


Review

The Microbial Diversity of Sherry Wines

Gustavo Cordero-Bueso * , Marina Ruiz-Muñoz, Mónica González-Moreno, Salvador Chirino, María del Carmen Bernal-Grande and Jesús Manuel Cantoral

Department of Biomedicine, Biotechnology and Public Health, University of Cádiz, Puerto Real, 11510 Cádiz, Spain; marina.ruiz@uca.es (M.R.-M.); monica.gonzalez@uca.es (M.G.-M.); salvachirino@gmail.com (S.C.); mariadelcarmen.bernal@uca.es (M.d.C.B.-G.); jesusmanuel.cantoral@uca.es (J.M.C.)

* Correspondence: gustavo.cordero@uca.es; Tel.: +34-956-01-64-24

Received: 15 February 2018; Accepted: 16 March 2018; Published: 19 March 2018

Abstract: The principal role of wine yeast is to transform efficiently the grape-berries' sugars to ethanol, carbon dioxide, and other metabolites, without the production of off-flavors. Wine yeast strains are able to ferment musts, while other commercial or laboratory strains fail to do so. The genetic differences that characterize wine yeast strains in contrast to the biological ageing of the veil-forming yeasts in Sherry wines are poorly understood. *Saccharomyces cerevisiae* strains frequently exhibit rather specific phenotypic features needed for adaptation to a special environment, like fortified wines with ethanol up to 15% (*v/v*), known as Sherry wines. Factors that affect the correct development of the veil of flor during ageing are also reviewed, along with the related aspects of wine composition, biofilm formation processes, and yeast autolysis. This review highlights the importance of yeast ecology and yeast metabolic reactions in determining Sherry wine quality and the wealth of untapped indigenous microorganisms co-existing with the veil-forming yeast strains. It covers the complexity of the veil forming wine yeasts' genetic features, and the genetic techniques often used in strain selection and monitoring during fermentation or biological ageing. Finally, the outlook for new insights to protect and to maintain the microbiota of the Sherry wines will be discussed.

Keywords: flor yeast; veil; taxonomy; Fino wine; Manzanilla wine

1. Introduction: The Origin of Sherry Wines

Biological ageing of wines is traditionally carried out in different regions, such as the wines of Jura (France), Szamorodni and Aszú (Tokaj-Hegyalja, Hungary) or Vernaccia di Oristano (Sardinia, Italy). However, the best-known biologically aged wines are produced in the Jerez-Xères-Sherry and Manzanilla-Sanlúcar de Barrameda D.O., in southern Spain [1,2].

The Greek geographer Strabo was the first who talked about Sherry wines, which were brought to the Jerez Region by the Phoenicians in the 1st Century, B.C. Phoenician ruins had been discovered at the excavations in Castillo de Doña Blanca (El Puerto de Santa María), located just 4 km from Jerez de la Frontera, where the remains of wine presses had been found. This finding confirmed that the same people who founded the ancient town of Gades, nowadays Cádiz, brought with them the art of cultivating the vine and wine making from the far-off lands of the current Lebanon. Since then, some Phoenicians traders produced wines in the former Xera (Jerez) and exported to Rome [3].

Around the year 138 B.C., the Baetica Region was conquered by the Roman Scipio Aemilianus, starting a substantial flow of trade between the region and the metropolis. People from this area exported local products to Rome, such as olive oil, wine from the Ceret region, and Garum (a kind of marinade sauce made from leftovers of salted fish).

By this time, both Rome and many other parts of the Empire became fascinated by Sherry wines, formerly called “Vinum Ceretensis”. This had been revealed through different archaeological remains of amphorae in those places.

Production of Sherry and Manzanilla wines follows two successive processes: firstly, an alcoholic fermentation of must by yeasts present on the surface of *Vitis vinifera* var. Palomino Fino in order to produce a “young” wine, and then, a biological ageing of this wine occurs by other veil-forming yeasts, the so-called yeasts of flor [4–6].

Biological ageing, in turn, involves two phases: a static one (‘añadas’) during which the wine is kept in a butt for a variable number of years, followed by a dynamic phase known as “criaderas-solera” or “soleraje”, consisting of a series of oak barrels of sherry in process of maturation divided into a varying number of stages. The first phase, when the wine is young and contains about 15% (v/v) of ethanol, is known as “sobretablas”. The middle phases are called “criaderas”, and the final stage (also called “solera”) contains the oldest wine; from it, the finished wine is withdrawn, but then the butts with a capacity of 500 L are only partially emptied, not more than one-third being removed in any one withdrawal. The transfer of wine from one oak barrel to the next is known as rocío [7]. This is usually made twice a year, and the complete process takes no less than three years and involves five or six “criaderas” (with a variable number of barrels). They have a common characteristic of a veil of flor of up to 1–3 cm thick growing on the free surface of the wine (barrels are only filled up to 5/6 parts of their total volume) which contains about 15–18% (v/v) of ethanol (Figure 1).

