Repeat (LTR) sequence flanking the retro-transposons Ty1/Ty2 present in several copies in the genome of the yeast *Saccharomyces cerevisiae* (Curcio *et al.,* 2015). These primers are used to better discriminate and interpret strain similarities and evolutionary or adaptive distance (Legras and Karst, 2003).

However, these delta sequences are concentrated in the genomic region between the tRNA genes. The number and position of these elements showed intraspecific variability that Ness *et al.* (1993) used to develop the PCR primers δ1 and δ2 useful for the differentiation of *S. cerevisiae* strains. Legras and Karst (2003) optimized this technique by designing two new primers: δ12 and δ21 that were close to δ1 and δ2. The use of δ12 and δ21 or δ12 with δ2 highlights a greater polymorphism if translated by a larger number of bands on electrophoresis gel (Xufre *et al*., 2011).

6.3.2. Typing with microsatellite markers

Microsatellites are short sequences, composed of repetitions in tandem from one to ten nucleotides. These sequences are disseminated in the genome of yeast, both in the coding and non-coding regions, but their concentration is lower in the coding regions (Saeed *et al.,* 2015). Microsatellite markers are loci polymorphs whose allelic diversity makes it possible to differentiate strains of the same species of yeast. They are frequently used as genetic markers in genetic mapping studies and in genetics populations (Borlin *et al*., 2016).

The combination of six microsatellite loci proves to be a highly discriminating and reproducible technique, and at the same time geographic and technological relationships between strains (Legras *et al.,* 2007). These polymorphic loci can be easily used to determine the profile of *S. cerevisiae* strains during fermentation, with the aim of identifying the most polymorphic loci with a high allelic diversity that can be used for both strain identification and the establishment of strain geographical or technological origin. Several studies used this approach to type *S. cerevisiae* strains of different geographical origins (Bradbury *et al.,* 2006; Legras *et al.,* 2005; Schuller *et al.,* 2007, 2012; Tofalo *et al.,* 2015; Peter *et al.,* 2018).

This genotyping method is effective for identifying very close genetic relationships between yeast strains (Viel *et al.,* 2017). The genetics differences and population structures among *S. cerevisiae* strains are derived from cumulative small microsatellite allele-frequency differences (Schuller *et al.,* 2012).

The instability of microsatellites can be explained by unequal recombination phenomena induced by the pairing of microsatellite repeats or by the slip of the polymerase during replication (Ayoub *et al*., 2006). Sometimes, the genotypes that could not be resolved by microsatellite profiles may be discriminate by interdelta PCR or by sequence analysis (Ayoub *et al.,* 2006).

7. Sulphite resistance in *Saccharomyces cerevisiae* strains

Sulfur dioxide SO_2 has been used for hundred years in winemaking process for its antimicrobial and antioxidant properties. It is added at different times throughout the winemaking process, including prior to inoculation with commercial yeast strains, in order to suppress any potential spoilage microorganisms from the grape must (Morgan *et al.,* 2019).

The application of high concentrations of SO_2 may influence the growth fermented yeasts growth by extending the lag phase (Zimmer *et al.,* 2014).

In addition, residual sulfur on grape has been observed to contribute an offensive odour and taste, and to the formation of H_2S during fermentation, and mercaptans under extended yeast lees ageing can increase at higher SO_2 concentrations.

During alcoholic fermentation, wine yeasts can produce in variable amounts undesirable sulfur compounds such as SO_2 and H_2S (Noble *et al.,* 2015). The formation of H_2S during fermentation has been suggested to be associated to the presence of elemental sulfur in the grape must, that may be spontaneously converted to sulfite under the anaerobic and low pH fermentation conditions (Muller and Rauhut 2018). Several translocation have been described as typical example of yeast domestication and have been detected among wine strains and not "natural isolates" (i.e; isolated from oak) (i.e; between chromosome VIII and XVI or XV and XVI). The 'biocontrol spontaneous fermentation' is a potential alternative to the use of SO_2 during winemaking process (De Ullivarri *et al.,* 2014). Selected starter/protective culture is usually added to control the winemaking process and to achieve specific desired oenological traits, and to assure worldwide food safety at all levels of fermented food production: household, traditional, and industrial (Capozzi *et al*., 2017; Ciani and Comitini, 2019).

Different yeast strains may respond differently to SO_2 addition, and it is important for winemakers to know which yeasts respond in favourable ways so they may select appropriate strains to use as inoculants. Usually commercial *Saccharomyces cerevisiae* strains are more tolerant than non-commercial (Morgan *et al.,* 2019).

Furthermore, yeasts usually produce low-to-medium SO_2 amounts, depending on their genetic features and on fermentation conditions. Wine yeasts can cope with SO_2 by different systems, such as: acetaldehyde production (that binds to the SO_2 inactivating it), production of glutathione, sulfite uptake and reduction or SO_2 export from the cell via a membrane transporter dedicated (SSU1 pump). It was also found that endogenous free SO_2 contributions are inadequate for long-term wine protection and appropriate SO_2 levels should be maintained utilizing exogenous SO_2 (Pezley, 2015).

8. Investment into the future of microbial resources: culture collection and BRC

The application of microorganisms in several industrial biotechnology, have produced high product value and may have a crucial role in ecosystems (Singh *et al.,* 2016; Vitorino and Bessa 2017). For this reason, microbial biotechnology requires the existence of microbial culture collections (MCC) since microorganisms maintained in them provide biomolecules as well as sources of compounds with a wide variety of research and industrial applications. It is an important network for conserving and preserving traditional microbial community (Smith *et al.,* 2014). The MCC's are a key component of life science research, biotechnology, and emerging global biobased economies. MCC's have a crucial role in maintaining, understanding conserving and utilizing the sustainable microbial diversity (Sharma and Shouche, 2014).

The first culture collection was established by Frantisek Král in 1890 at the German University of Prague. After Král's collection, many culture collections were established, such as the Mycothèque de l'Université Catholique de Louvain (MUCL) established in 1894, in Louvain-la Neuve, Belgium, and the Collection of the Central bureau voor Schimmel cultures (CBS) founded in 1906, in Utrecht, the Netherlands. Currently there are around 568 culture collections in 68 countries registered in the World Data Center for Microorganisms (WDCM) (ÇaktüK and Türkoğlu, 2011).

Culture collections still provide a significant degree of continuity with the past through the preservation and distribution of microbial strains described or cited in publications. Collections often maintain novel microorganisms awaiting future exploitation by industrial biotechnology (Boundy-Mills *et al*., 2016). The culture collection has been established to preserve and sustain biological resources in natural forms and ensure their proper usage (Smith 2012). The conservation of microbial community is present usually in BRC (Biological Resources Centers), which is a public infrastructure for culture collection, research and utilization of microorganisms and sustaining biotechnology innovations (Smith *et al.,* 2014). It has established collaborations with many culture collection centers worldwide, which can provide various services to institutes/universities and industries like supply of cultures, identification services and educational services, where the culture data is accessible to the microbiological research community via printed or online catalogues (Cánovas and Ibarra, 2003).

The main objectives of the microbial culture in BRC are the following:

- Focusing on basic research in the areas of microbial diversity, microbial taxonomy, microbial genomics and proteomics etc.
- Isolation and identification of microorganisms from various environmental niches.
- Preservation of microbial biodiversity from niche areas as metagenomic libraries.
- Development of new strategies for isolation of "not yet cultured" microbes.

To provide consultation services for patent deposits, preservation, propagation, bio deterioration, industrial problems, biosystematics and microbial biodiversity issues etc.

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AIM OF THE STUDY

Considering the limited knowledge of yeast diversity in Lebanese indigenous fermented foods and beverages, this study focuses on the isolation, identification and characterization of indigenous *Saccharomyces cerevisiae* strains isolated from Lebanese native wine 'Merwah'. The wine sector is currently showing an increased production in both quality and quantity. This could solve part of the actual economic crisis in Lebanon by favoring export of Lebanese wines.

Regarding the climate changes that affect the local microbial communities in oenology sector, it is important to select and inoculate indigenous *Saccharomyces cerevisiae* strains adapted to a specific wine region. This is a useful tool to preserve and exploit microbial diversity, to control alcoholic grape must fermentation, to improve the quality parameters and to safeguard the typical sensory characteristics of wine produced from specific regions.

For this purpose, genetic and technological characterization of the indigenous *S. cerevisiae* isolates was evaluated during spontaneous fermentation of 'Merwah' wine. The evaluation of the genetic diversity was carried out to select starter strains that can be useful in Lebanese wine production; in order to help traditional wineries to preserve the patrimony of yeast biodiversity. In addition, sulfite resistance, an important criterion in the selection of strain starter culture, has been evaluated genetically (Chromosomal translocation) and phenotypically in the studied *S. cerevisiae* strains. At the end of this study, selected indigenous Lebanese yeast strain(s) can be screened for its enological and aromatic potential as starters culture to produce wines with regional characteristics.

This study represents an important step to establish a Lebanese culture collection of indigenous *S. cerevisiae* strains isolated from a unique environment, such as 'Merwah' wine cultivar.

GENETIC AND PHENOTYPIC CHARACTERISATION OF A *SACCHAROMYCES CEREVISIAE* POPULATION OF MERWAH WHITE WINE (Published article)

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Article

Genetic and Phenotypic Characterisation of a *Saccharomyces cerevisiae* Population of 'Merwah' White Wine

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Abstract: The study of yeast biodiversity represents an important step in the preservation of the local heritage, and this work in particular has an innovative character since no further studies have investigated 'Merwah', one of the main grape varieties used in winemaking in Lebanon. To gain deeper knowledge of the genetic diversity and population structure of native *Saccharomyces cerevisiae* wine strains, 202 isolates were collected during spontaneous alcoholic fermentation of eight must/wine samples of cultivar 'Merwah', over two consecutive years (2016, 2017) in a traditional winery in Mount Lebanon (1400 m a.s.l.). The isolates were identified as *S. cerevisiae* on the basis of their morphology and preliminary sequence analysis of their internal transcribed spacer (ITS) PCR. They were then characterised at the strain level by interdelta PCR and genotyped using multiplex PCR reactions of 12 microsatellite markers. High genetic diversity was observed for the studied population. To select potential yeast starter strains from this population, micro-fermentations were carried out for 22 *S. cerevisiae* strains that were selected as representative of the 'Merwah' wine yeast population in order to determine their technological and oenological properties. Three indigenous yeast strains might represent candidates for pilot-scale fermentation in the winery, based on relevant features such as high fermentation vigour, low production of volatile acidity and H_2S and low residual sugar content at the end of alcoholic fermentation.

Keywords: native yeast; spontaneous fermentation; genotype–phenotype diversity; regional wine

1. Introduction

In recent years, many wineries have been using selected indigenous yeast strains, which might persist more easily and dominate over other yeast strains. The inoculation of fermentation with autochthonous *Saccharomyces cerevisiae* strains provides distinctive characteristics to the wine and helps to preserve the native yeast strains that are better adapted to the environment of the viticulture region and to the winemaking process [1,2]. In addition, *S. cerevisiae* strains identified in different wineries in the same wine region are considered to be representative of an oenological zone [3].

Several studies have shown that grape varieties, geographic location, climate conditions, the chemical composition of the initial must, biotic factors (e.g., microorganisms, killer factors, migratory birds, grape varieties), abiotic factors (e.g., pH, temperature, ethanol, osmotic pressure,

nitrogen, molecular sulphur dioxide) and anthropogenic factors (e.g., use of commercial starters) can affect the diversity of an *S. cerevisiae* population [4–11].

Lebanon is at the eastern extremity of the Mediterranean and is one of the oldest of the countries of the Old World. The land is particularly favourable for the cultivation of grapevines, which are among the oldest cultivated [12]. Lebanon has a rich heritage of native grape varieties, such as 'Merwah', 'Obeidi', 'Tfeifihi', 'Beitamouni' and 'Maghdouchi'. Of these, the main local grape varieties used in winemaking are 'Merwah' and 'Obeidi', which have attracted the attention of wine consumers [13]. The presence of such indigenous grape varieties allows the determination of the genetic map of Lebanon. To date, and to the best of our knowledge, no previous studies have investigated 'Merwah' wines. Therefore, in the present study, the genetic and phenotypic characteristics of Lebanese indigenous *S. cerevisiae* wine yeast were evaluated for yeast ecology during spontaneous fermentation, and the indigenous yeast were screened for use as potential starters for the production of wines with regional characteristics.

The characterisation of the autochthonous *S. cerevisiae* strains is an important step towards the conservation and exploitation of microbial biodiversity. The aim was therefore to evaluate the genetic diversity and relatedness among *Saccharomyces cerevisiae* strains isolated from 'Merwah' wine, in order to select starter strains that be useful in Lebanese wine production. For this objective, 202 isolates were collected from eight must/wine samples of 'Merwah' grapes during spontaneous alcoholic fermentation over two consecutive years (2016, 2017), from the 'Château Bybline' Lebanese winery.

Genetic characterisation was performed using PCR amplification of the interdelta sequences. In this large population of isolates, 22 yeast strains were selected as representative of the major strain clusters of the interdelta sequence profiles. The identification of *S. cerevisiae* was confirmed by PCR–restriction fragment length polymorphism of the 5.8S internal transcribed spacer (ITS) rDNA, with all of the 202 isolates confirmed as *S. cerevisiae* [14]. In addition, the yeast isolates were genotyped for 12 microsatellite markers to provide a deeper analysis of their genetic diversity and the population structure.

The technological characterisation of these yeast were evaluated in synthetic grape juice (SGJ) at the laboratory scale, according to the main parameters (i.e., residual sugar, fermentative vigour and kinetics, lowest production of sulphur products (H_2S, SO_2), acetic acid) in order to select the *S. cerevisiae* strains that might be used as starter cultures at an industrial scale.

2. Materials and Methods

2.1. Winery and Winemaking Process

This study was carried out at the 'Château Bybline' winery, where the 'Merwah' autochthonous white grape variety is planted around the estate (altitude, 1400 m a.s.l.). 'Merwah' grapes were harvested from two vineyards at Wata al Jawz (2000 m^2) and Bekaatet Achkout (2000 m^2), in Keserwan, Mount Lebanon region. This winery has been using traditional viticulture practices with old grapevines (for >100 years), with low yields (<30 hL/ha) and spontaneous fermentation.

The harvesting season was planned according to the acidity and sugar concentration of the grapes in 2016 and 2017. Before the grape pressing, the winemaker used 'skin maceration', where the crushed grapes were left in a vat as a macerate for a few hours, to extract the maximum aromas from the grapes. Sulphite was added as a gassed liquid solution of SO_2 during grape pressing (5 g/hL), and after the malolactic fermentation and racking (5 g/hL). 'Merwah' musts were fermented in French oak wood barrel during the process of fermentation. The temperature of the alcoholic fermentation varied from 15 to 18 °C.

2.2. Sample Collection, Isolation and Morphological Identification

Eight of the 'Merwah' must/wine samples were collected at the middle and final stages of their spontaneous alcoholic fermentation. Chemical analysis of the must was carried out to determine the density, sugar content, pH and total acidity during alcoholic fermentation. Yeast isolation and

morphological identification was performed after thawing must/wine samples previously collected and maintained at −18 °C with added 75% glycerol.

The samples were inoculated onto YPD agar (1% yeast extract, 2% peptone, 2% glucose, 2% Bacto-agar) and incubated at 25 °C for 48 h. The colonies were sampled and cultivated on fresh YPD agar. Then replica plating was carried out on differential Wallerstein Laboratory Nutrient media (WL media) at 25 °C for 2 days [15].

According to the colony morphologies and colours on WL agar and under light microscopy, 202 isolates were identified as *S. cerevisiae*. These isolates were maintained as frozen stocks (40% glycerol, *v*/*v*) as static culture in YPD liquid medium at −80 °C before use and are stored in the University of Sassari (Italy) strain collection. Twenty-two of these yeast strains were selected from this large population of isolates as representative of the major strain clusters of the interdelta sequence profiles [14]. This technique allows discrimination of the *S. cerevisiae* isolates, as no amplicon is generated for other *Saccharomyces* and non-*Saccharomyces* species [16]. Identification of the 22 *S. cerevisiae* isolates was confirmed by PCR of the internal transcribed spacer (ITS) region of ribosomal DNA [17].

2.3. DNA Extraction

The yeast isolates were cultivated overnight in YPD liquid at 25 °C. Then 1.5 mL samples of the cell biomass were centrifuged at 13,000× *g* for 5 s, with the supernatants eliminated. The pellets were resuspended in 200 µL extraction mix: (2% Triton 100×, 1% sodium dodecyl sulphate, 100 mM NaCl, 10 mM Tris-HCl, 1 mM Na_2EDTA, phenol: chloroform: isoamyl alcohol (25:24:1)) with 0.3 g 212–300-µm-diameter glass beads (Sigma-Aldrich, St Louis, MO, USA). The samples were vortexed for 2 min and then centrifuged at 13,000× *g* for 5 min. The DNA was precipitated from the supernatants by adding three vol. 100% ethanol and 0.1 vol. 3 M NaOH, with the samples cooled to −80 °C for 20 min. The samples were then centrifuged at 13,000× *g* for 15 min at 5 °C. The pellets were washed with 70% ethanol and centrifuged at 13,000× *g* for 15 min, and then vacuum dried. The DNA extracted was dried and suspended in 50 µL TE buffer (0.1 M Tris, 0.1 M EDTA, pH 8.0) and stored at −20 °C [18]. The efficiency of this DNA extraction procedure, and its purity and concentration, were measured using a spectrophotometer (NanoDrop; BMG Labtech, Offenburg, Germany) [19].

2.4. Interdelta PCR Typing

The DNA suspensions (50 ng/µL) were added to reaction mixtures (25 µL) that contained: Taq buffer (+1.5 mM Mg^{2+}) (1×), 25 mM $MgCl_2$, 0.2 mM primer 1 δ2 (5′-GTGGATTTTTATTCCAAC-3′), 0.2 mM primer 2 δ12 (5′-TCAACAATGGAATCCCAAC-3′) [20], 0.2 mM dNTP and 1 U Taq polymerase (Trans Gene Biotech, Beijing, China). Amplification reactions were performed on a PCR machine (Thermal Cycler T-100; BioRad, Milan, Italy) using the following programme: 95 °C for 30 s, 52 °C for 30 s, 72 °C for 90 s, and final extension at 72 °C for 10 min.

The products of the PCR reactions were analysed by electrophoresis on 1.5% agarose gels, which were then stained with SYBR safe and visualised under a UV transilluminator (Chemi Doc XRS imaging system; BioRad, Milan, Italy). All of the visible bands were assigned a number based upon their relative position in the DNA ladder. Each position was then assigned '0' or '1' to indicate the absence or presence of the band, respectively. The 0/1 matrix was then used to generate the dendrograms. The comparative cluster analysis of different strains integrated the banding pattern data over the two consecutive years (i.e., 2016, 2017).

2.5. ITS-PCR

Identification of the selected yeast was performed by amplification and sequencing of the ribosomal DNA ITS region. The DNA suspensions (50 ng/µL) were added to the PCR mixture (final volume, 50 µL) that included 1× buffer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.5 µM primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), and 1 U Taq

polymerase [17]. The reaction involved initial denaturation at 95 °C for 10 min, followed by 30 cycles of the series of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 90 s, with a final cycle at 72 °C for 10 min.

The amplicons were purified using PCR purification kits (QIAquick; Qiagen GmbH, Hilden, Germany), following the manufacturer instructions, and were sequenced by the BMR Genomics Laboratory (www.bmr-genomics.it; Padova, Italy). The sequences obtained were compared with those in the GeneBank database (NCBI) using the BLAST programme [21]. Sequences with ≥97% identity were considered to represent the same species.

2.6. Microsatellite Amplification

Each DNA sample was diluted and adjusted to 50 ng/μL DNA and genotyped using two multiplex PCRs of five and seven microsatellite loci for mixtures #1 and #2, respectively (Table 1). For each PCR reaction, a total volume of 1.9 μL was taken from each mix, then 6.25 μL Qiagen multiplex PCR master mix (2×) was added to 1 μL DNA, with MilliQ water added to take the final volume to 12.5 μL. The amplification reactions were performed on a PCR machine (Thermal Cycler T-100; BioRad, USA) using the following programme: initial denaturation at 95 °C for 15 min, followed by 35 cycles of 95 °C for 30 s, 57 °C for 2 min and 72 °C for 1 min, and final extension at 60 °C for 30 min.

Table 1. Characteristics of the 12 microsatellite loci for the *Saccharomyces cerevisiae* genotyping.