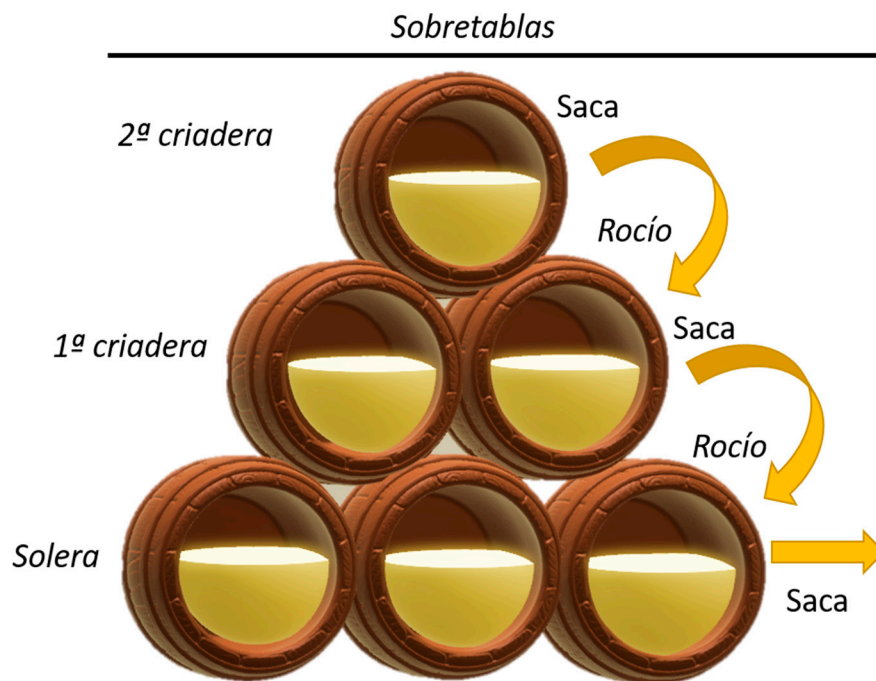


Figure 1. The way the solera and criadera system works means that the oldest wines benefit both from being refreshed (Saca-Rocío) with young wines and from characteristics acquired over years of crianza.

The veil of flor in Sherry wines consists of multicellular aggregates composed, in turn, by yeasts, bacteria, and fungi, with yeasts being the main microorganisms [4,6,8–12]. Within the different species of yeasts, *Saccharomyces cerevisiae* is the most popular species found in the different wines elaborated under biological ageing. The veil of flor is mainly responsible for the sensory characteristics of Sherry and Manzanilla wines through its oxidative metabolism; but also, the active consumption of oxygen and the isolation effect exerted by the yeast layer prevent wine oxidation. Development of veil-forming yeasts depends not only on a number of ambient factors, but also on the chemical composition of the wine. Acetaldehyde displays an inhibitory effect on veil formation when its concentration is above the tolerance threshold. Thus, different properties of industrial interest have been detected, such as

physiologic, genetic, and metabolic characteristics in the different identified strains, as well as clear differences using molecular analysis techniques [1,2,13–15].

2. Biodiversity of Flor Biofilm

Certain yeast species have the ability to develop on the surface of substrates that have previously fermented. To do this, firstly they must convert their enzymatic equipment and adapt it to the new aerobic conditions. In this stage of aerobiosis, yeasts produce remarkable physicochemical and organoleptic changes in such substrates. In these wines, special conditions are exerted as a consequence of the oxidative metabolism of yeasts and the reducing medium created inside, where the dissolved oxygen is consumed by the veil, and it is prevented by the contribution of new quantities from abroad. The biological ageing, which is defined as the biochemical transformation that the wine undergoes, previously headed at 15–15.5 °C, by the addition of certain aerobic veil-forming yeasts of the genus *Saccharomyces* at the beginning, according to data and descriptors in the middle of the last century in Jerez de la Frontera [4,6,8,9].

Wine yeasts have traditionally been identified based on their morphological attributes at macro- and microscopic levels, as well as on their physiological behavior. In 1963, Rocques, according to the previous observations of Louis Pasteur, described *Mycoderma vini* as the yeast species responsible of the veil formed in the yellow wines (vin jaune) from Arbois (Jura region, France). Since then, veil-forming yeast taxonomy and the repercussion of the physiology of the different species over the main components of Sherry wines, have been studied by numerous researchers [1,2,5,6,8,9,15,16]. In the case of *flor* yeasts, the most commonly analyzed substrates for their differential fermentation and assimilation capabilities are galactose, dextrose, lactose, maltose, melibiose, raffinose, and sucrose [17].

These conventional methods had firstly shown that veil-forming strains in southern Spain were composed of four *S. cerevisiae* races: *beticus*, *montuliensis*, *cheresiensis* and *rouxii* [1]. Moreover, other veil-forming yeast species were described such as *Saccharomyces hispanica*, *S. cordubensis* and *S. gaditensis* [18]. However, these races are not recognized by the last taxonomic study, as we discuss later.

The morphological characteristics are very similar in every species of *Saccharomyces cerevisiae*, without being aware of differences that justify a separation between species. However, if we take into account the physiological characteristics, such as the fermentation sugars mentioned above, they are very different, which allows differentiation between the species. Flor yeasts also have in common the use of ethanol as a carbon source, and the requirement of oxygen to restore the viability, and lipid metabolism to form the veil of flor [19]. However, both morphological and physiological characteristics may be influenced by culture conditions, and can provide ambiguous results. Thus, classical techniques, in some cases, can lead to an incorrect classification of species or a misidentification of strains [6].

Referring to yeast species belonging to non-*Saccharomyces* genera, they have little or no fermentative power, and they do not have the capacity to survive at ethanol concentrations higher than 15% *v/v*. However, several genera and species have been detected in wines at the “*sobretablas*” stage. Yeast of the genera *Rhodotorula*, *Candida*, *Hansenula*, and *Zygosaccharomyces* are the most abundant, especially the species *Wickerhamomyces anomalus*, *Pichia membranaefaciens*, *Rhodotorula mucilaginosa*, *R. minuta*, *Zygosaccharomyces bailii*, or the undesirable species *Dekkera bruxellensis* (anamorph *Brettanomyces bruxellensis*) [4,6,17].

During the biological ageing of Sherry wines, other microorganisms, such as lactic acid bacteria of the genera *Leuconostoc*, *Pediococcus*, and *Lactobacillus*, and opportunistic fungi, like *Botrytis cinerea*, can coexist with the veil-forming yeast strains mentioned above [12,17,20]. The presence of lactic acid bacteria, such as *Lactobacillus hilgardii*, *L. plantarum*, and *L. brevis* in Sherry veils is strongly related to the presence of high concentrations of gluconic acid coming from the filamentous fungi *B. cinerea* [12,17,21,22]. The presence of this microbiota, different to yeasts, results in undesirable wines, and the presence of high concentrations of biogenic amines.

3. Genetic and Biochemical Characteristics of the Flor Yeasts

According to their ability to ferment and assimilate different substrates, Sherry veil-forming yeasts have traditionally been divided into four varieties or races belonging to the *Saccharomyces cerevisiae* species: *beticus*, *cheresiensis*, *montuliensis*, and *rouxii* [1]. These biochemical properties were normally tested using inverted Durham tubes in a liquid medium, which rose out of the tube where fermentation to occur. However, according to the last taxonomic study [17], the former race *montuliensis* has been classified as *Torulaspota delbrueckii*, while the race *rouxii* is now catalogued as *Zygosaccharomyces rouxii*. On the other hand, all other previously identified races are currently considered as *S. cerevisiae* synonyms, based on their nuclear DNA similarity.

The development of molecular biology has given rise to a considerable number of techniques for yeast identification based on their genetic information. These include electrophoretic karyotyping, RFLP (restriction fragment length polymorphism) of mitochondrial DNA, random amplified polymorphic DNA analysis (RAPD), ribosomal internal transcribed spacers (ITS-PCR) or studies of simple sequences repeats (SSR-multiplex PCR). Some of these methods have been of great interest in enology, since they allow one to obtain a high level of resolution in yeast strain characterization.

As discussed above, flor yeasts are mainly *Saccharomyces cerevisiae*, but they show a high level of variability regarding nuclear and mitochondrial genome [11,23–25]. Studies on Spanish wine fermentations using restriction patterns generated from the PCR-amplified regions spanning the internal transcribed spacers (ITS 1 and 2) and the 5.8S rRNA gene demonstrated that flor yeasts which carry out the biological ageing in the Jerez-Xères Sherry and Montilla-Moriles D.O. showed a 24 bp deletion located in the ITS1 region [5,9,23]. This deletion is fixed in flor yeasts, and it may have originated from a slipped-strand mispairing during replication, or it might stem from unequal crossing-over [23]. Furthermore, said deletion may also be related to a nuclear gene involved in ethanol tolerance.

Moreover, veil-forming yeasts exhibit some chromosome alterations. Especially, they usually have a considerable number of additional copies of some chromosomes, mainly of chromosome XIII, that contain certain genes related to the oxidative conversion of ethanol to acetaldehyde during biological ageing [24,26]. Flor yeasts are characterized by a high degree of heterozygosity [27]. Therefore, chromosomal analysis of yeasts isolated in sherry wines show a high level of variability, as it is not possible to correlate between a specific pattern and a former race described above [11]. On the other hand, mitochondrial DNA restriction analysis of natural isolates of sherry wines show a small level of variability due to the conditions of the biological ageing [5,6,28].

As stated by [1,29], comparative population genomics between wine yeasts and biofilm-forming flor yeasts have disclosed some genomic regions, including multiple genes, involved in adaptation to biological ageing conditions, since they are fixed in the analyzed flor yeasts. Some of these genomic regions are involved in ethanol tolerance, hexose transport, cell–cell adhesion, or zinc transport. This finding could put, on show, the phylogenetic origin of flor yeasts.

However, it would also be worth emphasizing that molecular techniques are not enough to understand the diversity and dynamics of yeasts during biological ageing in Sherry wines. Some studies [6] revealed that biochemical tests are necessary to identify and classify the different flor yeasts involved in Sherry wine, since each yeast strain might bring to the Sherry wines a different organoleptic characteristic.

4. Factors Which Affect Veil Formation

The development of yeast on the surface of the wine forming the veil of flor occurs after alcoholic fermentation, and this process produces important changes in the characteristics of the wine due to the metabolism of the flor yeasts. The air–liquid interface plays a fundamental role, since under these conditions, cell growth is dependent on oxygen availability [15].

Flor yeasts are capable of growing on non-fermentable carbon sources, such as glycerol, ethyl acetate, and even ethanol [16]. On the other side, biofilm formation is limited by the presence

of high contents of succinic acid, lactic acid, and acetic acid. Veil formation is affected also by environmental factors in wineries, such as nutrient composition, growth temperature, osmotic or ionic stress, and the presence of toxic drugs, oxidizing agents, or heavy metals [30]. Thus, veil formation is favored by agitation before inoculation of veil [31], whereas it is limited by the presence of high concentrations of ammonium [32], due to the decrease of *FLO11* expression, a key gene for veil development [33].

Although there is a large number of factors which affect veil formation, flor yeast development is essentially influenced by the ethanol content in base wine (15%, *v/v*) and the temperature in the cellar (below 22 °C). If the temperature is increased, the veil of flor is deteriorated, which causes a delay in the ageing process. Elevated temperatures and inhibitory ethanol concentrations have a lethal effect on yeast cells, and both act upon the mitochondria [28]. Thermal and ethanol tolerance are genetically determined properties that may diverge from strain-to-strain. Therefore, these properties are susceptible to biological improvement, although the deterioration may be attributed to other environmental factors, such as limited amount of essential nutrients or the opportunistic proliferation of polluting yeasts [34].