Mix	Site Name	Multiplexed Primers	Motif and Type	Fluorescent Dye	Reference
#1	C5-F	TGACACAATAGCAATGGCCTTCA	GT	5′-YAKYE	[22]
	C5-R	GCAAGCGACTAGAACAACAATCACA			
	SCYOR267C-F	TACTAACGTCAACACTGCTGCCAA	TGT	5′-YAKYE	[23,24]
	SCYOR267C-R	GGATCTACTTGCAGTATACGGG			
	C8-F	CAGGTCGTTCTAACGTTGGTAAAATG	TAA	5′-FAM	[22]
	C8-R	GCTGTTGCTGTTGGTAGCATTACTGT			
	C11-F	TTCCATCATAACCGTCTGGGATT	GT	5′-FAM	[25]
	C11-R	TGCCTTTTTCTTAGATGGGCTTTC			
	SCAAT2-F	CAGTCTTATTGCCTTGAACGA	TAA	5′-AT565	[24]
	SCAAT2-R	GTCTCCATCCTCCAAACAGCC			
#2	C9-F	AAGGGTTCGTAAACATATAACTGGCA	TAA	5′-AT550	[22]
	C9-R	TATAAGGGAAAAGAGCACGATGGC			
	C4-F	AGGAGAAAAATGCTGTTTATTCTGACC	TAA + TAG	5′-AT550	[22]
	C4-R	TTTTCCTCCGGGACGTGAAATA			
	SCAAT5-F	AGCATAATTGGAGGCAGTAAAGCA	TAA	5′-AT550	[22]
	SCAAT5-R	TCTCCGTCTTTTTTGTACTGCGTG			
	SCAAT1-F	AAAGCGTAAGCAATGGTGTAGATACTT	TTA	5′-YAKYE	[22–24]
	SCAAT1-R	CAAGCCTCTTCAAGCATGACCTTT			
	C6-F	GTGGCATCATATCTGTCAATTTTATCAC	CA	5′-YAKYE	[22]
	C6-R	CAATCAAGCAAAAGATCGGCCT			
	YKL172W-F	CAGGACGCTACCGAAGCTCAAAAG	GAA	5′-FAM	[25]
	YKL172W-R	ACTTTTGGCCAATTTCTCAAGAT			
	YPL009c-F	AACCCATTGACCTCGTTACTATCGT	CTT	5′-FAM	[23,24]
	YPL009c-R	TTCGATGGCTCTGATAACTCCATTC			

F, forward; R, reverse. YAKYE, Yakima yellow; FAM, carboxyfluorescein green; AT550, Cyanin bleu; AT565, Phycoerythrin (PE) red.

PCR product sizes were obtained for 12 microsatellite loci on a capillary DNA sequencer (ABI3730xl DNA analyser; Applied Biosystem; Singapore, Republic of Singapore) with DS-33 Matrix Standard kits using the polyacrylamide Pop 7, and the size standard 600LIZ (GeneScan 600 LIZ Size Standard v2.0; Thermo Fisher, San Diego, CA, USA). Raw sizes were assigned into classes of alleles of similar size using GeneMarker version 2.6.3 (Demo). The sizes of microsatellite amplicons were used to investigate the genetic relationships between the strains.

After microsatellite typing, the presence of missing values was authorised to a maximum of three markers per sample, and they were taken into account in the analyses considering that they might

reflect part of the diversity. Only individuals with more than 5/12 microsatellite data were kept in the end, 194 yeast strains were genotyped according to the 12 microsatellite markers.

The recorded allele sizes for the 12 microsatellite markers were analysed using the R software V3.2.5. [26]. A dendrogram was constructed using Bruvo's distances [27] and neighbour-joining clustering [28], with the *poppr* (V2.8.0) [29,30] and *ape* (V3.2.5.) [31] packages. Multidimensional scaling was performed with Bruvo's distance matrix, with the *cmd scale* function in R. As this analysis does not allow for missing data, the nearest neighbour method (on the basis of Bruvo's distances) was applied to impute the missing data.

Population structure analysis was performed using the package LEA built under R V3.2.5. [32] and the non-negative matrix factorisation algorithm [33] to estimate individual ancestry coefficients. Models with populations (K) from 1 to 45 were tested in 100 repetitions.

In order to compare the studied population to other population origins, 138 *S. cerevisiae* strains from different origins (i.e., bioprocess, wild, wine strains), described in Table S4 in supplementary data, were added to this study.

The differentiation among the different yeast populations (i.e., bioprocess, wild, wine, Lebanon 'Merwah' wine strains) was analysed through the fixation index (*Fst*), computed with the polysat package [34]. Here, 100 bootstraps were computed, and the confidence intervals were calculated for the *Fst* indices.

Diversity indices were calculated using the *poppr* package [29,30], with Simpson's index and Shannon's equitability index (e.g., Shannon's index taking into account the population size). The Shannon index is calculated as follows: $H' = -\sum p_i \cdot \ln p_i$; where p_i is the proportion of individuals in species i. For a well-sampled community, we can estimate this proportion as $p_i = n_i/N$, where n_i is the number of individuals in species i and N is the total number of individuals in the community. As by definition p_{is} will all be between zero and one, the natural log makes all of the terms of the summation negative, which is why we take the inverse of the sum. Typical values are generally between 1.5 and 3.5 in most ecological studies, and the index is rarely >4. The Shannon index increases as both the richness and the evenness of the community increase. The Simpson index is based on the probability of any two individuals drawn at random from an infinitely large community belonging to the same species: $D = \sum p_i^2$; where again p_i is the proportion of individuals in species i. For a finite community, this is: $D = \sum n_i(n_i - 1)/N(N - 1)$. As species richness and evenness increase, diversity also increases. The values of D lie between 0 and 1.

2.7. Microfermentation and Phenotypic Analysis

After genetic characterisation, 22 *S. cerevisiae* strains were selected based on the main clusters obtained from the interdelta PCR results and the ancestral clustering of microsatellites analysis. To select yeast strain(s) with interesting fermentative performance and technological features, oenological characterisation was carried out for the 22 selected strains (Table 2).

The yeast strains were pre-cultured in 100 mL synthetic media (50 g/L glucose, 1 g/L yeast extract, 2 g/L $(NH4)_2SO_4$, 0.3 g/L citric acid, 5 g/L L-malic acid, 5 g/L L-tartaric acid, 0.4 g/L $MgSO_4$, 5 g/L KH_2PO_4) [35] for 2 days at 22 °C in an incubator-shaker at 250 rpm (Multi Stack, shaking; LabTech, Sorisole (BG), Italy). To evaluate the strain-specific fermentation performances, micro fermentations were carried out by inoculation of each yeast strain in triplicate to the final concentration of 3×10^6 cell/mL in 250 mL synthetic grape juice (SGJ) (100 g/L glucose, 100 g/L fructose, 1 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$, 0.3 g/L citric acid, 5 g/L L-malic acid, 5 g/L L-tartaric acid, 0.4 g/L $MgSO_4$, 5 g/L KH_2PO_4) [35]. The flasks were locked with Müller valves to allow only the CO_2 to escape from the system and incubated at 22 °C in the incubator-shaker at 250 rpm (Multi Stack, shaking; LabTech) for all of the alcoholic fermentations.

Table 2. Description of the 22 Lebanese indigenous *S. cerevisiae* strains used for the technological characterisation. EF = end of alcoholic fermentation.

Sample Code	Sample Name	Isolate Number	Harvesting Year
M.1.16	2016-1EF	3	2016
M.2.16	2016-1EF	16	2016
M.3.16	2016-1EF	6	2016
M.4.16	2016-2EF	1	2016
M.5.16	2016-1EF	2	2016
M.6.16	2016-2EF	3	2016
M.7.16	2016-2EF	5	2016
M.8.16	2016-2EF	8	2016
M.9.16	2016-3EF	9	2016
M.10.16	2016-3EF	6	2016
M.1.17	2017-1EF	3	2017
M.2.17	2017-1EF	20	2017
M.3.17	2017-1EF	21	2017
M.4.17	2017-1EF	39	2017
M.5.17	2017-1EF	49	2017
M.6.17	2017-1EF	54	2017
M.7.17	2017-1EF	66	2017
M.8.17	2017-2EF	4	2017
M.9.17	2017-2EF	5	2017
M.10.17	2017-2EF	9	2017
M.11.17	2017-2EF	16	2017
M.12.17	2017-2EF	17	2017

The kinetics of the fermentations were monitored daily using gravimetric determinations to evaluate the loss of weight due to the production of CO_2 (g/100 mL) [36]. In addition, daily measurements of the cell concentrations (cells/mL), OD 600 nm and biomass (g/L) were carried out [37]. Furthermore, the biochemical parameters were determined at the end of the alcoholic fermentation to select the pertinent strain(s) for the technological properties for wine fermentation. Several parameters were evaluated as follow:

- H_2S production on BiGGY agar (Bismuth Sulphite Glucose Glycine Yeast, Difco, Sparks (MD), USA). The quantities of H_2S produced by the yeast strains were evaluated qualitatively by colony colour formation, with scoring of the degree of browning (1–6) associated with the yeast growth, according to the following scale: white, 1; cream, 2; light brown, 3; brown, 4; dark-brown, 5; black, 6 [38].
- CO_2 production (g/100mL) using the gravimetric method [36].
- Volatile acidity, using enzymatic reaction kits (Cat. No. 10148261035; Boehringer Mannheim, R-Biopharm, Darmstadt, Germany) with a double-beam UV/Vis spectrophotometer (UV S100; Shimadzu, Duisburg, Germany)), and expressed as g/L acetic acid.
- Residual sugar by UV-visible spectrophotometry, with the dinitrosalicylic acid method, and expressed as g/L [39].
- Ethanol concentration by considering the theoretical yeast yield ~16.83 g/L to produce 1% alcohol: (initial sugar concentration—Residual sugar)/16.83 [40] and expressed as %.
- Total and free SO_2 using the modified Ripper iodometric method and expressed as mg/L [41].
- Total acidity, with titration using 0.1 M NaOH to pH 7.00 ± 0.05, with the concentration determined here by acid-base titration and expressed as g/L sulphuric acid.
- The pH, with a pH meter (ST3000; Ohaus Co., Parsippany, NJ, USA).

2.8. Statistical Analysis of the Micro-Fermentations

All of the experimental measurements were conducted in triplicates. Means and standard deviations of the assays were calculated using conventional statistical methods. The ability of CO_2 production at the

end of the fermentation was analysed statistically using ANOVA followed by Tukey HSD test ($p < 0.01$). The relationships among yeast strains and their qualitative and quantitative phenotypic characteristics (fermentation vigour, ethanol production, H_2S production, free and total SO_2 production, volatile acidity, total acidity, pH and residual sugar) structured into groups, were summarized and visualized using Multiple factor analysis (MFA) (R package 'FactoMineR').

3. Results

3.1. Genetic Characterisation

A total of 112 and 90 isolates were collected from spontaneous alcoholic fermentations during the two consecutive years of 2016 and 2017, respectively, at the middle and the end of the alcoholic fermentations (Table 3). The isolates were initially classified as *S. cerevisiae* based on colony morphology on WL nutrient media.

Table 3. Sample repartition and chemical analysis of the 'Merwah' samples collected at the middle and the end of the alcoholic fermentations over the two harvest seasons (2016, 2017).

Sample Name *	Vineyard Location	Number of Isolates	Mean Fermentation Temperature (°C)	Density	Sugar Concentration (g/L)	pH	Total Acidity (g/L H_2SO_4)
2016-1MF	Wata el Jozz	25	17	1.038	95	3.20	4.12
2016-1EF	Wata el Jozz	19	17	0.998	8	3.28	4.31
2016-2MF	Bekaatet Achout	40	17	1.038	103	3.28	3.62
2016-2EF	Bekaatet Achout	12	17	0.998	8	3.29	3.32
2016-3MF	Wata el Jozz Bekaatet Achout	1	17	1.040	96	3.32	3.68
2016-3EF	Wata el Jozz Bekaatet Achout	15	17	0.994	4	3.13	3.23
2017-1EF	Wata el Jozz Bekaatet Achout	71	18	0.996	8	3.15	4.21
2017-2EF	Wata el Jozz Bekaatet Achout	19	16	0.994	4	3.29	4.26

* The sample names refer to the year of the yeast isolation from the must (2016, 2017), the number of the batch (1, 2, 3), which differs according to vineyard location and grape maturity, and the stage of the alcoholic fermentation (MF, middle fermentation; EF, end fermentation).

3.1.1. Genotyping by Interdelta PCR

The interdelta sequence patterns obtained after the gel electrophoresis were used to cluster the 202 yeasts isolates. As a first step, all of the isolates (112 from 2016, 90 from 2017) were subjected to cluster analysis together to test for similarities between the isolates from one year to the other. This analysis highlighted three main groups according to the year of isolation (one cluster for 2016, two clusters for 2017; Table S1). No genetic similarities were seen between the yeast isolates from 2016 and 2017.

For this reason, further cluster analysis was carried out separately for the isolates of each harvesting season. The dendrograms show the genetic dissimilarities of the various *S. cerevisiae* strains identified for both seasons (2016, 2017) (Figure 1). On the basis of agglomerative hierarchical clustering, four major clusters were identified for each season. High dissimilarities were seen across the isolates of 2016 and 2017 for the same cluster number (cluster 4; Tables S2 and S3, for 2016, 2017, respectively). It should be noted that the degree of dissimilarity in 2016 was twice that in 2017, which means that for 2016, higher differentiation was seen between the isolates, with greater similarity among the isolates in 2017.

From the initial 202 isolates, 22 of the yeast strains that were representative of the main clusters after the interdelta sequence profiles of 2016 and 2017 were confirmed as *S. cerevisiae* after sequencing of their ITS rDNA. Therefore, and according to previous studies, all 202 isolates can be considered as *S. cerevisiae* [14].

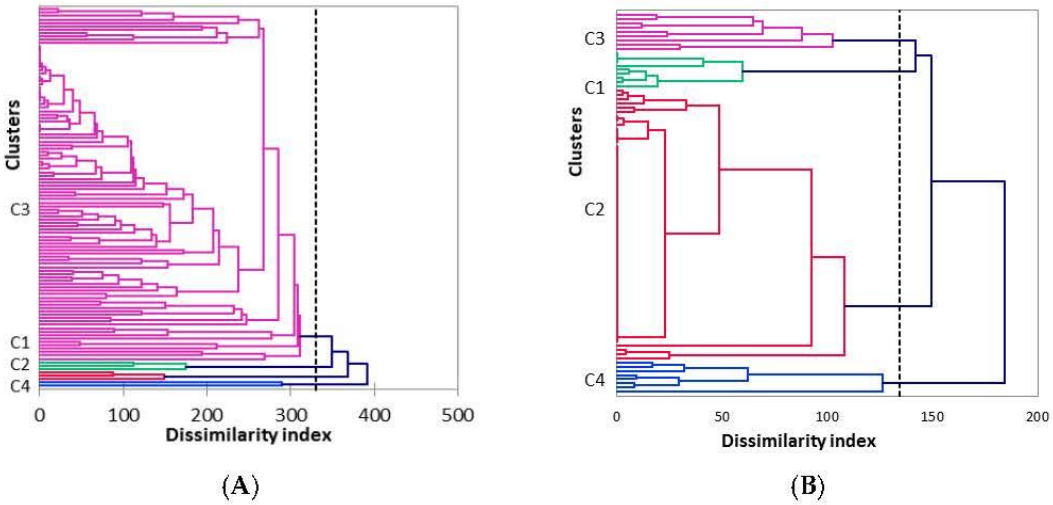


Figure 1. Dendrogram of the agglomerative hierarchical clustering (AHC) of the interdelta PCR for the 112 and 90 yeast isolates from 2016 (**A**) and 2017 (**B**), respectively. The dissimilarity is based on Euclidean distances (pbarret.net, 2005), and clusters are shown in four colours (C1: green; C2: red; C3: purple and C4: blue). In 2016, some of the isolates of the first batch were separated from the others (three isolates from the middle (2016-1MF) and five from the end (2016-1EF) of the alcoholic fermentation). For 2017, the isolates from the first and second batches were mixed in four clusters. Heavy blue line represents the relevant clusters considered. The dotted line corresponds to the minimum dissimilarity level considered in the analysis in order to define the relevant cluster.

3.1.2. Biodiversity of *S. cerevisiae* Strains According to Microsatellite Markers

Among the 202 *S. cerevisiae* isolates, 194 autochthonous isolates were analysed at 12 microsatellite loci (it was not possible to cultivate eight of the isolates), to study their genetic diversity and population structure. Two diversity indices that used the *poppr* package [29,30] were evaluated: Shannon's equitability index (H', also termed the Shannon-Wiener index), which measures the diversity within a population and takes into account the population size; and the alternate of Simpson's index of diversity ($1\text{-}D$) that is used to compare diversity among communities and gives more weight to common or similar species [42]. These different indices were evaluated on the basis of the number of different genotypes and the standard deviations of H' and D.

Only samples with more than five isolates were considered for this analysis. The Shannon index was high and similar, regardless of sample or fermentation stage, thus showing high diversity in the Lebanese 'Merwah' yeast population. In addition, the inverse Simpson index was high (>0.98; Figure 2).

From the initial 194 Lebanese *S. cerevisiae* isolates, 180 different genotypes were identified, thus revealing high genetic diversity in the fermentations studied. To investigate the relationships between these 'Merwah' wine strains and those from other origins, a total of 138 strains of *S. cerevisiae* isolated from different sources were added to this analysis (e.g., from bioprocess, wild, wine industrial strains; Table S4), using the data from the 12 microsatellite markers [43]. Due to the clonal individual genotypes during fermentation, it was necessary to remove identical genotypes from the sample.

The microsatellite patterns for the indigenous 'Merwah' wine yeast were different from those of the *S. cerevisiae* strains of the industrial wines. Multidimensional scaling analysis showed that the vineyard origin, vintage and fermentation stage had no significant impacts on the genetic diversity of these Lebanese *S. cerevisiae* strains (Figure 3).

To examine the genetic linkage between all of the genotyped *S. cerevisiae* isolates (194 yeast isolates from 'Merwah' wine, and 134 from other origins), a dendrogram was constructed using Bruvo's distance and a clustering neighbour-joining tree (Figure 4A). The results show differentiation among the yeast strains isolated from the different sources (i.e., single nucleotide polymorphisms) and good

distribution of these 'Merwah' wine yeast throughout the tree. Also, as shown in Figure 4B, some of the 'Merwah' wine yeast isolates were more coordinated to 'wine' strains. In another part, a group of 'Merwah' wine yeast were greatly differentiated from other population origins (i.e., bioprocess, wild, and wine strains).

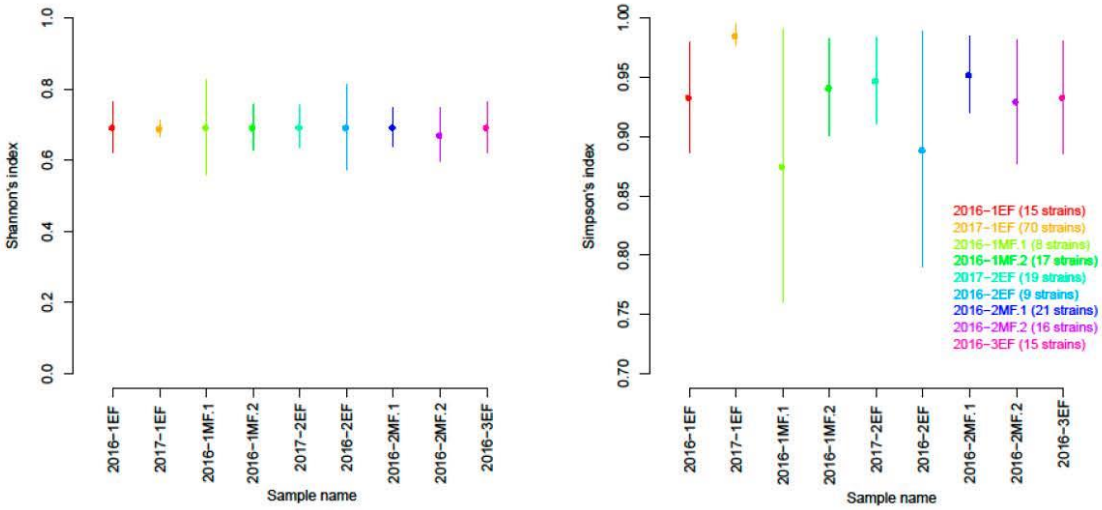


Figure 2. Shannon's equitability and Simpson's diversity indices for the *S. cerevisiae* isolates during the 'Merwah' winemaking. The indices were calculated using the *poppr* package.