5. Chemistry and Biochemistry of the Biological Ageing

The genetic analysis of flor strains with microsatellite typing performed by [35] revealed that most flor strains of Spain, Italy, France, and Hungary belong to the same genetic group of *S. cerevisiae*. This subclustering might be related to the differences between them in the ability to produce a biofilm. Moreover, the Sherry wines have the peculiarity of refreshing the wine each year through the soleraje process. This process is what makes this kind of wine very singular, and sets it apart from other wines. During biological ageing, the wine also goes through several changes in its chemical composition that take place over time. Those changes are carried out by the yeasts, which use their metabolism to release a number of compounds. Moreover, other reactions occur during ageing such as chemical reactions between the components of the wine, crystal precipitation, and the extraction of wooden compounds from the casks. The veil-forming yeasts need a widely list of nutrients to grow, and they are able to use many sources of carbon, such as ethanol, glycerol, and acetic acid. Oxygen is necessary for assimilation of L-proline and the synthesis of unsaturated fatty and sterols [32].

Autolysis has been defined as the hydrolysis of intracellular biopolymers under the effect of hydrolytic enzymes associated with cell death, forming low molecular weight products. This process is accompanied by a loss of dry matter, a decrease in the percentage of protein and nucleic acids, and by an intracellular proteolytic activity. Flor yeasts may suffer autolysis process during biological ageing, due to the special conditions to which they are subjected, such as contact with yeasts for long periods during storage. It may fluctuate depending on the number of rows in the *criaderas* and *soleras* system, number of *rocios*, veil surface, volume of wine extracted per year, and climatic conditions. This autolysis releases a lot of compounds in wines, such as amino acids, peptides, nucleotides, mannoproteins, esters, alcohols, aldehydes, acids, and lactones. All these compounds have an effect, in the sensorial way [36–38].

Ethanol is used by the veil-forming yeasts as a source of carbon and energy. A part of the alcohol is converted into compounds such as acetaldehyde, acetic acid, butanediol, diacetyl, and acetoin. The rest are metabolized via the tricarboxylic pathway as carbohydrates, lipids, and proteins, into cellular material [37]. The veil formation stage and the scales that contain the youngest wine are the most ethanol-consuming phases [39,40].

On the other side, glycerol is the third compound of wine, after water and ethanol. Yeasts are able to use glycerol as a carbon source, decreasing its amount along the ageing process. This is an effective way to measure the degree of ageing in Sherry wines [40]. The amount of glycerol is one of the main differences between wines with biological and oxidative ageing, since the second one has no veil-forming yeasts, and the amount of glycerol remains at levels of 6.5–8 g/L, while the first one has below 0.5 g/L [41].

Acetaldehyde is one of the compounds that makes this kind of wine especially different, since it influences the sensory properties, giving them a typical pungent aroma. This compound reaches concentrations from 350 to 450 mg/L, achieving around 1000 mg/L [42]. Yeasts produce it by oxidation of ethanol using alcohol dehydrogenase II (ADH II), which produces NADH. This isoenzyme is repressed by glucose, so it plays an active role in the biological ageing. The acetaldehyde amount increases as the wine ages, but the biggest changes occur in the rows that contain the youngest wine, in which yeasts have a more active metabolism [7,43]. Acetaldehyde influences the synthesis of sotolon, which is an important aromatic compound in sherry wines, providing a typical nut fragrance [18].

The two main amino acids in must are L-arginine and L-proline [44], and they are used by yeasts during the fermentation process. Thus, the content of amino acids is reduced during biological ageing, especially L-proline content [44,45], because it is not degraded by the yeasts during the fermentation process, since the transformation process requires the presence of molecular oxygen [46].

One of the most important aromatic influencers are the higher alcohols. In spite of that they do not usually change their overall content during biological ageing, some individual alcohols may change during the process. Isobutanol, 2-phenylethanol, and isoamyl alcohols (2-methyl-1-butanol and 3-methyl-butanol) change slightly, whereas concentrations of propanol can be doubled during the process. Moreover, the autolysis of yeasts plays an important role in the content of higher alcohols. This fact can be checked by looking the content of propanol, isobutanol, and isoamyl alcohols in the yeast extract of wines under biological ageing [2].

As stated above, "Sobretablas" wine is located in the top row of the *soleras* system, and is where the malolactic fermentation took place. Thus, the ageing system contains no appreciable concentration of malic acid. During biological ageing, the amount of tartaric acid decreases, since the crystal precipitates. Gluconic acid is an indicator of wine quality, because it comes from rotten grapes (and they can be affected especially by fungus *Botrytis cinerea*). Having said that, wines that contain below 1 g/L of this acid are allowed to biologically age. Veil-forming yeasts can metabolize such acids without altering the final quality of the product [14,47]. Regarding acetic acid, biological ageing has a very important advantage for the winemakers, because it is metabolized by veil-forming yeasts during the process, and its concentration is reduced through consumption via acetyl-CoA.

6. Why Are Fino Wines Different from Manzanilla Wines?

Manzanilla is a dry white wine made from the same grape-berries of Fino (*Vitis vinifera* cv. Palomino) and it also goes through biological ageing due to the presence of veil-forming yeasts. It is produced exclusively in the coastal town of Sanlúcar de Barrameda (located 12 km from Jerez). Despite that the elaboration process of this wine is the same as Fino, winemakers and people from this area claim that Manzanilla wine differs from Fino because of its uniquely and distinctive sensory characteristics. The special climatic conditions of the town, situated at the mouth of the river Guadalquivir, favor the formation of a special kind of veil of flor, which gives the wine its uniquely distinctive characteristics. While Fino wine sensory characteristics ranges from bright straw yellow to pale gold in color and a delicate bouquet slightly reminiscent of almonds with a hint of fresh dough and wild herbs, Manzanilla wines denotes a very bright, pale straw colored wine, and a sharp bouquet with predominant floral aromas reminiscent of chamomile, almonds, and dough.

It should be noted that in Jerez area, the veil of flor thickness fluctuates considerably during every season of the year, due to the effect of the changes in temperature, and therefore, the wine alternates biological and oxidative ageing periods. On the other hand, the veil in Sanlúcar lasts all year, due to the mild climate, owing the Guadalquivir River. However, according to the results obtained by [6], the genetic characterization of the veil-forming yeast strains from Manzanilla and Fino is highly variable: some patterns appear repeatedly in both kinds of Sherry wines, while others are exclusives to each wine.

According to [48], Manzanilla wine has its origins from the village of Manzanilla, in the Condado de Huelva D.O., placed 40 km from Sanlúcar de Barrameda. Oak and *Abies pinsapo* barrels containing

wines from Condado de Huelva were transported during the Centuries XVI–XVII to Sanlúcar de Barrameda, and then to the *Indias* (nowadays Central and South America). Thus, this evidence will open new frontiers for a better understanding of the differences between Manzanilla and Fino wines of the Jerez-Xères-Sherry winemaking framework.

7. The Outlook for the Future of the Veil-Forming Yeasts

The wine market is in constant improvement and renewal. It demands modern conditions under which to operate, including social security and protection for the environment. On the other side, consumer demands for newer styles of wines and improvements of the process efficiency, the sensory quality, and the wholesomeness of the wine is required.

These will be carried out by further refinements of existing technologies and products, but also by the development of new products based on the exploitation of new strains of *Saccharomyces* and non-*Saccharomyces* yeast strains. In this regard, genetic improvement and metabolic engineering technologies are applied to construct strains with interesting properties [49]. Furthermore, new hybrid strains have been developed with an increased tolerance to the stresses of winemaking, such as temperature of fermentation and ethanol concentration, and the pool of strains available to enhance diversity in wine flavor has increased [50].

Along the same lines, the wide variety of genetic tools available has made it possible to improve the processes by studying genes involved in the control of the subcellular localization of different proteins, such as BTN2. Its deletion affects the biofilm formation ability of flor yeasts, and it increases its sliding motility, resulting in increased mat formation. This correlates with an increased transcription of the *FLO11* gene, essential for biofilm formation [10]. Similar studies have concentrated on overexpressing genes *SOD1*, *SOD2*, and *HSP12* in different veil-forming yeast strains showing that the veil formation by these strains took place more rapidly, and the veil was thicker and with higher percentages of viable cells. The result was an acceleration of both metabolism and wine ageing, thus reducing the time needed for wine maturation was obtained [51].

Why are wine ageing and maturation so important? Biological ageing is a slow process to ensure homogeneity and quality of the wine, and this raises the production costs of Sherry wines. Some authors have tried to find an effective way of shortening the ageing time without altering the quality of the resulting wines. Periodic microaeration of Fino wines is effective in shortening their biological ageing time without affecting the veil-forming yeasts' integrity [52]. On the other hand, a method based on aerating wine under biological ageing on a monthly basis was studied, resulting in an increase of ADH II activity, rising production of acetaldehyde and its derivatives [53]. It has been proven that by combining the microaeration in stainless-steel vessels and the traditional biological ageing procedures, the ageing time can be substantially shortened [54].

The optimization of wine maturation has been studied through the presence of proteins involved in respiration, translation, amino acid metabolism, glycolysis/gluconeogenesis process, biosynthesis of vitamin B9 (folate), and stress damage prevention and repair under biofilm formation conditions. In this study, proteins like Bgl2p, Gcv3p, Hyp2p, Mdh1p, Suc2p, and Ygp1p were quantified in very high levels [55], paving the way for the design of conditions and genetic constructions of flor yeasts to improve the cellular survival and, thus, to optimize biological ageing of Sherry wine production.

There must be adequate conditions surrounding the flor yeasts' growth to ensure that the process mentioned above is as easy and efficient as possible. As discussed above, lactic acid bacteria coexist with our interesting yeast strains, triggering organoleptic deviations and deterioration of the wine. Under these conditions, a lysozyme might be used to prevent the development of lactic bacteria. More recently, there has been evidence showing that if yeast inoculation is carried out under submerged culture conditions during biological ageing, then low doses of lysozyme could affect cell multiplication and the membrane hydrophobicity of the yeast, inhibiting their aggregation and flotation, and the subsequent development of veil of flor [12].

As stated above, low ethanol content, along with a visually pleasing appearance in terms of color in wine, are some of the demanding requirements of the current wine market. A more uniform color in red wine has been obtained by using veil-forming yeasts over short periods [56]. Relevant studies suggest that veil-forming yeasts can be used as fining agents, increasing the percentage of red color and decreasing astringency and ethanol content [57], therefore supporting new perspectives for the elaboration of a new wine type.

Tolerance to ethanol and acetaldehyde of veil-forming yeasts could be useful for producing bioethanol [58], but they have also been used in terms of food control. The combined lysozyme and submerged culture of veil-forming yeast treatment is very effective at removing bacteria from the wine, reducing volatile acidity and preventing acetification [59]. In this context, a new method that would prevent the deleterious biofilm formation in wineries is studied by the transcription of the *FLO11* gene in a veil-forming *S. cerevisiae* strain repressed by glucose [59].

New potential applications are moving towards the yeast cell immobilization on *Penicillium chrysogenum* for sweet wine production [60]. Previously, this fungus has been used in a forced symbiosis with *Saccharomyces cerevisiae* in the absence of physicochemical modifications [61]. Recent research in the same row has revealed a gradual adaptation to the fermentation conditions and increasingly uniform behavior, in terms of the fermentation kinetics and production of metabolites during the process [62].