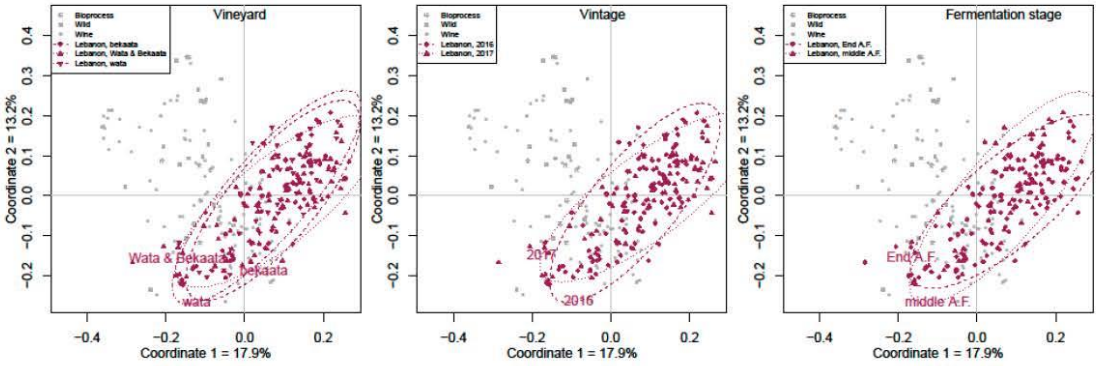


Figure 3. The MULTIDIMSCALE factors. Multidimensional scaling of the Lebanon 'Merwah' *S. cerevisiae* wine strains according to vineyard, vintage and fermentation stage. Dotted circle regroup strains population refer to their factorial group.

To better compare the populations of the 'Merwah' wine yeast with the other populations (i.e., bioprocess, wild, wine strains), *Fst* statistics were calculated through the fixation index (*Fst*) (Table 4). Pairwise *Fst* indicated low and moderate differentiation between the Lebanon 'Merwah' wine strains and the bioprocess, wild and wine strains, respectively.

Table 4. Pairwise fixation indices calculated using the distance matrix.

Population	Fixation Index (*Fst*) According to Yeast Strain Population			
Bioprocess	NA	0.056	0.026	0.017
Wild	0.056	NA	0.041	0.028
Wine (industrial)	0.026	0.041	NA	0.100
Lebanon 'Merwah' wine	0.017	0.028	0.100	NA

Fst <0.05, no genetic differentiation; 0.05 < *Fst* < 0.15, moderate genetic differentiation; 0.15 < *Fst* < 0.25, important genetic differentiation; *Fst* >0.25, high genetic differentiation [44]. NA, not applicable.

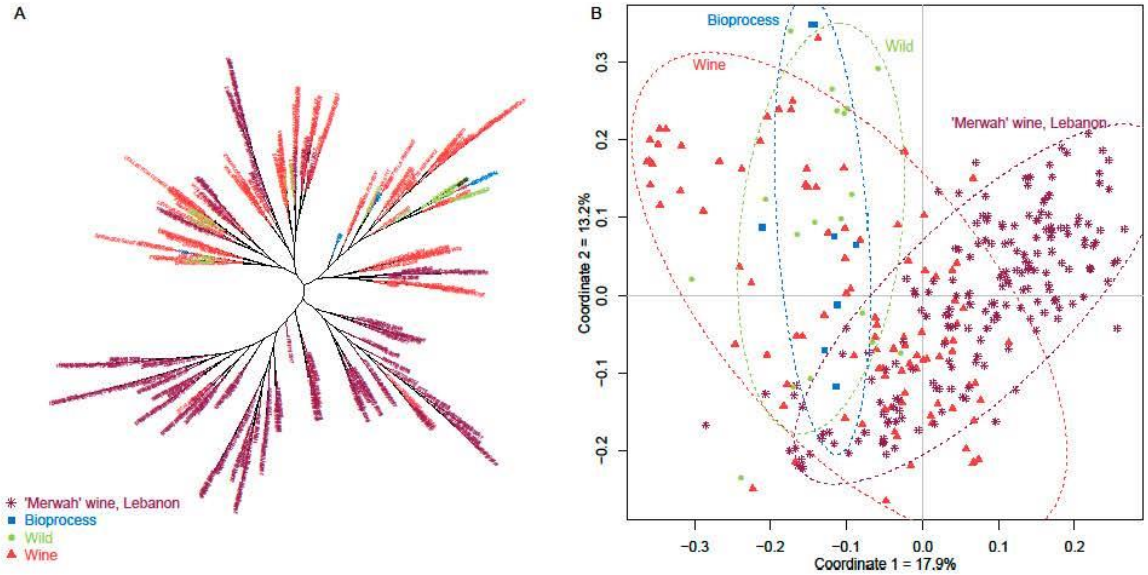


Figure 4. TREE-MULTIDIMSCALE. Population analysis of the 334 *S. cerevisiae* strains using 12 microsatellites. (**A**) Dendrogram using Bruvo's distance and neighbour-joining clustering. (B) Multidimensional scaling. Coordinates 1 and 2 explain 17.9% and 13.2% of the variation. The strains from the different isolations are represented by different colours: bioprocess strains (blue), wild strains (green), wine strains (red) and Lebanon 'Merwah' wine strains (purple).

The population structure from shared ancestry was evaluated for the 332 individuals (194 Lebanon 'Merwah' wine strains; 138 from other population origins: bioprocess, wild and wine strains, including industrial strains). The model with 22 ancestral populations (Figure 5) was the one with the lowest cross-entropy, as determined using Kruskal–Wallis tests ($\alpha = 0.05$; package agricolae, V1.2-8) [45].

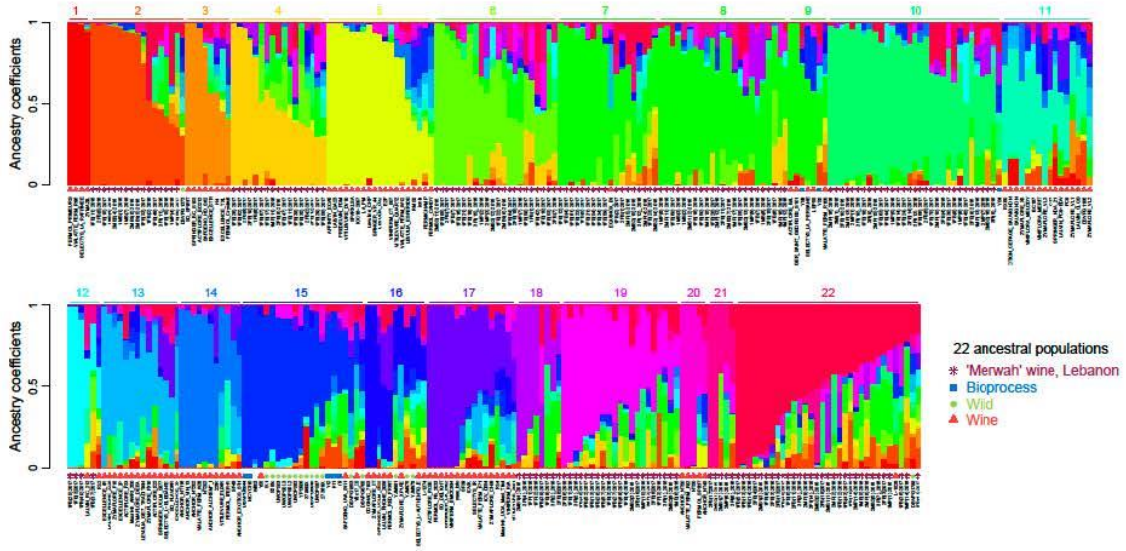


Figure 5. Barplots showing the population structure from the shared ancestry for the optimal $K = 22$. The population structure inferred using ADMIXTURE analysis for 332 populations and differentiated by four colours: purple, Lebanon 'Merwah' wine strains; blue, bioprocess strains; green, wild strains; red, wine strains.

The 'Merwah' wine strains were assigned to 11 ancestral clusters, with numerous isolates composed of mosaic ancestral subpopulations. Ancestry profile analysis provided evidence that the 'Merwah' wine population and the wine strains are related.

3.2. *Technological Characterisation*

3.2.1. Kinetics of the 22 'Merwah' Wine *S. cerevisiae* during Alcoholic Fermentation in Synthetic Grape Juice

The fermentation kinetics of the 'Merwah' wine yeast strains were determined in triplicate by following their production of CO_2 during fermentation in SGJ—Where not visible, error bars lie under the strain symbols (Figure 6). The ability of CO_2 production was analysed through ANOVA followed by Tukey HSD test only at the end of the fermentation. Results are shown in Table S5 (as supplementary data).

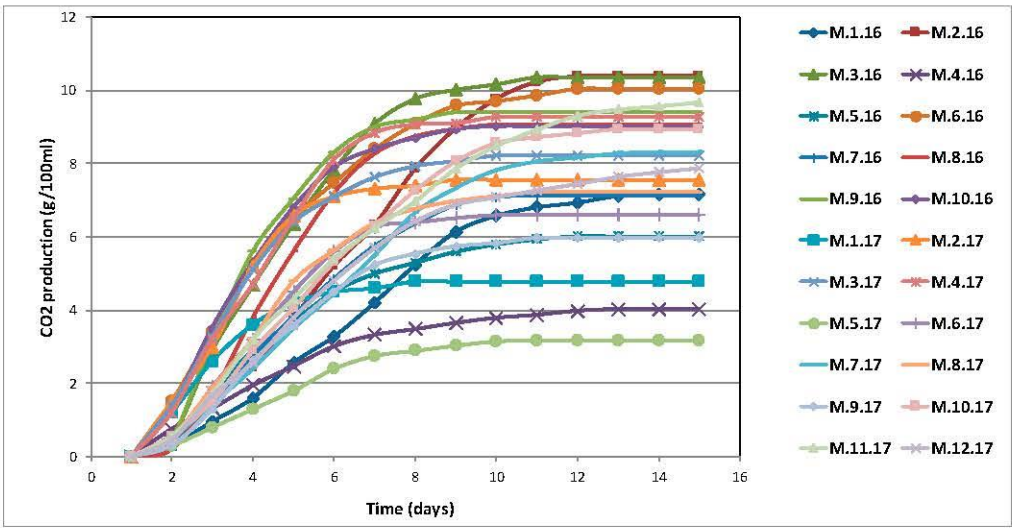


Figure 6. Carbon dioxide production by the 22 Lebanese *S. cerevisiae* isolates during alcoholic fermentation in synthetic grape juice (SGJ).

These strains underwent fermentations over variable periods of 9 days to 15 days at 22 °C, and released high levels of CO_2, which indicated their significant metabolic activities. Strain M.5.17 showed the lowest production of CO_2. In contrast, nine strains showed a high production of CO_2 (>8.9 g/100 mL): M.2.16, M.3.16, M.6.16, M.8.16, M.9.16, M.10.16, M.4.17, M.10.17 and M.11.17.

3.2.2. Phenotypic Analysis

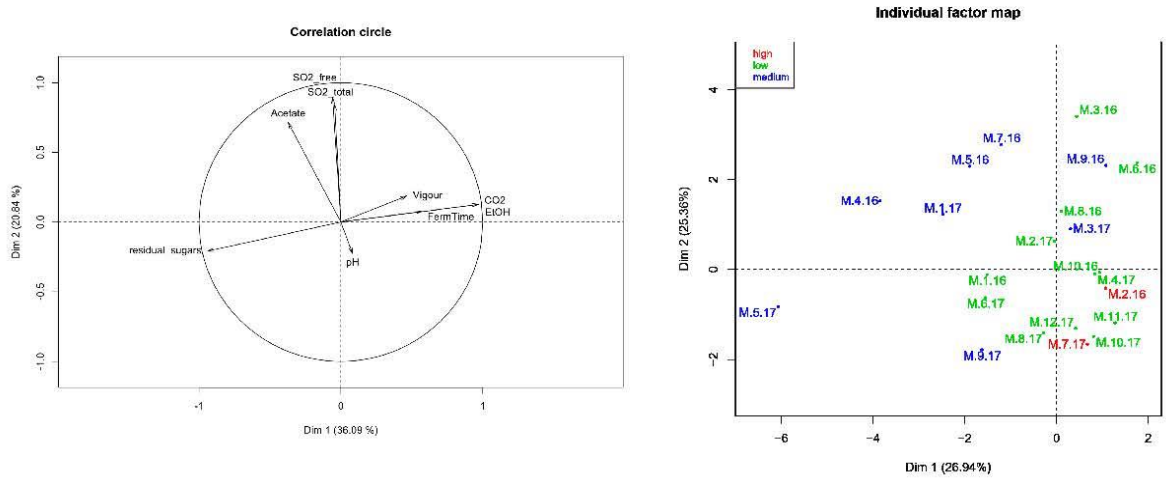
Twenty-two *S. cerevisiae* strains were fermented in the SGJ at 22 °C in order to evaluate the relevant parameters: fermentation vigour, ethanol production, H_2S production, free and total SO_2 production, volatile acidity, total acidity, pH and residual sugar (Table S6). Twelve of these 22 yeast strains consumed all of the sugar, as seen by their very low residual sugar (<2 g/L, whereas the others indicate incomplete fermentation. Among these six indigenous strains were low producers of H_2S (M.3.16, M.6.16, M.4.17, M.10.17, M.11.17), which is an undesired compound during fermentation as it can confer unpleasant off flavours to the wine. This avoids yeast using their own amino acids (which contain the sulphur molecule) as a source of nitrogen [46].

Volatile acidity ranged from 0.01 to 0.69 g/L acetic acid. These concentrations are low and acceptable from an oenological point of view, and also according to European legislation (<0.9 g/L acetic acid) [47]. High variability was seen for the levels of SO_2 produced by the 22 strains studied. At the end of the fermentation, these yeast strains produced ethanol from 11.10 to 11.88%. The final pH of the SGJ after fermentation was 3.2 to 3.5. Usually, low final pH (~3.1) can be explained by the release of organic acids by the strains during alcoholic fermentation, but this was not the case here. Indeed, acetic acid combined with ethanol can affect yeast fermentative behaviour, by decreasing the cell pH and the fermentation rate [48].

Finally, the fermentation vigour of these tested yeast strains was from 0.8 g CO_2/L (M.5.17) to 3.53 g CO_2/L (M.10.16). The fermentation vigour defines the speed at which the yeast starts their fermentation. This is an important criterion for dominance by starter yeast [48]. The most vigorous yeast strains are selected as dominant strains during wine fermentation.

The phenotypic data were evaluated by multiple factor analysis, with the aim to study correlations among groups of variables and how these can influence the co-variation among the different strains.

Figure 7A shows the weight (or contribution) of each variable to the two axes. The degree of variability explained by the X axis and the Y axis was 26.94% and 25.36%, respectively. Then Figure 7B shows the positions of the strains on the axes, where their colours represent H_2S production. The strains in green in Figure 7B are those that produced the lowest levels of H_2S, which is recommended in the selection of wine yeast strains as starter cultures. The two yeast strains shown in red in Figure 7B (M.2.16, M.7.17) show high production of H_2S. A high sulphite producer will not be chosen for starter culture due to the risk of off-flavour production during alcoholic fermentation.



(A) (B)

Figure 7. Correlation plots. (**A**) Relationship between the quantitative variables. (**B**) Individual factor map evaluating the dispersion of the yeast strains depending on the qualitative variable of 'H_2S production'.

The dispersion of these strains in the individual factor map (Figure 7B) shows the heterogeneity of these phenotypes. Among these strains, only one, which is far from the others, showed high residual sugar (M.5.17). This strain was thus eliminated as an unacceptable yeast starter for wine production, due to the risk of sluggish or incomplete fermentation.

Three strains, namely M.6.16, M.10.16 and M.4.17, may be selected as starters for microvinification assays as they showed the lowest residual sugar content at the end of alcoholic fermentation, the lowest production of volatile acidity and H_2S, and the higher fermentation vigour.

4. Discussion

In this study, we analysed a population of *S. cerevisiae* yeast from the spontaneous fermentation of 'Merwah' wine to select for suitable potential autochthonous starter cultures for the improvement of the oenological production of this typical regional wine. At the end of fermentation, none of the isolate samples here belonged to non-*Saccharomyces* species, which will be due to the high ethanol in, and SO_2 additions to, these wines [2].

High diversity across these *S. cerevisiae* strains was identified in the interdelta PCR analysis. This dissimilarity explains the absence of resident yeast from one year to the next and reveals the high genetic diversity within the populations from the same year. It is known that a low dominance of a

certain species/strain can generate high diversity [16]. It has also been demonstrated that *S. cerevisiae* strains isolated during spontaneous fermentations from the same wine can be genetically distinct [49].

Many surveys have demonstrated high genetic diversity within the *S. cerevisiae* species that carry out spontaneous fermentations in different wine-producing regions [5,50]. These suggest that specific native *S. cerevisiae* strains might be associated with a given *terroir*, and that they can have an influence on the *terroir*-associated wine characteristics [3].

In addition, microsatellite marker analysis was used to determine the genetic linkage between these isolates, the ancestral alleles, and the population genetic structure. In this study, multilocus microsatellite analysis was performed with 12 loci, which allowed the evaluation of the genetic diversity among the Lebanese strains and between the Lebanese strains and strains of other origins. A total of 180 genotypes were revealed from an initial population of 194 *S. cerevisiae* isolates (eight of the strains among the 202 isolates had missing data) from this Lebanon 'Merwah' white wine, thus confirming high genetic diversity of the native *S. cerevisiae* throughout the alcoholic fermentation. These strains were different each year, and very few common strains were detected across these years, which indicate that there were no resident strains in this winery over the 2 years of the study.

The data obtained by the microsatellite analysis showed that this spontaneous wine fermentation is not driven by commercial isolates, but by a diversity of natural isolates. In the region studied, low dissemination of commercial strains is associated with high autochthonous genotype diversity, as also shown in a previous study [51]. However, based on the results of multidimensional scaling analysis, the diversity of the Lebanese yeast populations was not impacted by the vineyard location, production year or fermentation stage factors. Otherwise, geographic locations and ecological niches are both believed to have significant roles in *Saccharomyces* strain diversity [11,14].

Some wild yeast strains of Lebanon 'Merwah' wine clustered with the wine strains (including the industrial wine strains). This similarity may be explained by the fact that the winemaker received red grapes from other regions. For this reason, the oenological practices applied in the winery and the distribution of yeast populations from other winery areas or surrounding vineyard regions might affect the yeast diversity [52–55]. Also, the other part of the Lebanon 'Merwah' wine strain population was differentiated from the populations with other origins and formed an independent group. This differentiation reveals that these indigenous Lebanese yeast strains of the autochthonous 'Merwah' grape must have a different origin. This supports the concept that indigenous yeast strains selected in a winery can be associated with a *"terroir"*, and thus reflect the biodiversity of a particular area [56,57]. It has been demonstrated that genetic characterisation of wide groups of *S. cerevisiae* strains from different geographic origins and technological groups are related to their ecologically important phenotypic traits [51].

Based on the data from cluster analysis of interdelta PCR and the data obtained in ancestral analysis (11 ancestries for the Lebanon 'Merwah' wine yeast strains), 22 *S. cerevisiae* strains were defined as representative of this 'Merwah' wine yeast population, and these were selected for phenotypic characterisation. The strains characterised by molecular analysis were also used in micro-fermentation assays to evaluate their technological and oenological properties, with the aim of defining a starter culture. The selection of these yeast isolates was performed according to their characteristics: first, low production of H_2S during fermentation, as H_2S is highly undesirable and produces a rotten eggs odour, and then, low production of volatile acidity produced at the end of fermentation (as expressed by g/L acetic acid: <0.7 g/L), which can also negatively affect the wine aroma [2]. The acetic acid is affected by the addition of SO_2 (i.e., less acetic acid is produced when SO_2 is added), the turbidity (i.e., more acetic acid is produced when the turbidity is lower) and pre-fermentation temperature (i.e., stronger effects at low temperature) [37]. In addition, the endogenous SO_2 levels produced depend strongly on the yeast strain, availability of nutrients, such as nitrogen, pH, and temperature [58].

All of these studied strains produced low concentrations of acetic acid and H_2S, which are positive characters in a fermenting strain. The most important factor in the selection of a yeast strain is the consumption of sugar, as the presence of residual sugar (i.e., >4 g/L) is indicative of incomplete

fermentation [59]. The strains M.2.16, M.3.16, M.6.16, M.7.16, M.8.16, M.9.16, M.10.16, M.3.17, M.4.17, M.7.17, M.10.17 and M.11.17 almost entirely consumed the sugars during the alcoholic fermentation and showed acceptable alcohol yields. For these strains, the selection was based on the fermentation vigour and kinetics.