It would also be relevant to highlight the analysis of veil-forming yeasts using omic tools, which are also expanding and offering interesting possibilities. Proteome remodeling is extended by comparative genomics, proteomics, and metabolomics of flor and wine yeast strains [1]. These are some of the infinite ways in which we can deepen our knowledge regarding veil-forming yeast.

Finally, a new line of research could be the development of products that do not affect veil formation or volatile compounds which are characteristic of biological ageing (e.g., acetaldehyde or ethyl acetate), normally used to indicate the stage and quality of the industrial process.

8. Conclusions

Sherry wines are unique, due to the metabolism of some yeasts capable of growing on their surface during biological ageing, granting them some organoleptic characteristics. We will be able to optimize and improve Sherry wine production by studying the genetic and biochemical characteristics of the veil-forming yeasts. Thus, it is also necessary to study all the factors that might affect the formation of the veil of flor, which is now possible due to the wide variety of techniques already developed, and the potential for new ones to be discovered.

The Sherry wine market must be profitable for both producers and consumers, and this may be achieved by looking for new methods to allow a faster wine maturation process without damaging the quality of the final product. This would in turn allow the obtention of a homogeneous product, despite the environmental changes that are gradually occurring due to climate change.

Author Contributions: Gustavo Cordero-Bueso, Jesús Manuel Cantoral and María del Carmen Bernal-Grande wrote “Introduction: the origin of Sherry wines”; Gustavo Cordero-Bueso wrote “The veil of Flor microbial diversity” and “Why are different Fino wines from Manzanilla wines?”; Marina Ruiz-Muñoz wrote “Genetic and biochemical characteristics of the Flor yeasts” and “Factors which affect veil formation”; Mónica González-Moreno wrote “The outlook for the future of the veil-forming yeasts” and Salvador Chirino wrote “Chemistry and biochemistry of the biological ageing”. Gustavo Cordero-Bueso coordinated the work, Marina Ruiz-Muñoz revised the English and all authors critically revised the manuscript before submission.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Legras, J.L.; Moreno-Garcia, J.; Zara, S.; Zara, G.; Garcia-Martinez, T.; Mauricio, J.C.; Mannazzu, I.; Coi, A.L.; Zeidan, M.B.; Dequin, S.; et al. Flor yeast: New perspectives beyond wine aging. *Front. Microbiol.* **2016**, *7*, 503. [[CrossRef](#)] [[PubMed](#)]
2. Peinado, R.A.; Mauricio, J.C. Biologically Aged Wines. In *Wine Chemistry and Biochemistry*; Moreno-Arribas, M.V., Polo, M.C., Eds.; Springer: New York, NY, USA, 2009; pp. 82–96.