5. Conclusions

The exploitation of the biodiversity of indigenous strains has great importance for the characterisation and selection of strains with particular phenotypes, and for biodiversity preservation and exploitation in terms of the Lebanese culture collection of *S. cerevisiae* strains, which can be certified following international standards. The selection of native yeast can provide strains that are characterised by oenological traits different from those of the starter strains commercially available, such as reduced alcohol levels and the increased production of secondary metabolites. For this reason, as a result of this study, three wine yeast strains from this large representative population, namely M.6.16, M.10.16 and M.4.17, may be used as starters based on their favourable oenological properties: high fermentation vigour, low production of volatile acidity and H_2S and low residual sugar content at the end of alcoholic fermentation. Alone or together, the selected strains appear to be promising for use at the winery scale to produce different types of 'Merwah' wine.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/7/11/492/s1. Table S1: Clustering data for the 202 'Merwah' yeast strains isolated in 2016 and 2017 based on interdelta-PCR analysis, Table S2: Statistical analysis of the interdelta-PCR data for the 112 'Merwah' wine yeast isolates from 2016, Table S3: Statistical analysis of the interdelta-PCR data for the 90 'Merwah' wine yeast isolates from 2017, Table S4: *Saccharomyces cerevisiae* strains of different origins (138) added to the 'Merwah' wine yeast population in the microsatellite analysis, Table S5: Statistical differences in total CO2 production of 22 *S. cerevisiae* strains as determined by ANOVA followed by Tukey -HSD test ($p<0.01$). Strain showing not significant differences share common group letters, Table S6: Technological parameters of the 22 *Saccharomyces cerevisiae* strains during fermentation of the synthetic must.

Author Contributions: Conceptualization, N.F. and M.B.; Investigation, N.F., W.A., Z.R. and A.B.; Methodology, W.A., Z.R., A.B. and G.Z.; Resources, M.B.; Software, G.Z.; Supervision, I.M.-P. and M.B.; Visualization, E.T.; Writing—Original Draft, N.F.; Writing—Review and Editing, M.B., N.F. and I.M.-P. All authors have read and approved the final manuscript.

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STUDYING SULFITE RESISTANCE AND MECHANISMS OF TRANSLOCATION IN *SACCHAROMYCES CEREVISIAE* STRAINS OF MERWAH WINE

1. Introduction

The use of sulfite has become a general practice in winemaking, because of the ability of sulfite to limit the oxidation of must and wines, and especially to preserve aroma compounds (e.g., volatile Thiols) particularly sensitive to oxidation, and the color of the wines (Ribéreau-Gayon *et al*., 2006; Oliveira *et al.,* 2011). Sulfur dioxide (SO_2) also inhibits the action of enzymes such as polyphenol oxidases tyrosinase, and laccase produced by the grapes and fungi respectively. Sulfur dioxide also display antimicrobial activity towards yeasts and bacteria and is thus used for microbiological stabilization of wine (Pezley, 2015). According to the EU Regulation, the limit concentration of total SO_2 up to 150 mg/L in red wines and 200 mg/L in white and rosé wines containing a maximum of 5 g/L of reducing sugars (EU Regulation No. 606/2009).

Besides, *Saccharomyces cerevisiae* strains can ensure complete fermentation at a concentration greater than 30 mg/L of free sulfur dioxide and 50 mg/L of total sulfur dioxide (Divol *et al*., 2012).

An acidic pH favors the molecular form and therefore the import of SO_2 and high temperature also increases its toxicity (Zimmer *et al.,* 2014). When the molecular SO_2 penetrates by simple diffusion rather than by active transport in yeast cells (*Saccharomyces cerevisiae*) (Lisanti *et al.,* 2019), the higher internal pH converts molecular SO_2 to bisulfite (HSO_3^-), which can be incorporated into the sulfur amino acid biosynthesis (SAAB) pathway (Figure 1) (Nadai *et al.,* 2016). Once it is in this pathway, bisulfite is reduced to sulfide (S^{2-}) and then either used to produce sulfur-containing amino acids or exported from the cell as hydrogen sulfide (H_2S). This response to SO_2 is undesirable for winemakers and wine consumers alike because H_2S has a low detection threshold and can lend a rotten egg or cooked cabbage aroma to the wine (Huang *et al.,* 2017). Finally, acetaldehyde is produced by yeasts as an intermediate in many metabolic pathways, including alcoholic fermentation. Acetaldehyde has an extremely high affinity for SO_2, with one mole of acetaldehyde able to bind approximately one mole of SO_2. Bound SO_2 is no longer active as an antimicrobial agent, so this is an effective method of sulfite resistance. Like the sulfite efflux pump, highly resistant yeast strains tend to have higher constitutive production of acetaldehyde, even in the absence of SO_2 (Morgan *et al.,* 2019).

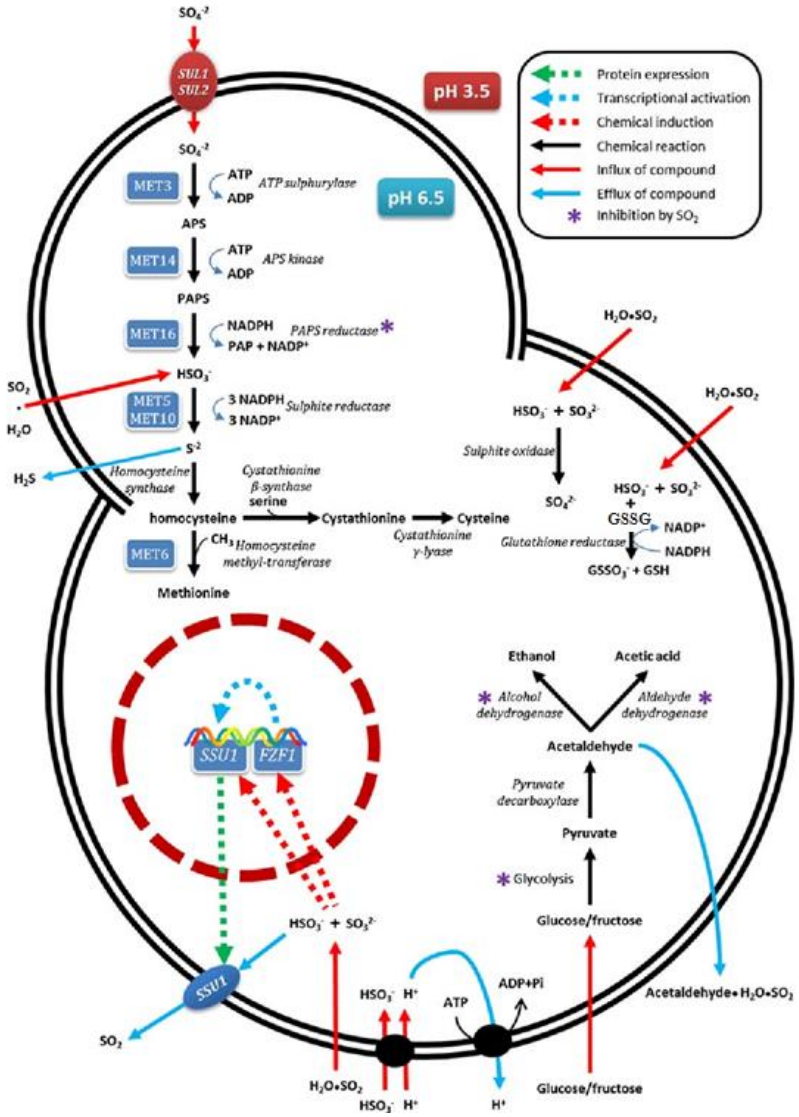

Figure 1: A summary of the sulphate assimilation pathway and the cellular and molecular responses of *S. cerevisiae* to the presence of SO_2. SAAB sulphur amino acid biosynthesis, SR sulphur reduction. (Divol *et al.,* 2012 modified).

The use of sulfite has led to the selection of mutants having an improved ability to resist to the toxic agent, and especially several mutants carrying a translocation permitting higher sulphite export (Pérez-Ortin *et al.,* 2002; Zimmer *et al.,* 2014; Treu *et al.,* 2014).

The most efficient sulfite detoxification mechanism in *Saccharomyces cerevisiae* uses a plasma membrane protein called SSU1 to efflux sulfite (Nadai *et al.,* 2016). The first zinc finger of Fzf1p as well as the 11 N-terminal amino acids has been shown to be essential to ensure the binding of Fzf1p to the SSU1 promoter (Avram *et al.,* 1999). SSU1 is a trans-membrane protein located on chromosome XVI (Liu *et al.,* 2018).

SO_2 resistant strains of *Saccharomyces cerevisiae* possess a specific allele of SSU1 called SSU1-R, which has been seen to be 97 % identical to SSU1. As wine strains of *S. cerevisiae* exhibit different

degrees of ploidy and different levels of heterozygosity, the number of SSU1 and SSU1-R could potentially explain the diverse range of resistance observed between strains. In wine yeast strains, two chromosomal translocations (VIII-t-XVI and XV-t-XVI) (Figure 2) involving the SSU1 promoter region have been shown to up-regulate SSU1 expression and, as a result, increase sulfite tolerance (Lisanti *et al.,* 2019).

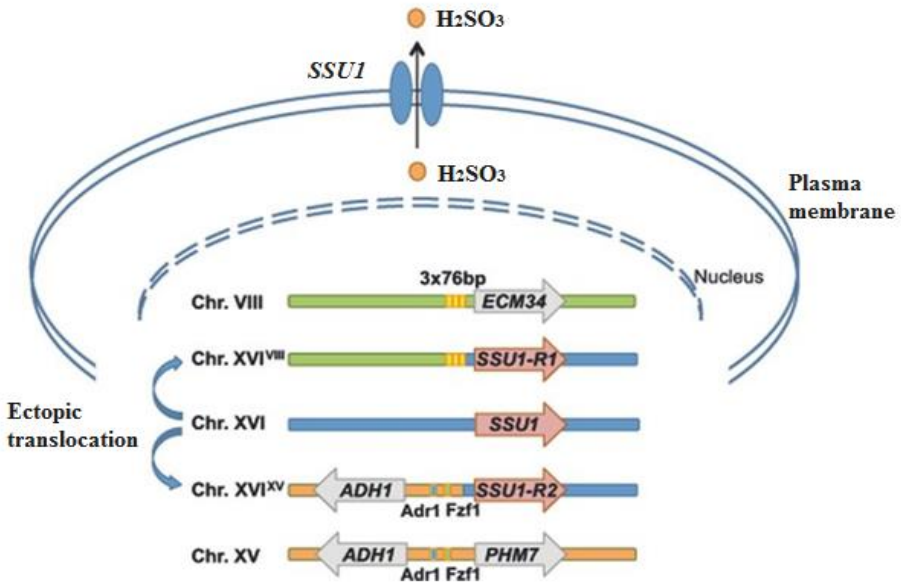

Figure 2: Mechanisms of sulfite resistance through reciprocal translocations (Marsit and Dequin 2015 modified).

A well-documented example of chromosomal rearrangement with an adaptive advantage is the reciprocal translocation between chromosome VIII and XVI, which is widespread among wine yeasts (Nardi *et al.,* 2010; Borneman *et al.,* 2011).

Recently, another translocation between chromosome XV and XVI was identified in several wine strains through quantitative trait loci (QTL) mapping for lag phase duration in the alcoholic fermentation of grape juice, and this translocation increased the expression of this gene (Zimmer *et al.,* 2014). The presence of chromosomal translocation XV-t-XVI in *S.cerevisiae* confers a higher expression of SSU1p by modifying the upstream region of the gene, which can led to a reduction of the lag phase in presence of sulfite (Ferreira *et al.*, 2017).

A first translocation (VIII-t-XVI, the most frequent form) was mediated through crossing-over mediated by microhomology within the promoters of ECM34 and SSU1 (Figure 3) (Guillamon and Barrio, 2017). Several 76-bp repeats (3–6 tandem repeats) were found in the promoters of non-recombinant ECM34 and recombinant SSU1-R1. A direct relationship between the number of 76-bp repeats and sulfite tolerance has been described (Pérez-Ortín *et al*., 2002). A second reciprocal

translocation (XV-t-XVI) involves Adr1 and Fzf1 binding regions of the promoter of ADH1 and SSU1, respectively, resulting in the SSU1-R2 allele having increased expression during the first hours of alcoholic fermentation (Figure 4) (Zimmer *et al*. 2014). Both translocations VIII-t-XVI and XV-t-XVI conferred a selective advantage by shortening the growth lag phase in a medium containing SO_2 (Treu *et al.,* 2014; Ferreira *et al*., 2017).

More recently, a new chromosomal rearrangement that triggers an adaptation of wine yeast sulfite has been identified. An inversion in chromosome XVI (inv-XVI), increases the expression of SSU1 and sulfite resistance of a commercial yeast strain, which is probably due to the microhomology of the sequences, and involving regulatory regions SSU1 and GCR1 (Rios *et al.,* 2019).

Here, as a complementary analysis to previous genetic characterization, the presence of chromosomal rearrangement involved in the adaptive evolution of *Saccharomyces cerevisiae* strains has been studied in order to understand the strain attitude towards sulfites. In addition, phenotypic analysis identified the dominant type of chromosomal translocation (VIII-t-XVI, XV-t-XVI) of the gene SSU1 present in 202 *S. cerevisiae* strains of 'Merwah' white wine during spontaneous alcoholic fermentation. In addition, phenotypic test has been occurred in order to determine the resistance strains to sulfite when exposed to different SO_2 concentrations.

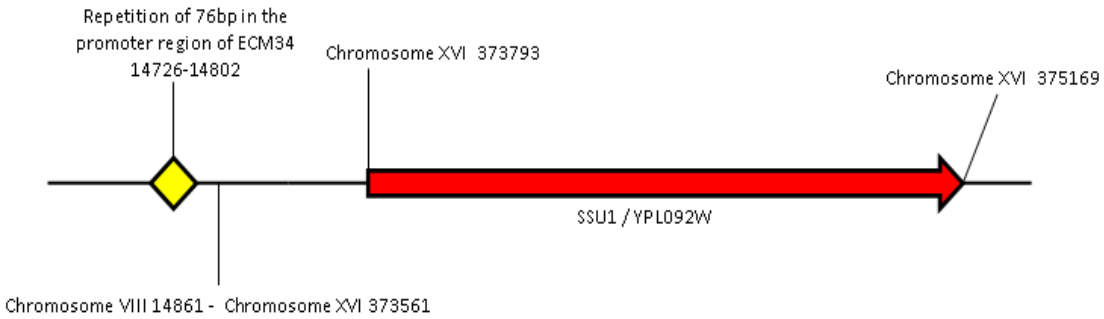

Figure 3: Translocation between chromosome VIII and XVI (Perez-Ortin, 2002).

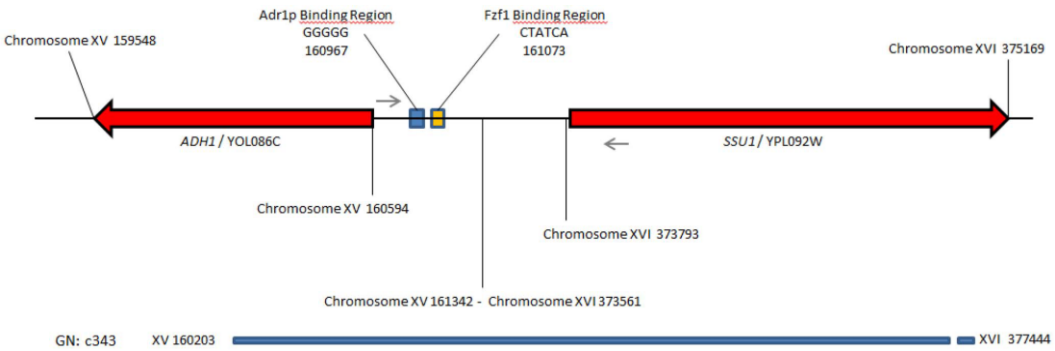

Figure 4: Description of the XV-t-XVI translocation (Zimmer *et al.,* 2014).

2. Materials and Methods

2.1. Sample collection

Eight 'Merwah' musts/ wine samples were collected 'Château Bybline' winery at the middle and final stages of their spontaneous alcoholic fermentation during two consecutive years (2016, 2017). Sulphite was added as a gased liquid solution of SO_2 during grape pressing (50 mg/L), and after the malolactic fermentation and racking (50 mg/L). 'Merwah' musts were fermented in French oak wood barrel during all the process of fermentation. The temperature of the alcoholic fermentation varied from 15 °C to 18 °C.

2.2. Yeast isolation

As described in previous genetic and technological characterization (Chapter 2), 202 isolates collected from these samples were identified as *S. cerevisiae*, according to the colony morphologies and colors on WL agar and under light microscopy, than approved by PCR of the internal transcribed spacer (ITS) region of ribosomal DNA (Kumar and Shukla, 2005). Yeast isolation and morphological identification was performed after thawing must/wine samples previously collected and maintained at -18 °C with added 75% glycerol.

These isolates were maintained as frozen stocks (40% glycerol, v/v) as static culture in YPD liquid medium at -80 °C before use and are stored in the University of Sassari (Italy) strain collection.

2.3. DNA extraction

The yeast isolates were cultivated overnight in YPD liquid at 25 °C. Then 1.5 mL samples of the cell biomass were centrifuged at 13,000× g for 5 s, with the supernatants eliminated. The pellets were resuspended in 200 µL extraction mix: (2% Triton X100, 1% sodium dodecyl sulphate, 100 mM NaCl, 10 mM Tris-HCl, 1 mM Na2EDTA, phenol: chloroform: isoamyl alcohol [25:24:1]) with 0.3 g 212-300-µm-diameter glass beads (Sigma-Aldrich, St Louis, MO, USA). The samples were vortexed for 2 min and then centrifuged at 13,000× g for 5 min. The DNA was precipitated from the supernatants by adding three vol. 100% ethanol and 0.1 vol. 3 M NaOH, with the samples cooled to -80 °C for 20 min. The samples were then centrifuged at 13,000× g for 15 min at 5 °C. The pellets were washed with 70% ethanol and centrifuged at 13,000× g for 15 min, and then vacuum dried. The DNA extracted was dried and suspended in 50 µL TE buffer (0.1 M Tris, 0.1 M EDTA, pH 8.0) and stored at -20 °C (Harju *et al*., 2004). The efficiency of this DNA extraction procedure, and its purity and concentration, were measured using a spectrophotometer (NanoDrop; BMG Labtech, Germany) (Sambrook *et al.,* 2006)

2.4. Translocation PCR

The sulfite resistance capacity of the selected strains and the presence or absence of the chromosomal translocations was tested by PCR translocation.

The DNA suspensions (50 ng/µL) were added to the PCR mixture (final volume, 10 µL) including 1 µl of mixed primers diluted to 1/50 (Table 1) and 5 µL Qiagen multiplex PCR master mix (2×). The amplification reactions were performed on a Thermal Cycler T-100 (BioRad, USA) using the following program: 95 °C for 15 min followed by 94° C for 30 sec of 34 cycles, 55 °C for 90 sec, 72°C for 90 sec with a final extension at 60 °C for 30 min. The PCR amplicons were diluted to 1/20 and 2 µL of the dilution was added to 0.2 µl of standard 1200LIZ® (GeneScanTM, V2.0 Thermo Fisher, CA, USA) and 9.8 µL of formamide.

Raw sizes were assigned into classes of alleles of similar size using GeneMarker V2.6.3 (Demo).

Table 1: The primers used for the Translocation PCR –SSU1 (Zimmer *et al.,* 2014).

Chromosome type	Sequence	Marker
8 F_8 R	Fw—TCGAACATCGAGCATGCA Rv—CCATATTTGTGATGATATCG	FAM (blue)
15 F _15 R	Fw—ACCTATCGAGTCTCCCAC Rv—GACACCCATGACCATCAC	NED (yellow)
16 F _16 R	Fw—AAAGAAGTTGCATGCGCCTA Rv—GCCCCTCCATGTTCTACTATT	VIC (green)

2.5. Phenotypic analysis for sulfite resistance

In order to study the influence of chromosomal translocation on phenotypic sulfite resistance, three *S. cerevisiae* strains (VIII-t-XVI; XV-t-XVI; non Transloc) were subjected to different dose of sulfite: 0, 50 and 200 mg/L of SO_2 during fermentation in synthetic grape juice (Bely *et al.,* 1990) at laboratory scale. The production of SO_2 were determined at the end of alcoholic fermentation by using the modified Ripper iodometric method and expressed as mg/L (Rizk *et al.,* 2016). The evaluation of SO_2 sulfite resistance were registered and compared to the chromosomal translocation data.

3. Results and Discussion

3.1. Chromosomal translocation identify sulfite resistance in *Saccharomyces cerevisiae*

In this study, to better understand the strain-specific mechanisms of resistance, 202 *Saccharomyces cerevisiae* strains genotyped previously, have been investigated for the presence of translocations that are known to confer SO_2 resistance (Zimmer *et al*., 2014; Ferreira *et al*., 2017).