3. González Gordon, M.M. *Jerez-Xeres "Scherish": Apuntes Sobre el Origen de la Ciudad, Sobre su Historia y su vino*; Gráficas del Exportador: Jerez de la Frontera, Spain, 1935; ISBN CABP 0045594.
4. Esteve-Zarzoso, B.; Peris-Torán, M.J.; García-Maiquez, E.; Uruburu, F.; Querol, A. Yeast Population Dynamics during the Fermentation and Biological Aging of Sherry Wines. *Appl. Environ. Microbiol.* **2001**, *67*, 2056–2061. [[CrossRef](#)] [[PubMed](#)]
5. Esteve-Zarzoso, B.; Fernández-Espinar, M.T.; Querol, A. Authentication and identification of *Saccharomyces cerevisiae* "flor" yeast races involved in sherry ageing. *Antonie Leeuwenhoek* **2004**, *85*, 151–158. [[CrossRef](#)] [[PubMed](#)]
6. Ruíz-Muñoz, M.; Bernal-Grande, M.; Cordero-Bueso, G.; González, M.; Hughes-Herrera, D.; Cantoral, J. A Microtiter Plate Assay as a Reliable Method to Assure the Identification and Classification of the Veil-Forming Yeasts during Sherry Wines Ageing. *Fermentation* **2017**, *3*, 58. [[CrossRef](#)]
7. Berlanga, M.T.; Peinado, R.; Millán, C.; Mauricio, J.C.; Ortega, J.M. Influence of blending on the content of different compounds in the biological aging of sherry dry wines. *J. Agric. Food Chem.* **2004**, *52*, 2577–2581. [[CrossRef](#)] [[PubMed](#)]
8. Rodríguez, M.E.; Infante, J.J.; Mesa, J.J.; Rebordinos, L.; Cantoral, J.M. Enological behavior of biofilms formed by genetically-characterized strains of sherry flor yeast. *Open Biotechnol. J.* **2013**, *7*, 23–29. [[CrossRef](#)]
9. Marin-Menguiano, M.; Romero-Sanchez, S.; Barrales, R.R.; Ibeas, J.I. Population analysis of biofilm yeasts during Fino sherry wine aging in the Montilla-Moriles D.O. region. *Int. J. Food Microbiol.* **2017**, *244*, 67–73. [[CrossRef](#)] [[PubMed](#)]
10. Espinazo-Romeu, M.; Cantoral, J.M.; Matallana, E.; Aranda, A. Btn2p is involved in ethanol tolerance and biofilm formation in flor yeast. *FEMS Yeast Res.* **2008**, *8*, 1127–1136. [[CrossRef](#)] [[PubMed](#)]
11. Mesa, J.J.; Infante, J.J.; Rebordinos, L.; Cantoral, J.M. Characterisation of yeasts involved in the biological ageing of sherry wines. *LWT Food Sci. Technol.* **1999**, *32*, 114–120. [[CrossRef](#)]
12. Roldán, A.; Lasanta, C.; Caro, I.; Palacios, V. Effect of lysozyme on "flor" velum yeasts in the biological aging of sherry wines. *Food Microbiol.* **2012**, *30*, 245–252. [[CrossRef](#)] [[PubMed](#)]
13. Lasanta, C.; Roldán, A.; Caro, I.; Pérez, L.; Palacios, V. Use of lysozyme for the prevention and treatment of heterolactic fermentation in the biological aging of sherry wines. *Food Control* **2010**, *21*, 1442–1447. [[CrossRef](#)]
14. Peinado, R.A.; Mauricio, J.C.; Moreno, J. Aromatic series in sherry wines with gluconic acid subjected to different biological aging conditions by *Saccharomyces cerevisiae* var. *capensis*. *Food Chem.* **2006**, *94*, 232–239. [[CrossRef](#)]
15. Zara, S.; Bakalinsky, A.T.; Zara, G.; Demontis, M.A.; Budroni, M.; Pirino, G. FLO11-Based Model for Air-Liquid Interfacial Biofilm Formation by *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **2005**, *71*, 2934–2939. [[CrossRef](#)] [[PubMed](#)]
16. Zara, S.; Gross, M.K.; Zara, G.; Budroni, M.; Bakalinsky, A.T. Ethanol-independent biofilm formation by a flor wine yeast strain of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **2010**, *76*, 4089–4091. [[CrossRef](#)] [[PubMed](#)]
17. Kurtzman, C.; Fell, J.W.; Boekhout, T. *The Yeasts: A Taxonomic Study*; Elsevier: London, UK, 2011; p. 2363. ISBN 9780080542690.
18. Suárez Lepe, J.A. *Levaduras Vínicas: Funcionalidad y uso en Bodega*; Grupo Mundi-Prensa: Madrid, Spain, 1997; ISBN 8471146851.
19. Zara, G.; Angelozzi, D.; Belviso, S.; Bardi, L.; Goffrini, P.; Lodi, T.; Budroni, M.; Mannazzu, I. Oxygen is required to restore flor strain viability and lipid biosynthesis under fermentative conditions. *FEMS Yeast Res.* **2009**, *9*, 217–225. [[CrossRef](#)] [[PubMed](#)]
20. Marcilla, J.; Alas, G.; Feduchy, E. *Contribución al Estudio de las Levaduras que Forman velo sobre Ciertos vinos de Elevado Grado Alcohólico*; Anales Centro Inv. Vitivinícolas: Madrid, Spain, 1939; Volume 1, p. 1.
21. Moreno-Arribas, M.V.; Carmen Polo, M. Occurrence of lactic acid bacteria and biogenic amines in biologically aged wines. *Food Microbiol.* **2008**, *25*, 875–881. [[CrossRef](#)] [[PubMed](#)]
22. Perez, L.; Valcarcel, M.J.; González, P.; Domecq, B. Influence of Botrytis infection of the grapes on the biological aging process of Fino Sherry. *Am. J. Enol. Vitic.* **1991**, *42*, 58–62.
23. Fernández-Espinar, M.T.; Esteve-Zarzoso, B.; Querol, A.; Barrio, E. RFLP analysis of the ribosomal internal transcribed spacers and the 5.8S rRNA gene region of the genus *Saccharomyces*: A fast method for species identification and the differentiation of flor yeasts. *Antonie Leeuwenhoek* **2000**, *78*, 87–97. [[CrossRef](#)]