A dominant translocation between chromosomes VIII and XVI (Allele 540) (Table S7) has been shown in 88% of the studied *Saccharomyces cerevisiae* population (Figure 5). In addition, chromosome VIII wild type has been found at the allele 596, which is demonstrated to be found in the industrial strains especially in white wine. The translocation XV-t-XVI was detected only in two *Saccharomyces cerevisiae* strains (2016-3FI). This result has been justified previously, where less translocation forms between chromosome XV and XVI has been found in white wine (Ferreira *et al.,* 2017). In contrast, 9.5% of the 202 *S. cerevisiae* strains have not shown a chromosomal translocation (Figure 5). Thirteen and six non chromosomal translocated strains isolated from Merwah wine of 2016 and 2017 respectively, were not considered in the selection of enological starter culture strains.

Indeed, a translocation between chromosome VIII and XVI increases sulfite resistance due to the creation of a new genetic environment regulating differentially the sulfite membrane pump, SSU1p (Nardi *et al.,* 2010; Zimmer *et al.,* 2014).

Perez-Ortin (2002) was the first to describe the translocation VIII-t-XVI in the natural population. The new type of translocation XV-t-XVI has been empirically selected by human activity and found only in wine strains adapted to the prolonged use of sulfite in winemaking (Zimmer *et al.,* 2014). Translocation events also play a role in environmental adaptation as described in wine yeast. The increasing of sulfite resistant in wine yeast revealed to the domestication in wine strains (Warringer *et al*., 2011).

The presence of these translocations could play a fundamental role during wine fermentation lag phase, as SO_2 is frequently used in musts and wines as a preservative and an antioxidant, and may represent an important source of stress.

As far as a mutation event may lead to the replacement of SSU1 promoter by more active/regulated differently promoter we can assess that these mutations should lead to an overexpression of SSU1. In addition different independent translocations events should not occur exactly at the same branching point. The fact that only three translocations have been identified in distant countries favor the presence of few ancestral mutations spread in population. Population genetics indicates that before a mutation is fixed, the time it co-exists with the wild type allele depends on the fitness gained by the mutation, and on the theorical population size (high for yeast). Last it is also possible that the advantage offered by these translocations may lead to a detrimental growth under non wine environment (i.e. in the vineyard…).

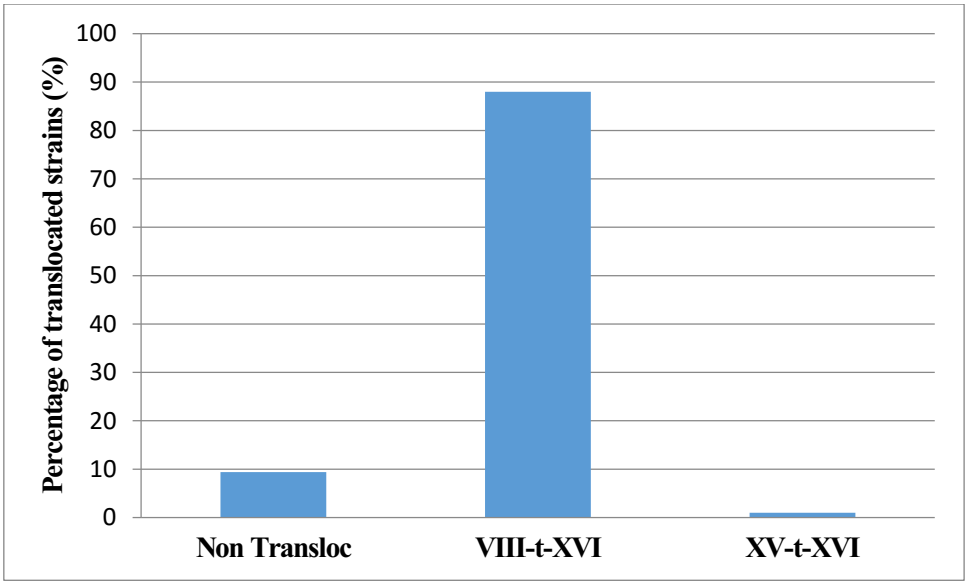

Figure 5: Results of the chromosomal translocation of indigenous *S. cerevisisae* strains isolated from 'Merwah' wine, Lebanon.

3.2. Phenotypic test determining sulfite resistance in *Saccharomyces cerevisiae* strains

Based on the above results of translocation, 3 *Saccharomyces cerevisiae* strains have been selected from different type of translocation and subjected to phenotypic sulfite resistance test. These strains were grown in synthetic must under laboratory conditions with different sulfite concentrations (0, 50 and 200 mg/L of SO_2). Sulfite productions were registered at the end of fermentation for each strain at different level, in order to evaluate their sulfite resistance. Both translocated strains (VIII-t-XVI and XV-t-XVI) produced similar total SO_2 levels in the absence and added SO_2 (Figure 6).

No significant differences have been observed in both type of translocation in terms of phenotypic sulfite resistance. Both translocated strains (VIII-t-XVI and XV-t-XVI) have been able to resist to sulfite doses and to produce high concentration of total SO_2 (free + bound SO_2) compared to non-chromosomal translocation. This is due to the presence of SSU1 gene that mediates sulfite efflux and provides high sulfite resistance to the wine yeast (Yuasa *et al.,* 2004). The presence of free SO_2 in the fermentations suggests that the responsible strains have a higher constitutive expression of FZF1 or SSU1/SSU1-R, which encodes and regulates the sulfite efflux pump SSU1p (Morgan *et al.,* 2019). This expression could lead to more free SO_2 being exported back outside the yeast cell.

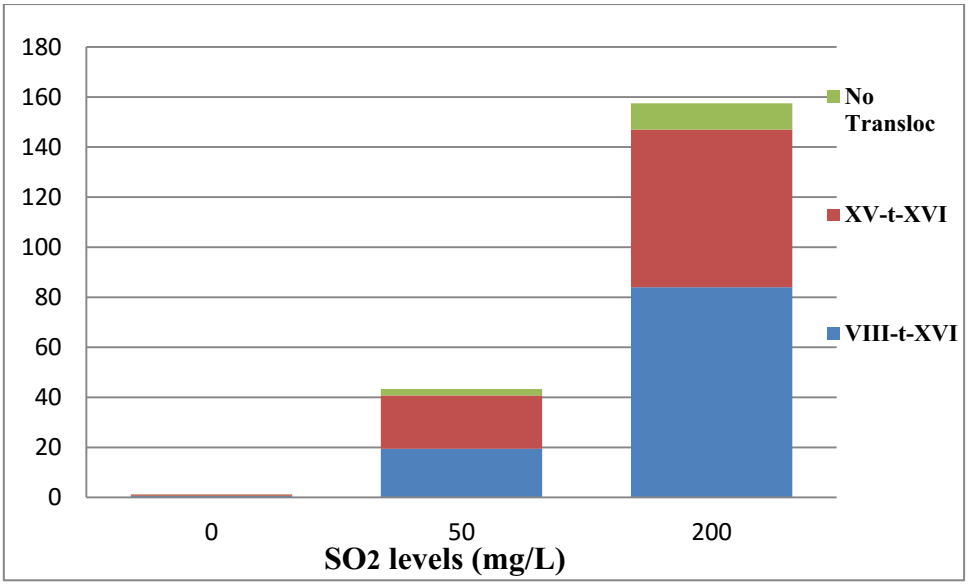

Figure 6: SO_2 level at the end of fermentation conducted by 3 *Saccharomyces cerevisiae* strains of different chromosomal translocation type and in the presence of different initial SO_2 concentrations (0, 50, 200 mg/L).

In addition, yeast growth depends on SO_2 dose added initially. However, non-chromosomal translocation strain has demonstrated sulfite sensitivity and display a decrease in cell growth during the first few days of fermentation when SO_2 was added.

It has been demonstrated, that translocation is way particularly manner to provide a fast and stable adaptation in yeast (Koszul *et al*., 2006; Ferreira *et al.,* 2017).

This adaptation was observed through the evolution of the SSU1 gene leading to SO_2 resistance (Aa *et al.,* 2006). Here, most of *S. cerevisiae* strains presented chromosomal translocation confers increased resistance to sulfite. Yeast resistance to sulfite is of great interest and represents an important technological character for winemaking (Nadai *et al.,* 2016). This criterion was taken into consideration in the selection of starter culture.

As identified, the presence of chromosomal translocation in the Lebanese indigenous strains can be explained by its adaptive value in wine-making environments, where sulfite is widely used as a preservative. *S. cerevisiae* strain exposed to high concentration of sulfite, produce undesirable sulfur compounds during alcoholic fermentation, such as SO_2 and H_2S, in variable amounts (Noble *et al.,* 2015). These compounds produced during fermentation may affected by several factors such as, availability of sulfur compounds, fermentation conditions and the nutritional status of the environment (Cordente *et al.,* 2009).

4. Conclusion

Despite proven toxicity of sulfur dioxide, even for low SO_2 doses, it is still widely used as preservative during winemaking stages. Sulphur dioxide had an impact on yeast cell growth, sporulation and recovery after exposure.

Yeast resistance to sulfite is of great interest and it still represents an important challenge for winemaking. This study, supposed high reciprocal chromosomal translocations in the Lebanese indigenous *Saccharomyces cerevisiae* strains of 'Merwah' wine expressed by a phenotypic sulfite resistance. A complementary analysis may be occurred by adding the Lebanese data to other population wine strains coming from different country, in order to test the hypothesis of a new translocation event by controlling the negatives samples to chromosome XVI.

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PILOT FERMENTATION BY SELECTED *SACCHAROMYCES CEREVISIAE* STRAINS IN WINERY

1. Introduction

In recent years, in line with consumer preferences, and due to the effects of global climate change, new trends have emerged in wine fermentation and wine technology. Consumers are looking to wine that express their *terroir* with a good balance in terms of acidity and mouth feels (Vilela, 2019). Some winemakers argue that authentic expression of *terroir* and vintage can only be achieved through fermentation with indigenous yeasts. Other winemakers prefer the greater security and controlled variability of specialized strains (Ganucci *et al.,* 2018). There are in fact, advantages and disadvantages for both indigenous and cultured yeasts.

One of the major advantages of fermentation with indigenous yeast lies in the timing and duration of fermentation. A delay in the onset of vigorous fermentation allows oxygen to react with anthocyanins and other phenols present in the must to enhance color stability and accelerate phenolic polymerization which enhances texture and mouthfeel (Sevenich, 2010).

However, the production of wines by inoculated fermentations by selected indigenous strains are useful tools to avoid sluggish and stuck fermentations, to increase the microbial diversity, to enhance the wine character, and may be an interesting tool to improve the quality of wines (Andorrà *et al.,* 2019).

Inoculation fermentation with a starter culture is intended to establish a high population of a selected strain of *Saccharomyces cerevisiae* from the beginning of fermentation to ensure its dominance (Ciani *et al.,* 2016). This is one of the most important features determining the starter ability to dominate the process and persist over other yeast strains of the natural microbiota (Granchi *et al*., 2019).

In addition, the use of selected indigenous *Saccharomyces cerevisiae* strains has been introduced in many wineries for several reasons as it (i) provides distinctive characteristics to the wine, (ii) enhances the organoleptic and sensory properties of typical regional wines, and (iii) helps to preserve the native yeast strains that are better adapted to the environment of the viticulture region and to the winemaking process (Vigentini *et al.,* 2017; Capece *et al.,* 2019; De Celis *et al*., 2019).

It was reported the existence of a correlation between specific indigenous strain origin and the characteristics of inoculated wines obtained from strains isolated of different wine regions (Capece *et al.,* 2019). In this way, it might be possible to associate specific indigenous strains with a specific region, or with a *terroir*.

It is well recognized that wines made with indigenous *S. cerevisiae* strains are perceived to be more complex by showing a greater diversity of flavours; where these yeasts produce variable amounts of fermentative by-products, with desirable or undesirable effects on wine bouquet (Capozzi *et al.,* 2015).

In the present study, three indigenous *Saccharomyces cerevisiae* starter strains, previously selected from indigenous populations of 'Merwah' wine during spontaneous fermentation (chapter 2), were used to inoculate 'Merwah' must/wine of 2019 vintage at the same winery Château Bybline. The aim of this work was to produce wines using selected *Saccharomyces cerevisiae* strains and compare their characteristics to the wine produced by indigenous yeasts, using the same harvest Merwah grape.

The wines obtained from these strains were compared to a reference wine fermented spontaneously with wild microflora. Pilot-fermentations were carried out in duplicate for inoculated and un-inoculated wines.

The dominance ability of the inoculated starters and their influence on the aromatic and sensorial qualities of the wines were evaluated in order to test the suitability of these indigenous starters to be used at the cellular scale. In addition, chemical constituents in wine were evaluated at the end of alcoholic fermentation based on the relevant features: fraction of ethanol, residual sugar, free and total SO_2, tartaric, malic, citric, lactic, acetic acids, total acidity and volatile acids.

The assays performed showed a significant difference between the studied wines; where the inoculated wines confirm that the uses of selected yeasts contribute to improve sensorial wine quality and enhance the wine character.

2. Materials and Methods

To determine the effects of three selected *Saccharomyces cerevisiae* strains "M.6.16, M.10.16 and M.4.17" (Table 1) (previously isolated from 'Merwah' wine, Chapter 2) on wine characteristics, pilot-scale fermentations were carried out on 'Merwah' must during the 2019 vintage. The grapes of 'Merwah' were harvested from Bekaatet Achout, Mount Lebanon region (1400 m a.s.l.), proceed to cold maceration at 4°C for 2 days, then crushed and rested 24 hours with sulfite (40 mg/L). The temperature of the alcoholic fermentation was stable during all of the pilot-fermentation at 19 °C.

The strain performances were compared to the same 'Merwah' must/wine but fermented spontaneously (Control).

Table 1: Strains starters used to inoculate 'Merwah' must of 2019 season in pilot fermentation.

Strain name	Experimental wine
M.6.16	A
M.10.16	B
M.4.17	C
Merwah must without inoculation (Control)	M

2.1. Culture preparation and inoculation

Prior to inoculation, one colony of each *S. cerevisiae* strain was aseptically transferred to separate flasks containing 100 mL liquid YEPD media which were incubated in an incubator-shaker at 250 rpm (Multi Stack, shaking; LabTech, Sorisole (BG), Italy) at 25°C.

A small homogeneous subsample from each of these flasks was diluted 10 times, and cells concentrations were counted using the Thoma cell counting chamber to determine the cells/mL of each flask. Approximately 2.5×10^6 cells/mL was inoculated from the YEPD medium into a culture of 2 L of 'Merwah' must (sugar 223 g/L, pH 3.45) of the season 2019 and incubated at 25°C for 48 h. Each pre-culture was inoculated into 20 L of 'Merwah' must fermented in glass tank.

2.2. Microbiological analysis

Three samples (start, middle, and end of alcoholic fermentation) taken from four fermented wines (in duplicate) were inoculated after a series of dilution in differential Wallerstein Laboratory Nutrient media (WL media) (Oxoid) and incubated at 25 °C for 48h. The colonies were evaluated based on colony color and morphology on WL nutrient media and under light microscopy to identify the dominant fermented yeast *Saccharomyces cerevisiae.* Around 10 white yeast colonies were isolated, cultured and stored into YPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% Bacto-agar) (Oxoid). The selected colonies were purified and maintained in slants at 4°C. The results obtained from inoculated wines were compared to isolates obtained in spontaneous 'Merwah' fermentation.

In addition, the viability was evaluated by microscopy by counting yeast cells using a Thoma counting chamber after staining the sample (v/v) with methylene blue for 10 minutes. Blue cells were considered as dead cells (Lange *et al.,* 1993). The percentage of viability is calculated by dividing the number of living cells over the total counted cells multiplying by 100.

2.3. Kinetic of pilot-fermentations

Fermentations were monitored by measuring the decrease of density (Hydrometer), the residual sugar (DNS method) and cell concentration through the alcoholic fermentation.

2.4. Chemical analysis

Ethanol, total acidity, volatile acidity, pH, free and total SO_2, glycerol, organic acids [eg. tartaric, malic, succinic and citric acids] and sugars [glucose and fructose]) were evaluated at the end of alcoholic fermentation using wine scan (Foss, Denmark).

2.5. Sensorial analysis

In order to test the consumer preference and acceptability of the tested wines, a hedonic scaling method (Lim, 2011) was conducted for evaluating wines with a small panel (20 persons). This was examined for reliability, and as a way of comparing the merits of the various wines. According to Cheng *et al*., (2016) 5-point hedonic scale was performed, by giving a score to the studied wines according to the Table 2.

Table 2: 5-point hedonic scores given for each wine.

Dislike very much	Dislike somewhat	Neither like nor dislike	Like somewhat	Like very much

In addition to the preference test, a qualitative analysis was also made to evaluate the same studied wines, by giving a score to the relatively set system (Table 3) (Civille and Oftedal, 2012). At the end, the quality perception of the three inoculated wines was compared to the spontaneous wine tested.

Table 3: Principle aspects of wine tested with given score scale during the sensorial analysis

Parameters	**1**	**2**	**3**	**4**	**5**
Fines and complexity	Ordinary	Simple	Fine	Elegant	Refined
Aromatic power	very low	Low	Medium	High	Very high
Oxidation odor	very low	Low	Medium	High	Very high
Acidity	very low	Low	Medium	High	Very high
Bitterness	very low	Low	Medium	High	Very high
Balance	very low	Low	Medium	High	Very high

2.6. Statistical data analysis

All analytical measurements were performed in duplicate. The values of the parameters are expressed as the mean ± standard deviation.

In order to evaluate which is the category (like or dislike) "significant" for each wine, a binomial test was applied to the results of preference test. In this case the following link: (https://www.socscistatistics.com/tests/binomial/default2.aspx) was used to calculate the p-value for the categories of each wine. The data obtained from sensorial analyses were scored depending on the scale for each parameter and their means were subjected to Radar analysis in order to compare the different tested parameters for each wine. In addition, the results of sensorial analysis have been generated using ANOVA test (95% confidence interval) through XLSTAT to evaluate the significant difference for each parameter in each wine.

Also, the relation between chemical variables and the results of sensorial analysis were evaluated using the Pearson correlation coefficient through XLSTAT (Codină *et al.,* 2013).

3. Results and Discussion

3.1. Microbiological analysis

Three inoculated and one un-inoculated grape must were fermented in duplicate. In total 8 must samples have been taken at the initial, middle and end of pilot fermentations and cultivated on WL medium agar. A dominant yeast colony was observed in the early stages of fermentation (as early as 3 days). As observed visually on the microscope and on WL medium, all the samples showed a dominant white cream colony, similar to *Saccharomyces cerevisiae*, at all fermentation stages.

The viability as determined by microscopy, varied from 98% to 77% at the initial point of fermentation. After viable yeast counting, from each sample and each fermentation phase, 10 colonies showing *Saccharomyces* morphology were randomly isolated than stored in YPD agar (in slant at 4°C) to be subjected to further molecular analyses. This allowed to identify genetically the predominant strain and to compare the inoculated fermentations to the spontaneous one.

3.2. Kinetic of pilot-fermentation

After strains inoculation, must samples were taken from each experimental wine at the initial, middle and end of alcoholic fermentation. Sugar concentration of the initial fermented musts/wines (A, B, C and M) was registered respectively 224.14 g/L; 221.46 g/L; 221.91 g/L; 224.11 g/L.

Monitoring fermentation in the winery scale was based on a daily measurement of the wine density decreases (Figure 1). In addition, sugar consumption (g/L) was measured in order to evaluate fermentation capacity of the inoculated wines (A, B and C) compared to the spontaneous wine fermentation (M: Control). The consumption of sugar was faster in wine A and B compared to the other experimental wines (Figure 2). The wine fermented spontaneously (M) resumes slow speed fermentation respective to inoculated wines by selected *S. cerevisiae* strains, except for the wine C.

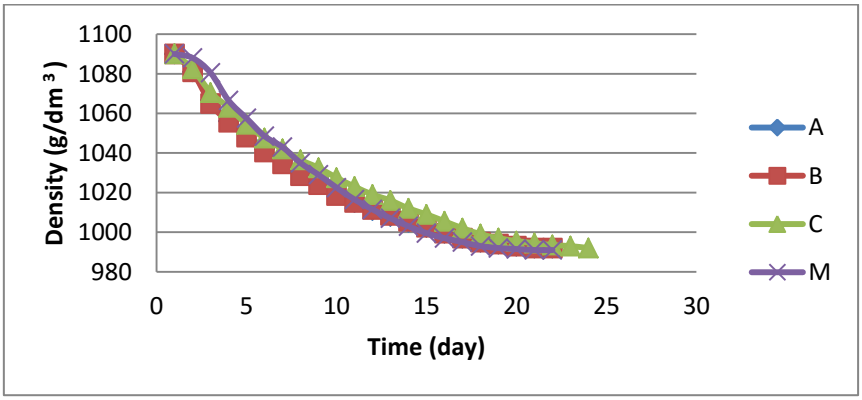

Figure 1: Density evaluation of 4 experimental Merwah wines during alcoholic fermentation at winery Château Bybline.

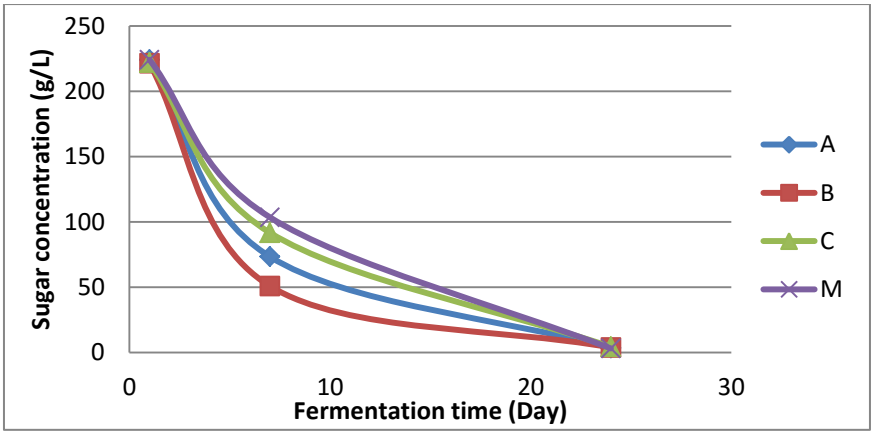

Figure 2: Sugar consumption (g/L) during pilot-fermentation in the winery.

3.3. Enological and chemical analysis

Certain criteria have been met in order to guarantee the desirable features of the selected yeast strains. The most important ones were: low residual sugar, tolerance to ethanol, high fermentation activity, low volatile acidity, low production of sulfur dioxide and hydrogen sulfide (Nikolaou *et al.,* 2006; Capece *et al.,* 2019). The resulting wines were compared based on inoculated and spontaneous wine fermentation.

Usually, fermenting grape must contain a high amount of sugars (about 200 g/L) and low pH (3–3.5). After its fermentation, the chemical composition of base wines is characterized by a considerable amount of ethanol (about 10%–12% v/v), low pH (2.8–3.5), high total acidity (5–7 g/L H_2SO_4), and total SO_2 contents (50–80 mg/L) (Garofalo *et al.,* 2018).

The enological parameters cited in Table 4 were employed discriminately for the selection of autochthonous yeast strains for their use in controlled fermentations.

The tested wines had been fermenting between 22 and 24 days, with low residual sugar, at the end of alcoholic fermentation. High correlations were found between volatile acidity, acetic acid and sugar.

Usually, grapes from cooler climate wine regions have higher levels of acidity, due to the slower ripening process (Kemp *et al.,* 2018). This may confirm the presence of slightly high acidity level (> 4.45 g/L) in the tested wines correlated to the initial acidity concentration in 'Merwah' grape cultivar. Grapes contain several acids in different ratios, including tartaric, acetic, malic, citric, and lactic acids. The predominant acids are tartaric and malic, which together may account for over 90% of the total acidity in the berry, existing at crudely a 1:1 to 1:3 ratio of tartaric to malic acid (Ford, 2012).

At a typical wine pH (3.4), tartaric acid will be three times acidic as malic acid (Bayraktar, 2013). Here, the four wines (A, B, C and M) contain a concentration of tartaric acid below to 3g/L with a stable pH around 3.

Organic acids in wine play an important role in the taste and quality of wines. Perhaps, wine insufficient acidity with low content of organic acids, loses its fullness and roundness of taste and becomes characterless and expressionless (Muratova *et al.,* 2007).

The concentration of acetic acid higher than 0.8-0.9 g/L is immediately recognized due to the vinegar smell causing spoiled wine (Ribéreau-Gayon *et al*., 2006). The maximum acceptable limit for volatile acidity in most wines is 1.2 g/L of acetic acid (OIV, 2010). All the tested wines have demonstrated low production of acetic acid (<0.5 g/L) (Table 3) which is an important criterion for the selection of wine strain.

The strain A has produce less sulfur dioxide (3.5 mg/L of SO_2) than the others. But regarding to the free SO_2, all strains presented the same concentration. This is a normal level, since the winemaker has to use SO_2 before pressing.

Based on the results of chemical analysis, the inoculated (A, B, C) and spontaneous (M) wines have not seen a chemical difference. This may suggest the presence of predominant yeast strain in the environment that may produce the same chemical wine composition.

For this reason, sensorial analysis was performed for the 4 types of wines (in duplicate) in order to choose the best fermented 'Merwah' wine in point of aromatic profile.

Table 4: Chemical parameters of experimental wines registered at the end of pilot-fermentation.

	Experimental wine			
Parameters	A	B	C	M
Ethanol production (g/L)	13.115±0.035	12.93±0.3	12.935±0.29	13.135±0.09
Free SO_2 (mg/L)	3.5±0.14	3.55±0.35	3.55±0.07	3.1±0.14
Total SO_2 (mg/L)	3.5±2.1	11±1.4	14	13.5±0.70
Total acidity (g/L)	4.45±0.07	4.65±0.07	4.75±0.07	4.65±0.070
Volatile acidity (g/L)	0.3	0.3	0.4	0.35±0.070
pH	3.12±0.01	3.095±0.007	3.09	3.105±0.02
Malic acid (g/L)	1.85±0.07	1.9	2	1.9
Reducing sugar (g/L)	2.5	3.5±0.63	3.3±3.11	3.05±0.21
citric acid (g/L)	0.355	0.37	0.385±0.02	0.37
Glucose+Fructose (g/L)	4.7	3.3±0.70	5.65±6	2.4±0.28
Glycerol (g/L)	6.8	7±0.14	7.1±0.14	7.1
Glucose (g/L)	0.4±0.14	0.3±0.14	0.4±0.28	0.3±0.14
Fructose (g/L)	5.2	3.8±0.56	6.05±5.58	3±0.28
Tartaric acid (g/L)	3	2.95±0.07	2.85±0.70	2.85±0.070
CO_2 (mg/L))	371.04±2.02	328.99±12.05	466.485±8.6	417.18±6.89

3.4. Sensorial analysis

Hedonic method was made as preference test in order to differentiate among the wines made from 'Merwah' grapes.

Small panel of twenty persons were tested, some of them were experts with a high degree of experience in wine judging and the others consisting of fine wine consumers with no special training. Judges were instructed to drink and swallow each sample and rate the intensity of each attribute using

a five-point scale. The sessions were performed on the same day at the research center of the Lebanese University Faculty of Agricultural Sciences, Ghazir, Lebanon.

The small panel hedonic method (Lawless *et al.,* 2007) is suitable for generating quality scores for consumer guidance in large scale wine surveys, as are commonly found in popular wine periodicals (Pagliarini *et al.,* 2013).

The results of the hedonic scaling show diversity in the responses. Some confusion has been detected by judges, where some of them considered the duplicate of the tested wine as a totally different wine.

A binomial test was applied to the hedonic results (Table 5) to evaluate which is the category "significant" for each wine. The calculation of p-value was based on the formula from the following link: https://www.socscistatistics.com/tests/binomial/default2.aspx. Statistically, the wine C was significantly preferred by the judges ($p < 0.05$), while the analysis of the other wines did not revealed any statistical significance.

Table 5: Number of persons/20 that 'like' and/or 'dislike' the wines respecting to hedonic scale.

	Categories respecting to hedonic scale				
Wines	Dislike very much	Dislike somewhat	Neither like nor dislike	Like somewhat	Like very much
A	1	4	6	4	5
B	6	5	3	2	4
C	0*	4	5	3	8*
M	3	2	5	3	7

*Significant difference in the wine C for both categories "dislike very much" and "like very much" (p-value = 0.0252).

According to the Figure 3, the wine 'C' was very much liked by the majority of the wine judges. Meanwhile, the wine 'B' was very much disliked by judges. This may induce to strain-effect on sensorial wine quality. In addition, the wine 'M' fermented spontaneously was accepted by the most judges. It seems low perception differences were detected between the wine M and C, which may be due to the presence of some common strains in the fermented environment.

For a better knowledge about yeast dominance and resistance in both wines, genetic analysis will be suggested to do it further.

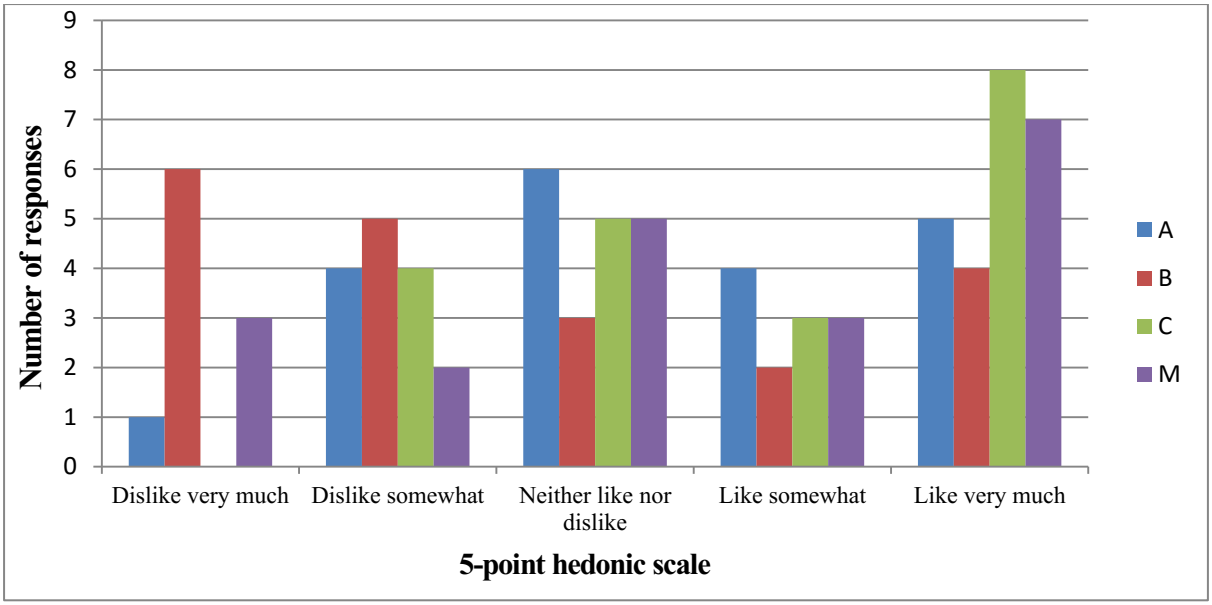

Figure 3: Distribution of consumer acceptance scores for inoculated (A, B and C) and un-inoculated (M) Merwah's wines.

In addition to the preference test, a sensorial evaluation was made for each wine considering the following parameters: finesse and complexity, oxidation odor, aromatic power, acidity, bitterness and balance in wine; and scored according to the table 3.

The following table (Table 6) present the score of each parameter for each duplicated wines and the p-values of each parameter were obtained by ANOVA test. Only the 'aromatic power' has demonstrated a significant difference ($p<0.05$) between the four wines.

Table 6: Mean of score (/5) values taken by judges about the four duplicated wines

	Wines tested in replicate				
Parameters	**A**	**B**	**C**	**M**	**p-value**
Acidity	2.4 2.7	3 3.25	2.2 3.2	2.8 3	p=0.541
Aromatic power	3 3.5	2.7 2.4	3.8 3.7	3 2.7	p=0.026
Oxidation	1.6 2.2	2.5 2.3	2 3.4	2.7 2.4	p=0.560
Fines and complexity	2.4 2.7	2.3 2.25	2.64 2.7	2.5 2.8	p=0.169
Balance	2.8 3	2.6 3.2	2.6 3.2	2.6 3	p=0.986
Bitterness	2.5 2.3	2 2.3	1.3 1.8	2.2 1.8	p=0.116

Regarding the radar results (Figure 4), the wine C has shown higher aromatic power with a complexity of flavors respective to other wines.

It was found a strict interaction between grapes must composition and yeast strains performing the fermentation according to aromatic wine quality (Capece *et al.,* 2019). These findings confirm that the aromatic quality is related to 'Merwah' must/wine and the type of inoculated strain.

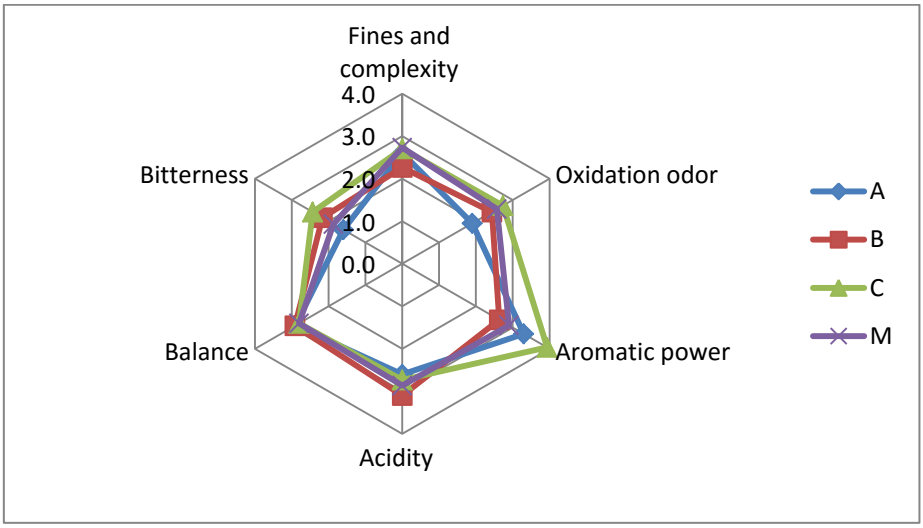

Figure 4: Sensory analysis of three inoculated wines and one spontaneous wine.

In order to determine the correlation between the sensorial perception and the chemical parameter of wine perceived by the wine tasters, Pearson correlation coefficient was made (Figure 5).

With regard to other findings, we found few correlations between chemical characteristics and sensory results. For example, there is a high correlation between aromatic feel and the amount of glucose and fructose. It seems that the fruity aroma was considered based on the level of sugar (glucose+fructose) in the wine. Instead, judges say bitterness highly correlated with the acids. Also, the acidity is only correlated to tartaric acid. This confirms the importance aspect of aroma in wine quality hence, for consumer acceptance (Wu *et al*., 2016).

Inoculated wine yeast strain isolated for its desirable fermenting characteristics might be able to produce pleasant fruity aromas, a high level of alcohol, an attractive mouth texture, and an ability to ferment in low temperatures or high acid (Andorrà *et al.,* 2019).

Spontaneous fermentations are likely to produce a wider palette of sensory characteristics than are usually found in inoculated fermentations, but whether those characteristics are positive or negative is dictated by factors we don't yet fully understand (Mansfield and Tahim, 2016).

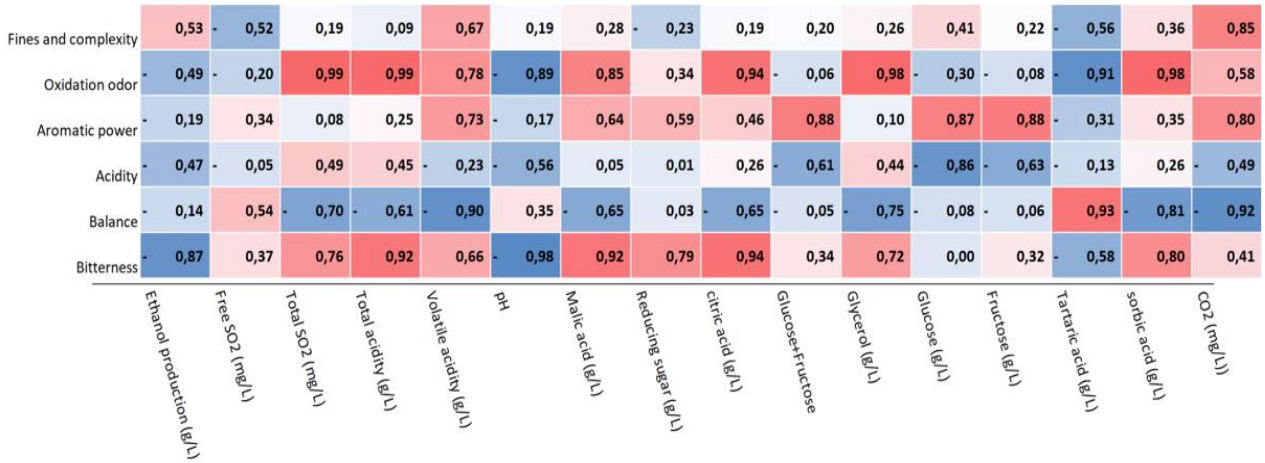

Figure 5: Correlation between chemical parameters and sensorial perception of wine.
The red and blue colors mean positively and negatively correlated respectively.
The color intensity means the grade of correlation (light color: low correlation; intense color: high correlation).

4. Conclusion

This study has evaluated the fermentative activity of three *Saccharomyces cerevisiae* strains, previously isolated, during inoculated fermentation of 'Merwah' grape must.

Several analysis have been evaluated in order to choose which strain can be better adapted to the 'Merwah' must environment revealing interested technological features and producing aromatic compounds. Technological profile of the inoculated wines (A, B and C) has been compared to the wine fermented spontaneously (M).

Considering the results of the preference test, the wine tasters classified the wine C in a first place and the wine fermented spontaneously (M) as second place of preference by almost judges.

The results obtained in this study showed high aromatic quality of wine related to the 'Merwah' grape cultivar and the inoculated strain. Moreover, the wine obtained from the spontaneous fermentation has shown a good quality perception like as inoculated wines.

In fact, inoculated wine with selected indigenous strain from a specific wine region can help the winemaker to reduce the risk of fermentation and to increase the stability production of wine quality. Among the studied strains, the strain (C) can be selected as a starter to produce wine characterized by peculiar oenological and organoleptic features.

As a preliminary analysis, isolated colonies from the fermented wines identify a dominance of *Saccharomyces cerevisiae.* In order to evaluate the microbial stability during inoculated fermentation, which is an important step to check the dominant fermentative strain, molecular analyses will be carried out using interdelta PCR in order to verify the dominant strain throughout the fermentation and to determine the responsible strain of 'Merwah' wine fermentation.

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GENERAL CONCLUSION

Nowadays, many wineries and regions are carrying out programs of isolation and selection of yeasts that are typical of their vineyards/wineries.

The evaluation of yeast biodiversity during spontaneous alcoholic fermentation has widely studied as an important prior step in the research to understand the winegrowing area before selecting and inoculating isolated strains from that area as starters.

This research studied the diversity of *Saccharomyces cerevisiae* strains isolated from 'Merwah' grape cultivar, collected during spontaneous fermentation in a unique winery, in order to establish the existence of typical strains belonging to the winery, or regional ecosystems.

The results obtained from the genetic characterization showed a great diversity among the isolated 202 *S. cerevisiae* strains and no resident strain was observed during the two studied consecutive years.

In addition, technological characterization was performed for 22 *S. cerevisiae* strains selected previously as representative of the studied population; where also shown a phenotypic diversity. The correlation between genotypic and phenotypic groups and the geographical origin may support the concept that there can be a microbial aspect to *terroir.*

Further genetic and phenotypic characterizations were evaluated in order to determine the presence of chromosomal translocations related to sulfite resistance in the studied *Saccharomyces cerevisiae* strains. An important adaptation to sulfite was observed due to the presence of chromosomal translocation (88% of the studied *S. cerevisiae* population) through the evolution of the SSU1 gene leading to SO_2 resistance.

Among the studied strains, three indigenous *Saccharomyces cerevisiae* strains were selected to be used as starter cultures in pilot-fermentation of 'Merwah' must/wine of 2019 vintage at cellar level (same winery Château Bybline).

It has been observed in the results of the chemical and sensorial analyses of pilot fermentation, that inoculated wine C by the strain (M.4.17) had interested oenological features compared to other wines and slightly similar to spontaneous fermentation. In this case, the application of the selected indigenous *S. cerevisiae* strain may help the winemaker to ferment without risk of spoilage and produce an aromatic wine. It will be more important to control the dominant fermented strains during inoculated fermentation to verify their dominance capacity during fermentation.

Different starter implantation throughout inoculated fermentation represents an additional character, which might be considered during the selection program for wine starter cultures.

The characterization and selection of indigenous *Saccharomyces cerevisiae* strains adapted to a specific wine region, has great importance in the biodiversity preservation and exploitation in terms of the starter culture collection. Selecting indigenous strains with particular phenotypes appear as a valuable tool for differentiation, diversification, and quality improvement of regional wines.

At the end of this project, it will be possible to establishing a Lebanese microbial culture collection network in order to preserve microbial *terroir* to facilitate the safe and responsible utilization of microbial resources for research, education, industry, medicine, and agriculture for the improvement of human kind.

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Finally, I would express my gratitude for my parents who supported me during all the ups and downs of my PhD.

SUPPLEMENTARY DATA

Table S1: Clustering data for the 202 'Merwah' yeast strains isolated in 2016 and 2017, based on interdelta-PCR analysis.

A significant cluster difference that splits 2016 from 2017. This dendrogram regroup the isolates into 3 clusters as following: C1= 112 observations from Merwah 2016 + 3 observations from Merwah 2017; C2= 50 observations from Merwah 2017; C3= 37 observations from Merwah 2017

Sample	Isolate	Observation number	Cluster number
2016-1MF	1	Obs1	C1
2016-1MF	2	Obs2	C1
2016-1MF	3	Obs3	C1
2016-1MF	4	Obs4	C1
2016-1MF	5	Obs5	C1
2016-1MF	6	Obs6	C1
2016-1MF	7	Obs7	C1
2016-1MF	8	Obs8	C1
2016-1MF	9	Obs9	C1
2016-1MF	10	Obs10	C1
2016-1MF	11	Obs11	C1
2016-1MF	12	Obs12	C1
2016-1MF	13	Obs13	C1
2016-1MF	14	Obs14	C1
2016-1MF	15	Obs15	C1
2016-1MF	16	Obs16	C1
2016-1MF	17	Obs17	C1
2016-1MF	18	Obs18	C1
2016-1MF	19	Obs19	C1
2016-1MF	20	Obs20	C1
2016-1MF	21	Obs21	C1
2016-1MF	22	Obs22	C1
2016-1MF	23	Obs23	C1
2016-1MF	24	Obs24	C1
2016-1MF	25	Obs25	C1
2016-1EF	1	Obs26	C1
2016-1EF	2	Obs27	C1
2016-1EF	3	Obs28	C1
2016-1EF	4	Obs29	C1
2016-1EF	5	Obs30	C1
2016-1EF	6	Obs31	C1
2016-1EF	7	Obs32	C1
2016-1EF	8	Obs33	C1
2016-1EF	9	Obs34	C1
2016-1EF	10	Obs35	C1
2016-1EF	11	Obs36	C1

2016-1EF	12	Obs37	C1
2016-1EF	13	Obs38	C1
2016-1EF	14	Obs39	C1
2016-1EF	15	Obs40	C1
2016-1EF	16	Obs41	C1
2016-1EF	17	Obs42	C1
2016-1EF	18	Obs43	C1
2016-1EF	19	Obs44	C1
2016-2MF	1	Obs45	C1
2016-2MF	2	Obs46	C1
2016-2MF	3	Obs47	C1
2016-2MF	4	Obs48	C1
2016-2MF	5	Obs49	C1
2016-2MF	6	Obs50	C1
2016-2MF	7	Obs51	C1
2016-2MF	8	Obs52	C1
2016-2MF	9	Obs53	C1
2016-2MF	10	Obs54	C1
2016-2MF	11	Obs55	C1
2016-2MF	12	Obs56	C1
2016-2MF	13	Obs57	C1
2016-2MF	14	Obs58	C1
2016-2MF	15	Obs59	C1
2016-2MF	16	Obs60	C1
2016-2MF	17	Obs61	C1
2016-2MF	18	Obs62	C1
2016-2MF	19	Obs63	C1
2016-2MF	20	Obs64	C1
2016-2MF	21	Obs65	C1
2016-2MF	22	Obs66	C1
2016-2MF	23	Obs67	C1
2016-2MF	24	Obs68	C1
2016-2MF	25	Obs69	C1
2016-2MF	26	Obs70	C1
2016-2MF	27	Obs71	C1
2016-2MF	28	Obs72	C1
2016-2MF	29	Obs73	C1
2016-2MF	30	Obs74	C1
2016-2MF	31	Obs75	C1
2016-2MF	32	Obs76	C1
2016-2MF	33	Obs77	C1
2016-2MF	34	Obs78	C1
2016-2MF	35	Obs79	C1
2016-2MF	36	Obs80	C1
2016-2MF	37	Obs81	C1
2016-2MF	38	Obs82	C1

2016-2MF	39	Obs83	C1
2016-2MF	40	Obs84	C1
2016-2EF	1	Obs85	C1
2016-2EF	2	Obs86	C1
2016-2EF	3	Obs87	C1
2016-2EF	4	Obs88	C1
2016-2EF	5	Obs89	C1
2016-2EF	6	Obs90	C1
2016-2EF	7	Obs91	C1
2016-2EF	8	Obs92	C1
2016-2EF	9	Obs93	C1
2016-2EF	10	Obs94	C1
2016-2EF	11	Obs95	C2
2016-2EF	12	Obs96	C2
2016-3MF	1	Obs97	C2
2016-3EF	2	Obs98	C2
2016-3EF	3	Obs99	C2
2016-3EF	4	Obs100	C2
2016-3EF	5	Obs101	C2
2016-3EF	6	Obs102	C2
2016-3EF	7	Obs103	C2
2016-3EF	8	Obs104	C2
2016-3EF	9	Obs105	C2
2016-3EF	10	Obs106	C2
2016-3EF	11	Obs107	C2
2016-3EF	12	Obs108	C2
2016-3EF	13	Obs109	C2
2016-3EF	14	Obs110	C2
2016-3EF	15	Obs111	C2
2016-3EF	16	Obs112	C2
2017-1EF	1	Obs113	C2
2017-1EF	2	Obs114	C2
2017-1EF	3	Obs115	C2
2017-1EF	4	Obs116	C2
2017-1EF	5	Obs117	C2
2017-1EF	6	Obs118	C2
2017-1EF	7	Obs119	C2
2017-1EF	8	Obs120	C2
2017-1EF	9	Obs121	C2
2017-1EF	10	Obs122	C2
2017-1EF	11	Obs123	C2
2017-1EF	12	Obs124	C2
2017-1EF	13	Obs125	C2
2017-1EF	14	Obs126	C2
2017-1EF	15	Obs127	C2
2017-1EF	16	Obs128	C2

2017-1EF	17	Obs129	C2
2017-1EF	18	Obs130	C2
2017-1EF	19	Obs131	C2
2017-1EF	20	Obs132	C2
2017-1EF	21	Obs133	C2
2017-1EF	22	Obs134	C2
2017-1EF	23	Obs135	C2
2017-1EF	24	Obs136	C2
2017-1EF	25	Obs137	C2
2017-1EF	26	Obs138	C2
2017-1EF	27	Obs139	C2
2017-1EF	28	Obs140	C2
2017-1EF	29	Obs141	C2
2017-1EF	30	Obs142	C2
2017-1EF	31	Obs143	C2
2017-1EF	32	Obs144	C2
2017-1EF	33	Obs145	C2
2017-1EF	34	Obs146	C2
2017-1EF	35	Obs147	C2
2017-1EF	36	Obs148	C2
2017-1EF	37	Obs149	C2
2017-1EF	38	Obs150	C2
2017-1EF	39	Obs151	C2
2017-1EF	40	Obs152	C2
2017-1EF	41	Obs153	C2
2017-1EF	42	Obs154	C2
2017-1EF	43	Obs155	C2
2017-1EF	44	Obs156	C2
2017-1EF	45	Obs157	C2
2017-1EF	46	Obs158	C2
2017-1EF	47	Obs159	C2
2017-1EF	48	Obs160	C2
2017-1EF	49	Obs161	C2
2017-1EF	50	Obs162	C2
2017-1EF	51	Obs163	C2
2017-1EF	52	Obs164	C2
2017-1EF	53	Obs165	C2
2017-1EF	54	Obs166	C3
2017-1EF	55	Obs167	C3
2017-1EF	56	Obs168	C3
2017-1EF	57	Obs169	C3
2017-1EF	58	Obs170	C3
2017-1EF	59	Obs171	C3
2017-1EF	60	Obs172	C3
2017-1EF	61	Obs173	C3
2017-1EF	62	Obs174	C3

2017-1EF	63	Obs175	C3
2017-1EF	64	Obs176	C3
2017-1EF	65	Obs177	C3
2017-1EF	66	Obs178	C3
2017-1EF	67	Obs179	C3
2017-1EF	68	Obs180	C3
2017-1EF	69	Obs181	C3
2017-1EF	70	Obs182	C3
2017-1EF	71	Obs183	C3
2017-2EF	1	Obs184	C3
2017-2EF	2	Obs185	C3
2017-2EF	3	Obs186	C3
2017-2EF	4	Obs187	C3
2017-2EF	5	Obs188	C3
2017-2EF	6	Obs189	C3
2017-2EF	7	Obs190	C3
2017-2EF	8	Obs191	C3
2017-2EF	9	Obs192	C3
2017-2EF	10	Obs193	C3
2017-2EF	11	Obs194	C3
2017-2EF	12	Obs195	C3
2017-2EF	13	Obs196	C3
2017-2EF	14	Obs197	C3
2017-2EF	15	Obs198	C3
2017-2EF	16	Obs199	C3
2017-2EF	17	Obs200	C3
2017-2EF	18	Obs201	C3
2017-2EF	19	Obs202	C3

Table S2: Statistical analysis of the interdelta-PCR data for the 112 'Merwah' wine yeast isolates from 2016.

Sample name	Observation number	Cluster number
2016-1MF	1	C1
2016-1MF	2	C1
2016-1MF	3	C1
2016-1MF	4	C1
2016-1MF	5	C1
2016-1MF	6	C1
2016-1MF	7	C1
2016-1MF	8	C1
2016-1MF	9	C1
2016-1MF	10	C1
2016-1MF	11	C1
2016-1MF	12	C1
2016-1MF	13	C1
2016-1MF	14	C1
2016-1MF	15	C1
2016-1MF	16	C1
2016-1MF	17	C1
2016-1MF	18	C1
2016-1MF	19	C1
2016-1MF	20	C1
2016-1MF	21	C1
2016-1MF	25	C1
2016-1EF	26	C1
2016-1EF	28	C1
2016-1EF	29	C1
2016-1EF	30	C1
2016-1EF	31	C1
2016-1EF	32	C1
2016-1EF	33	C1
2016-1EF	34	C1
2016-1EF	35	C1
2016-1EF	36	C1
2016-1EF	37	C1
2016-1EF	38	C1
2016-1EF	39	C1
2016-1EF	41	C1
2016-2MF	45	C1
2016-2MF	46	C1
2016-2MF	47	C1
2016-2MF	48	C1
2016-2MF	49	C1
2016-2MF	50	C1

2016-2MF	51	C1
2016-2MF	52	C1
2016-2MF	53	C1
2016-2MF	54	C1
2016-2MF	55	C1
2016-2MF	56	C1
2016-2MF	57	C1
2016-2MF	58	C1
2016-2MF	59	C1
2016-2MF	60	C1
2016-2MF	61	C1
2016-2MF	62	C1
2016-2MF	63	C1
2016-2MF	64	C1
2016-2MF	65	C1
2016-2MF	66	C1
2016-2MF	67	C1
2016-2MF	68	C1
2016-2MF	69	C1
2016-2MF	70	C1
2016-2MF	71	C1
2016-2MF	72	C1
2016-2MF	73	C1
2016-2MF	74	C1
2016-2MF	75	C1
2016-2MF	76	C1
2016-2MF	77	C1
2016-2MF	78	C1
2016-2MF	79	C1
2016-2MF	80	C1
2016-2MF	81	C1
2016-2MF	82	C1
2016-2MF	83	C1
2016-2MF	84	C1
2016-2EF	85	C1
2016-2EF	86	C1
2016-2EF	87	C1
2016-2EF	88	C1
2016-2EF	89	C1
2016-2EF	90	C1
2016-2EF	91	C1
2016-2EF	92	C1
2016-2EF	93	C1
2016-2EF	94	C1
2016-2EF	95	C1
2016-2EF	96	C1

2016-3MF	97	C1
2016-3EF	98	C1
2016-3EF	99	C1
2016-3EF	100	C1
2016-3EF	101	C1
2016-3EF	102	C1
2016-3EF	103	C1
2016-3EF	104	C1
2016-3EF	105	C1
2016-3EF	106	C1
2016-3EF	107	C1
2016-3EF	108	C1
2016-3EF	109	C1
2016-3EF	110	C1
2016-3EF	111	C1
2016-3EF	112	C1
2016-1MF	22	C2
2016-1MF	23	C2
2016-1MF	24	C2
2016-1EF	27	C3
2016-1EF	43	C3
2016-1EF	40	C4
2016-1EF	42	C4
2016-1EF	44	C4

Table S3: Statistical analysis of the interdelta-PCR data for the 90 'Merwah' wine yeast isolates from 2017.

Sample name	Observation number	Cluster number
2017-1EF	1	C1
2017-1EF	3	C1
2017-1EF	23	C1
2017-1EF	54	C1
2017-1EF	59	C1
2017-1EF	61	C1
2017-2EF	75	C1
2017-2EF	84	C1
2017-2EF	87	C1
2017-1EF	2	C2
2017-1EF	4	C2
2017-1EF	6	C2
2017-1EF	7	C2
2017-1EF	8	C2
2017-1EF	9	C2
2017-1EF	10	C2
2017-1EF	11	C2
2017-1EF	12	C2
2017-1EF	13	C2
2017-1EF	14	C2
2017-1EF	16	C2
2017-1EF	17	C2
2017-1EF	18	C2
2017-1EF	19	C2
2017-1EF	20	C2
2017-1EF	21	C2
2017-1EF	22	C2
2017-1EF	24	C2
2017-1EF	25	C2
2017-1EF	26	C2
2017-1EF	27	C2
2017-1EF	29	C2
2017-1EF	30	C2
2017-1EF	31	C2
2017-1EF	32	C2
2017-1EF	33	C2
2017-1EF	34	C2
2017-1EF	36	C2
2017-1EF	37	C2
2017-1EF	38	C2
2017-1EF	40	C2
2017-1EF	42	C2
2017-1EF	44	C2

2017-1EF	45	C2
2017-1EF	47	C2
2017-1EF	48	C2
2017-1EF	49	C2
2017-1EF	50	C2
2017-1EF	51	C2
2017-1EF	52	C2
2017-1EF	53	C2
2017-1EF	55	C2
2017-1EF	56	C2
2017-1EF	57	C2
2017-1EF	58	C2
2017-1EF	60	C2
2017-1EF	62	C2
2017-1EF	63	C2
2017-1EF	65	C2
2017-1EF	67	C2
2017-1EF	68	C2
2017-1EF	69	C2
2017-1EF	70	C2
2017-2EF	72	C2
2017-2EF	73	C2
2017-2EF	76	C2
2017-2EF	77	C2
2017-2EF	78	C2
2017-2EF	79	C2
2017-2EF	80	C2
2017-2EF	81	C2
2017-2EF	82	C2
2017-2EF	90	C2
2017-1EF	5	C3
2017-1EF	28	C3
2017-1EF	35	C3
2017-1EF	39	C3
2017-1EF	46	C3
2017-1EF	71	C3
2017-2EF	74	C3
2017-2EF	83	C3
2017-2EF	89	C3
2017-1EF	15	C4
2017-1EF	41	C4
2017-1EF	43	C4
2017-1EF	64	C4
2017-1EF	66	C4
2017-2EF	85	C4
2017-2EF	86	C4

Table S4: *Saccharomyces cerevisiae* strains of different origins added to the 'Merwah' wine yeast population in the microsatellite analysis.

Strain Name	Strain type	Origin	Country
YS2	Bioprocess	Bread	Australia
YS4	Bioprocess	Bread	The Netherlands
YS9	Bioprocess	Bread	Singapore
Y9	Bioprocess	Sake	Indonesia
Y12	Bioprocess	Palm wine	Ivory Coast
NCYC110	Bioprocess	Beer	West Africa
K11	Bioprocess	Sake	Japan
DBVP66044	Bioprocess	Beer	West Africa
223R	Wine	Industrial	NR
Actiflore 522	Wine	Industrial	NR
Actiflore BJL	Wine	Industrial	NR
Actiflore BO213	Wine	Industrial	NR
Actiflore F33	Wine	Industrial	NR
Actiflore ROSE	Wine	Industrial	NR
Affinity ECAS	Wine	Industrial	NR
ANCHOR ALCHEMY	Wine	Industrial	NR
ANCHOR EXOTICS SPH	Wine	Industrial	NR
ANCHOR NT116	Wine	Industrial	NR
ANCHOR NT202	Wine	Industrial	NR
ANCHOR NT45	Wine	Industrial	NR
ANCHOR NT50	Wine	Industrial	NR
ANCHOR VIN13	Wine	Industrial	NR
C17	Wine	Industrial	NR
COGNAC-7103	Wine	Industrial	NR
Collection cepage sauvignon	Wine	Industrial	NR
E2F	Wine	Industrial	NR
EQUINOX-81	Wine	Industrial	NR
EXCELLENCE SP	Wine	Industrial	NR
EXCELLENCE 8S	Wine	Industrial	NR
EXCELLENCE STR	Wine	Industrial	NR
EXCELLENCE TXL	Wine	Industrial	NR
EXCELLENCE XR	Wine	Industrial	NR
FC9	Wine	Industrial	NR
FERMICHAMP	Wine	Industrial	NR
FERMOL CANDY	Wine	Industrial	NR
FERMOL Cryofruit	Wine	Industrial	NR
FERMOL IPER-R	Wine	Industrial	NR
FERMOL PB2033	Wine	Industrial	NR
FERMOL PRIMEURS	Wine	Industrial	NR
FERMOL RED FRUIT	Wine	Industrial	NR
FERMOL SPIRIT	Wine	Industrial	NR
H4	Wine	Industrial	NR
ICV D254	Wine	Industrial	NR
ICVOK	Wine	Industrial	NR

IOC18-2007	Wine	Industrial	NR
I onis	Wine	Industrial	NR
LA-PM	Wine	Industrial	NR
LALVIN CLOS	Wine	Industrial	NR
LALVIN FC9 EDV	Wine	Industrial	NR
LALVIN QA23	Wine	Industrial	NR
LALVIN RC 212	Wine	Industrial	NR
LALVIN RHONE 2056	Wine	Industrial	NR
LALVIN RHONE 2226	Wine	Industrial	NR
LEP 55	Wine	Industrial	NR
LEVULIA ESPERIDE	Wine	Industrial	NR
LEVULIA GE7 TRADITION	Wine	Industrial	NR
LEVULIA U 32	Wine	Industrial	NR
LEVULINE CHP	Wine	Industrial	NR
MAURIVIN AWRI 350	Wine	Industrial	NR
MAURIVIN UOA MAXI	Wine	Industrial	NR
NT 202	Wine	Industrial	NR
NT 50	Wine	Industrial	NR
PREDELVIN PDM	Wine	Industrial	NR
RB 2	Wine	Industrial	NR
RB 4	Wine	Industrial	NR
RMS 2	Wine	Industrial	NR
RX 60	Wine	Industrial	NR
SAFOENO HD S62	Wine	Industrial	NR
SAFOENO UVA S111	Wine	Industrial	NR
SAUVIGNON	Wine	Industrial	NR
SELECTYS L-AUTHENT	Wine	Industrial	NR
SELECTYS L-ELEGANTE	Wine	Industrial	NR
SELECTYS LA PERSANE	Wine	Industrial	NR
SELECTYS LA RAFFINEE	Wine	Industrial	NR
SELECTYS SR	Wine	Industrial	NR
SO DELIGHT	Wine	Industrial	NR
SO FLAVOUR	Wine	Industrial	NR
SO SPIRIT	Wine	Industrial	NR
SP 39	Wine	Industrial	NR
SP 49	Wine	Industrial	NR
SPARKLSA	Wine	Industrial	NR
SPINGER VR 44	Wine	Industrial	NR
SPINGER BC S 103	Wine	Industrial	NR
SPINGER CK S 102	Wine	Industrial	NR
SPINGER NDA 21	Wine	Industrial	NR
SPINGER SAINT GEORGES-S 101	Wine	Industrial	NR
SPINGER UCLM S 325	Wine	Industrial	NR
SPINGER UCLM S 377	Wine	Industrial	NR
VIALATTE FERM R 100	Wine	Industrial	NR
VIALATTE FERM R 71	Wine	Industrial	NR

VIALATTE FERM R 82	Wine	Industrial	NR
VIALATTE FERM R 96	Wine	Industrial	NR
VIALATTE FERM W 12	Wine	Industrial	NR
VIALETTE FERM W 28	Wine	Industrial	NR
VINIFERM CT 007	Wine	Industrial	NR
VINIFERM EMOCION	Wine	Industrial	NR
VINIFERM RVA	Wine	Industrial	NR
VINIFLORA MELODY	Wine	Industrial	NR
VINIFLORA MERIT	Wine	Industrial	NR
VITILEVURE 3001	Wine	Industrial	NR
VITILEVURE DV10	Wine	Industrial	NR
VITILEVURE ELXIR	Wine	Industrial	NR
VITILEVURE QUARTZ	Wine	Industrial	NR
Yseo brio	Wine	Industrial	NR
ZYMAFLORE CH09	Wine	Industrial	NR
ZYMAFLORE DELTA	Wine	Industrial	NR
ZYMAFLORE F15	Wine	Industrial	NR
ZYMAFLORE FX10	Wine	Industrial	NR
ZYMAFLORE ST	Wine	Industrial	NR
ZYMAFLORE VL1	Wine	Industrial	NR
ZYMAFLORE VL2	Wine	Industrial	NR
ZYMAFLORE VL3	Wine	Industrial	NR
ZYMAFLORE XS	Wine	Industrial	NR
ZYMAFLORE XPURE	Wine	Industrial	NR
ZYMAZIL	Wine	Industrial	NR
BC 187	Wine	Wine	USA
DBVPG1106	Wine	Grape	Australia
273614 X	Wild	Clinical	UK
378604 X	Wild	Clinical	UK
DBVPG 1853	Wild	Cereal	Ethiopia
DBVPG 6765	Wild	Fruit	Indonesia
L-1374	Wine	Wine	Chile
L-1528	Wine	Wine	Chile
S288c	Wild	Fruit	USA
SK1	Wild	Soil	USA
UWOPSO3	Wild	Plant	Malaysia
UWOPSO5.2	Wild	Insect	Malaysia
UWOPS83	Wild	Fruit	Bahamas
UWOPS87	Wild	Plant	Hawai
W303	NA	Laboratory	NA
Y 55	Wine	Grape	France
YII C17 E5	Wine	Wine	France
YJM 975	Wild	Clinical	Italy
YJM 978	Wild	Clinical	Italy
YJM 981	Wild	Clinical	Italy
YPS 606	Wild	Plant	USA

NR, not recorded NA, not applicable

Table S5: Statistical differences in total CO_2 production of 22 *S. cerevisiae* strains as determined by ANOVA followed by Tukey -HSD test ($p<0.01$).

Strain	Mean CO_2 (g/100ml)	Significance groups ($p<0.01$)
M.3.16	10.47	A
M.2.16	10.4	A
M.6.16	10.05	Ab
M.11.17	9.68	Abc
M.9.16	9.4	Abcd
M.4.17	9.27	Abcd
M.8.16	9.08	Abcde
M.10.16	9.02	Abcde
M.10.17	8.95	Abcde
M.7.17	8.33	Bcdef
M.3.17	8.23	Bcdef
M.12.17	7.89	Cdefg
M.2.17	7.56	Defg
M.8.17	7.23	Efg
M.1.16	7.167	Efg
M.7.16	7.12	Efg
M.6.17	6.6	Fgh
M.5.16	6.02	Ghi
M.9.17	5.97	Ghi
M.1.17	4.84	Hij
M.4.16	4.03	Ij
M.5.17	3.18	J

Table S6: Technological parameters of the 22 *Saccharomyces cerevisiae* strains during fermentation of the synthetic grape juice

Sample name	Fermentation time (days)	Fermentation vigour (g/100mL)	CO_2 production (g/100 mL)	H_2S production	Free SO_2 (ppm)	Total SO_2 (ppm)	Total acidity (g/L sulfuric acid)	Volatile acidity (g/L acetic acid)	pH	Residual sugar (g/L)	Ethanol production (%)
M.1.16	14	0.95±0.02	7.17±0.21	1	3.40±0.02	0	3.6 ±0.24	0.69 ±0.23	3.16 ±0.01	3.83 ±0.36	11.66 ±0.02
M.2.16	13	1.45±0.16	10.40±0.43	3	4.20±0.03	0	3.8 ±0.05	0.02±0.002	3.2 ±0.03	0.94 ±0.04	11.83 ±0.02
M.3.16	11	2.95±0.18	10.37±0.24	1	5.50±0.02	34.50±0.02	3.5 ±0.4	0.50 ±0.06	3.265 ±0.05	0.12 ±0.04	11.88 ±0.003
M.4.16	13	1.32±0.01	4.03±0.05	2	4.80±0.05	23.50±0.07	3.56 ±0.23	0.55 ±0.24	3.18 ±0.08	8.04 ±1.35	11.41 ±0.08
M.5.16	12	1.68±0.07	6.02±0.06	2	5.30±0.04	32.00±0.03	4.0 ±0.06	0.52 ±0.04	3.39 ±0.06	4.54 ±0.43	11.61 ±0.03
M.6.16	12	3.40±0.22	10.05±0.24	1	5.30±0.04	19.50±0.02	4.7 ±0.17	0.53 ±0.004	3.54 ±0.04	0.40±0.07	11.86 ±0.004
M.7.16	11	1.45±0.01	7.12±0.06	2	5.80±0.13	42.00±0.14	3.9 ±0.3	0.31±0.025	3.4 ±0.041	1.90 ±0.22	11.77 ±0.01
M.8.16	10	1.68±0.06	9.08±0.02	1	4.95±0.03	40.00±0.36	3.75 ±0.25	0.31 ±0.043	3.39 ±0.03	0.91 ±0.02	11.83 ±0.01
M.9.16	9	3.20±0.22	9.40±0.22	2	3.9±0.23	8.00±0.03	4.8 ±0.08	0.33 ±0.081	3.23 ±0.12	0.45 ±0.06	11.86 ±0.03
M.10.16	10	3.53±0.21	9.02±0.09	1	0	0	3.97 ±0.16	0.021±0.09	3.40 ±0.16	2.3 ±0.242	11.75 ±0.01
M.1.17	8	2.60±0.37	4.78±0.17	2	5.00±0.14	0	4.6 ±0.59	0.32 ±0.003	3.17 ±0.23	7.6 ±0.864	11.43 ±0.05
M.2.17	9	2.97±0.62	7.56±0.29	1	4.90±0.02	1	4.2 ±0.03	0.02 ±0.005	3.23 ±0.05	3.06 ±0.03	11.70 ±0.002
M.3.17	10	3.27±0.05	8.23±027	2	3.60±0.05	5.00±0.10	3.3 ±0.04	0.05 ±0.01	3.53 ±0.06	0.94 ±0.02	11.83 ±0.001
M.4.17	10	3.20±0.25	9.27±0.22	1	0.23±0.03	0.53±0.03	3.5 ±0.06	0.06 ±0.002	3.21 ±0.13	0.74 ±0.03	11.84 ±0.002
M.5.17	11	0.80±0.29	3.18±1.08	2	0.33±0.20	0.60±0.01	3.62 ±0.23	0.34 ±0.048	3.36 ±0.06	13.6 ±1.33	11.07 ±0.19
M.6.17	10	1.90±0.43	6.60±0.67	1	0.13±0.04	0.53±0.16	3.35 ±0.15	0.24 ±0.10	3.19 ±0.02	3.96 ±1.76	11.65 ±0.105
M.7.17	14	1.44±0.06	8.33 ±1.25	3	0.47 ±0.06	0.38 ±0.18	3.78 ±0.46	0.04±0.02	3.37 ±0.60	1.07 ±0.16	11.82 ±0.01
M.8.17	11	1.87±0.13	7.23 ±0.35	1	0.38 ±0.07	0.41 ±0.03	4.10 ±0.22	0.01±0.002	3.61 ±0.04	4.30 ±0.32	11.63 ±0.02
M.9.17	11	1.3±0.31	5.97 ±0.22	2	0.5 ±0.023	0.65 ±0.03	3.36 ±0.07	0.15±0.009	3.51 ±0.17	4.85 ±0.25	11.60 ±0.015
M.10.17	13	1.51±0.19	8.95 ±0.79	1	0.53 ±0.003	0.80 ±0.12	3.58 ±0.05	0.13±0.002	3.42 ±0.38	0.39 ±0.17	11.86 ±0.01
M.11.17	15	1.72±0.11	9.68 ±0.16	1	0.64 ±0.002	0.93 ±0.02	3.84 ±0.06	0.17±0.03	3.26 ±0.06	0.63 ±0.09	11.85 ±0.006
M.12.17	15	1.67±0.09	7.89 ±0.23	1	0.65 ±0.03	0.60 ±0.04	4.27 ±0.18	0.07±0.009	3.50 ±0.05	3.45 ±0.11	11.68 ±0.007

Table S7: Results of chromosomal translocation of 202 *Saccharomyces cerevisiae* strains.

Sample	T8/16	8_WT1	T16/8	T16/15	16_WT	T15/16	15_WT
2016-1MP(1)	542	**	540	**	**	**	698
2016-1MP(2)	542	**	540	**	**	**	698
2016-1MP(3)	542	**	540	**	**	**	698
2016-1MP(4)	542	**	540	**	**	**	698
2016-1MP(5)	542	**	540	**	**	**	698
2016-1MP(6)	542	**	540	**	**	**	698
2016-1MP(7)	542	596	540	**	**	**	698
2016-1MP(8)	542	**	540	**	**	**	698
2016-1MP(9)	542	**	540	**	**	**	698
2016-1MP(10)	542	**	540	**	**	**	698
2016-1MP(11)	542	**	540	**	**	**	698
2016-1MP(12)	542	596	540	**	985	**	698
2016-1MP(13)	542	**	540	**	**	**	698
2016-1MP(14)	**	**	**	**	**	**	**
2016-1MP(15)	542	**	540	**	**	**	698
2016-1MP(16)	542	**	540	**	**	**	698
2016-1MP(17)	542	**	540	**	**	**	698
2016-1MB(1)	542	**	540	**	**	**	698
2016-1MB(2)	542	**	540	**	**	**	698
2016-1MB(3)	542	596	540	**	**	**	698
2016-1MB(4)	542	**	540	**	**	**	698
2016-1MB(5)	542	596	540	**	**	**	698
2016-1MB(6)	542	**	540	**	**	**	698
2016-1MB(7)	542	**	540	**	**	**	698
2016-1MB(8)	542	**	540	**	**	**	698
2016-1FP(1)	542	**	540	**	**	**	698
2016-1FP(2)	542	**	540	**	**	**	698
2016-1FP(3)	542	**	540	**	**	**	698
2016-1FB(1)	542	**	540	**	**	**	698
2016-1FB(2)	542	**	540	**	**	**	698
2016-1FB(3)	542	**	540	**	**	**	698
2016-1FB(4)	542	596	540	**	985	**	698
2016-1FB(5)	542	**	540	**	**	**	698
2016-1FB(6)	542	**	540	**	**	**	698
2016-1FB(7)	542	**	540	**	**	**	698

2016-1FB(8)	542	**	540	**	**	**	698
2016-1FB(9)	542	**	540	**	**	**	698
2016-1FB(10)	542	**	540	**	**	**	698
2016-1FB(11)	542	**	540	**	**	**	698
2016-1FB(12)	542	**	540	**	**	**	698
2016-1FB(13)	542	**	540	**	**	**	698
2016-1FB(14)	542	**	540	**	**	**	698
2016-1FB(15)	542	**	540	**	**	**	698
2016-1FB(16)	542	596	540	**	**	**	698
2016-2MP(1)	542	596	540	**	**	**	698
2016-2MP(2)	542	**	540	**	**	**	698
2016-2MP(3)	542	596	540	**	**	**	698
2016-2MP(4)	542	596	540	**	**	**	698
2016-2MP(5)	542	**	540	**	**	**	698
2016-2MP(6)	542	596	540	**	985	**	698
2016-2MP(7)	542	596	540	**	**	**	698
2016-2MP(8)	542	596	540	**	**	**	698
2016-2MP(9)	542	**	540	**	**	**	698
2016-2MP(10)	542	**	540	**	**	**	698
2016-2MP(11)	542	596	540	**	**	**	698
2016-2MP(12)	542	596	540	**	**	**	698
2016-2MP(13)	542	**	540	**	**	**	698
2016-2MP(14)	542	**	540	**	**	**	698
2016-2MP(15)	542	**	540	**	**	**	698
2016-2MP(16)	542	**	540	**	**	**	698
2016-2FP(1)	542	**	540	**	**	**	698
2016-2FB(3)	542	596	540	**	985	**	698
2016-2FB(4)	542	**	540	**	**	**	698
2016-2FB(5)	542	**	540	**	**	**	698
2016-2FB(6)	542	**	540	**	**	**	698
2016-2FB(7)	542	596	540	**	**	**	698
2016-2FB(8)	542	**	540	**	**	**	698
2016-2FB(9)	542	**	540	**	**	**	698
2016-2FB(10)	542	**	540	**	**	**	698
2016-2FB(11)	542	**	540	**	**	**	698
2016-2MB1(1)	542	**	540	**	**	**	698
2016-2MB1(2)	542	**	540	**	**	**	698

2016-2MB1(3)	542	**	540	**	**	**	698
2016-2MB1(4)	542	**	540	**	**	**	698
2016-2MB1(5)	542	596	540	**	**	**	698
2016-2MB1(6)	542	596	540	**	**	**	698
2016-2MB1(7)	542	**	540	**	**	**	698
2016-2MB1(8)	542	596	540	**	**	**	698
2016-2MB2(1)	542	596	540	**	985	**	698
2016-2MB2(2)	542	**	540	**	**	**	698
2016-2MB2(3)	542	596	540	**	**	**	698
2016-2MB2(4)	542	**	540	**	**	**	698
2016-2MB2(5)	542	596	540	**	**	**	698
2016-2MB2(6)	542	**	540	**	**	**	698
2016-2MB2(7)	542	**	540	**	**	**	698
2016-2MB2(8)	542	**	540	**	**	**	698
2016-2MB2(9)	542	**	540	**	985	**	698
2016-2MB2(10)	542	596	540	**	**	**	698
2016-2MB2(11)	542	596	540	**	**	**	698
2016-2MB2(13)	542	596	540	**	985	**	698
2016-2MB2(13)	**	596	**	**	**	**	698
2016-2MB2(14)	542	596	540	**	985	**	698
2016-2MB2(15)	542	596	540	**	**	**	698
2016-2MB2(16)	542	596	540	**	**	**	698
2016-3MI(1)	542	**	540	**	**	**	698
2016-3FI(1)	542	**	540	**	**	**	698
2016-3FI(2)	542	596	540	**	**	**	698
2016-3FI(3)	**	596	**	494	985	492	698
2016-3FI(4)	**	596	**	494	985	492	698
2016-3FI(5)	542	**	540	**	**	**	698
2016-3FI(6)	542	**	540	**	**	**	698
2016-3FI(7)	542	**	540	**	**	**	698
2016-3FI(8)	542	**	540	**	**	**	698
2016-3FI(9)	**	**	**	**	**	**	**
2016-3FI(10)	**	**	**	**	**	**	**
2016-3FI(11)	542	596	540	**	**	**	698
2016-3FI(12)	542	596	540	**	**	**	698
2016-3FI(13)	542	**	540	**	**	**	698
2016-3FI(14)	542	596	540	**	**	**	698

2016-3FI(15)	542	596	540	**	985	**	698
2017-1FB(2)	542	**	540	**	**	**	698
2017-1FB(3)	542	596	540	**	985	**	698
2017-1FB(4)	542	**	540	**	985	**	698
2017-1FB(5)	542	**	540	**	**	**	698
2017-1FB(6)	542	**	540	**	**	**	698
2017-1FB(7)	542	**	540	**	**	**	698
2017-1FB(8)	542	**	540	**	985	**	698
2017-1FB(9)	542	596	540	**	985	**	698
2017-1FB(10)	542	**	540	**	**	**	698
2017-1FB(11)	542	**	540	**	985	**	698
2017-1FB(12)	542	**	540	**	985	**	698
2017-1FB(13)	542	**	540	**	**	**	698
2017-1FB(14)	542	**	540	**	**	**	698
2017-1FB(15)	542	**	540	**	985	**	698
2017-1FB(16)	542	**	540	**	**	**	698
2017-1FB(17)	542	**	540	**	**	**	698
2017-1FB(18)	542	**	540	**	**	**	698
2017-1FB(19)	542	**	540	**	**	**	698
2017-1FB(20)	542	**	540	**	985	**	698
2017-1FB(21)	542	**	540	**	**	**	698
2017-1FB(22)	542	**	540	**	**	**	698
2017-1FB(23)	542	**	540	**	**	**	698
2017-1FB(24)	542	**	**	**	**	**	698
2017-1FB(25)	542	**	540	**	985	**	698
2017-1FB(26)	542	**	540	**	**	**	698
2017-1FB(27)	542	**	540	**	**	**	698
2017-1FB(28)	542	**	540	**	**	**	698
2017-1FB(29)	542	**	540	**	**	**	698
2017-1FB(30)	542	**	540	**	**	**	698
2017-1FB(31)	542	596	540	**	**	**	698
2017-1FB(32)	542	**	540	**	985	**	698
2017-1FB(33)	542	**	540	**	**	**	698
2017-1FB(34)	542	**	**	**	985	**	698
2017-1FB(35)	542	**	540	**	**	**	698
2017-1FB(36)	542	596	540	**	**	**	698
2017-1FB(37)	**	**	**	**	**	**	**

2017-1FB(38)	542	**	540	**	**	**	698
2017-1FB(39)	542	**	540	**	**	**	698
2017-1FB(40)	542	**	540	**	985	**	698
2017-1FB(41)	**	**	**	**	**	**	698
2017-1FB(42)	542	**	540	**	985	**	698
2017-1FB(43)	542	**	540	**	**	**	698
2017-1FB(44)	542	**	540	**	985	**	698
2017-1FB(45)	542	**	540	**	985	**	698
2017-1FB(46)	542	**	540	**	**	**	698
2017-1FB(47)	542	**	540	**	**	**	698
2017-1FB(48)	542	**	540	**	985	**	698
2017-1FB(49)	542	**	540	**	**	**	698
2017-1FB(50)	542	**	540	**	**	**	698
2017-1FB(51)	542	**	540	**	**	**	698
2017-1FB(52)	542	**	540	**	**	**	698
2017-1FB(53)	542	**	540	**	**	**	698
2017-1FB(54)	542	**	540	**	**	**	698
2017-1FB(55)	542	**	540	**	985	**	698
2017-1FB(56)	542	**	540	**	**	**	698
2017-1FB_57	542	596	540	**	985	**	698
2017-1FB(58)	542	**	540	**	**	**	698
2017-1FB(59)	542	**	540	**	**	**	698
2017-1FB(60)	542	**	540	**	**	**	698
2017-1FB(61)	542	**	540	**	**	**	698
2017-1FB(62)	542	**	540	**	**	**	698
2017-1FB(63)	542	**	540	**	**	**	698
2017-1FB(64)	542	**	**	**	985	**	698
2017-1FB(65)	542	**	540	**	**	**	698
2017-1FB(66)	542	**	540	**	**	**	698
2017-1FB(67)	542	**	540	**	**	**	698
2017-1FB(68)	542	**	540	**	**	**	698
2017-1FB(69)	542	**	540	**	**	**	698
2017-1FB(70)	542	**	540	**	**	**	698
2017-1FB(71)	**	**	**	**	**	**	**
2017-2FB(1)	542	**	540	**	**	**	698
2017-2FB(2)	542	596	540	**	985	**	698
2017-2FB(3)	542	596	540	**	985	**	698

2017-2FB(4)	542	596	540	**	985	**	698
2017-2FB(5)	542	**	540	**	**	**	698
2017-2FB(6)	542	**	**	**	**	**	698
2017-2FB(7)	542	**	540	**	**	**	698
2017-2FB(8)	542	**	540	**	**	**	698
2017-2FB(9)	542	**	540	**	985	**	698
2017-2FB(10)	542	**	540	**	985	**	698
2017-2FB(11)	542	596	540	**	985	**	698
2017-2FB(12)	542	**	540	**	**	**	698
2017-2FB(13)	542	596	540	**	985	**	698
2017-2FB(14)	542	**	540	**	**	**	698
2017-2FB(15)	542	**	540	**	**	**	698
2017-2FB(16)	542	**	540	**	**	**	698
2017-2FB(17)	542	596	540	**	985	**	698
2017-2FB(18)	542	**	540	**	**	**	698
2017-2FB(19)	542	**	540	**	**	**	698