24. Blandino, A.; Caro, I.; Cantero, D. Comparative study of alcohol dehydrogenase activity in flor yeast extracts. *Biotechnology* **1997**, *19*, 651–654. [[CrossRef](#)]
25. Infante, J.J.; Dombek, K.M.; Rebordinos, L.; Cantoral, J.M.; Young, E.T. Genome-Wide Amplifications Caused by Chromosomal Rearrangements Play a Major Role in the Adaptive Evolution of Natural Yeast. *Genetics* **2003**, *165*, 1745–1759. [[PubMed](#)]
26. Mauricio, J.C.; Moreno, J.J.; Ortega, J.M. In Vitro Specific Activities of Alcohol and Aldehyde Dehydrogenases from Two Flor Yeasts during Controlled Wine Aging. *J. Agric. Food Chem.* **1997**, *45*, 1967–1971. [[CrossRef](#)]
27. Budroni, M.; Giordano, G.; Pinna, G.; Farris, G.A. A genetic study of natural flor strains of *Saccharomyces cerevisiae* isolated during biological ageing from Sardinian wines. *J. Appl. Microbiol.* **2000**, *89*, 657–662. [[CrossRef](#)] [[PubMed](#)]
28. Ibeas, J.I.; Jiménez, J. Mitochondrial DNA loss caused by ethanol in *Saccharomyces* flor yeasts. *Appl. Environ. Microbiol.* **1997**, *63*, 7–12. [[PubMed](#)]
29. Coi, A.L.; Bigey, F.; Mallet, S.; Marsit, S.; Zara, G.; Gladieux, P.; Galeote, V.; Budroni, M.; Dequin, S.; Legras, J.L. Genomic signatures of adaptation to wine biological ageing conditions in biofilm-forming flor yeasts. *Mol. Ecol.* **2017**, *26*, 2150–2166. [[CrossRef](#)] [[PubMed](#)]
30. Kvittek, D.J.; Will, J.L.; Gasch, A.P. Variations in stress sensitivity and genomic expression in diverse *S. cerevisiae* isolates. *PLoS Genet.* **2008**, *4*, 31–35. [[CrossRef](#)] [[PubMed](#)]
31. Berlanga, T.M.; Atanasio, C.; Mauricio, J.C.; Ortega, J.M. Influence of aeration on the physiological activity of flor yeasts. *J. Agric. Food Chem.* **2001**, *49*, 3378–3384. [[CrossRef](#)] [[PubMed](#)]
32. Mauricio, J.C.; Valero, E.; Millán, C.; Ortega, J.M. Changes in nitrogen compounds in must and wine during fermentation and biological aging by flor yeasts. *J. Agric. Food Chem.* **2001**, *49*, 3310–3315. [[CrossRef](#)] [[PubMed](#)]
33. Zara, G.; Budroni, M.; Mannazzu, I.; Zara, S. Air-liquid biofilm formation is dependent on ammonium depletion in a *Saccharomyces cerevisiae* flor strain. *Yeast* **2011**, *28*, 809–814. [[CrossRef](#)] [[PubMed](#)]
34. Ibeas, J.I.; Jimenez, J. Genomic complexity and chromosomal rearrangements in wine-laboratory yeast hybrids. *Curr. Genet.* **1996**, *30*, 410–416. [[CrossRef](#)] [[PubMed](#)]
35. Legras, J.L.; Erny, C.; Charpentier, C. Population structure and comparative genome hybridization of European flor yeast reveal a unique group of *Saccharomyces cerevisiae* strains with few gene duplications in their genome. *PLoS ONE* **2014**, *9*, e108089. [[CrossRef](#)] [[PubMed](#)]
36. Charpentier, C.; Feuillat, M. *Wine Microbiology and Biotechnology*; Fleet, G.H., Ed.; Harwood Academic Publishers: Chur, Switzerland, 1993; pp. 225–242. ISBN 0-415-27850-31.
37. Suárez-Lepe, J.A.; Leal, I. *Microbiología Enológica: Fundamentos de Vinificación*; Mundi-Prensa: Madrid, Spain, 2004; pp. 673–716. ISBN 84-8476-184-3.
38. Martínez-Rodríguez, A.J.; Polo, M.C. Characterization of the nitrogen compounds released during yeast autolysis in a model wine system. *J. Agric. Food Chem.* **2000**, *48*, 1081–1085. [[CrossRef](#)] [[PubMed](#)]
39. Martínez, P.; Valcarcel, M.J.; Gonzalez, P.; Benítez, T.; Pérez, L. *Consumo de Etanol, Glicerina y Aminoácidos Totales en vinos Finos Durante la Crianza Biológica Bajo “Velo de Flor”*; Alimentación, Equipos y Tecnología; Reed Business Information S.A.: Bilbao, Spain, 1993; Volume 12, pp. 61–65. ISSN 0212-1689.
40. Martínez, P.; Valcárcel, M.J.; Pérez, L.; Benítez, T. Metabolism of *Saccharomyces cerevisiae* flor yeasts during fermentation and biological aging of Fino sherry: By-products and aroma compounds. *Am. J. Enol. Vitic.* **1998**, *49*, 240–250.
41. Cortes, M.B.; Moreno, J.; Zea, L.; Moyano, L.; Medina, M. Changes in Aroma Compounds of Sherry Wines during Their Biological Aging Carried out by *Saccharomyces cerevisiae* Races *bayanus* and *capensis*. *J. Agric. Food Chem.* **1998**, *46*, 2389–2394. [[CrossRef](#)]
42. Medina, M.; Mérida García, J.; Zea, L.; Moyano, L.; Moreno, J.; Mayén Riego, M.; Sherry Wines, J. Food Composition and Analysis. 2017. Available online: <http://helvia.uco.es/xmlui/bitstream/handle/10396/14301/Sherry.pdf?sequence=1> (accessed on 3 February 2018).
43. Martínez de la Ossa, E.; Caro, I.; Bonat, M.; Pérez, L.; Domecq, B. Dry extract in sherry and its evolution in the aging of sherry. *Am. J. Enol. Vitic.* **1987**, *38*, 321–325.
44. Mauricio, J.C.; Ortega, J.M. Nitrogen compounds in wine during its biological aging by two flor film yeasts: An approach to accelerated biological aging of dry sherry-type wines. *Biotechnol. Bioeng.* **1997**, *53*, 159–167. [[CrossRef](#)]
45. Botella, M.A.; Perez-Rodríguez, L.; Domecq, B.; Valpuesta, V. Amino acid content of Fino and oloroso sherry wines. *Am. J. Enol. Vitic.* **1990**, *41*, 12–15.

46. Ingledew, W.M.; Magnus, C.A.; Sosulski, F.W. Influence of oxygen on proline utilization during the wine fermentation. *Am. J. Enol. Vitic.* **1987**, *38*, 246–248.
47. Peinado, R.A.; Moreno, J.J.; Ortega, J.M.; Mauricio, J.C. Effect of gluconic acid consumption during simulation of biological aging of sherry wines by a flor yeast strain on the final volatile compounds. *J. Agric. Food Chem.* **2003**, *51*, 6198–6203. [[CrossRef](#)] [[PubMed](#)]
48. Robledo, M.J.L. *La Palma y los Vinos del Condado*. eDAP Documentos de Arquitectura y Patrimonio; 2010, pp. 11–24. Available online: http://arquitecturaypatrimonio.com/edap02_articulos/2_La_palma_y_los_vinos_del_condado.pdf (accessed on 4 February 2018).
49. Fleet, G.H. Wine yeasts for the future. *FEMS Yeast Res.* **2008**, *8*, 979–995. [[CrossRef](#)] [[PubMed](#)]
50. Belloch, C.; Orlic, S.; Barrio, E.; Querol, A. Fermentative stress adaptation of hybrids within the *Saccharomyces sensu stricto* complex. *Int. J. Food Microbiol.* **2008**, *122*, 188–195. [[CrossRef](#)] [[PubMed](#)]
51. Fierro-Risco, J.; Rincón, A.M.; Benítez, T.; Codón, A.C. Overexpression of stress-related genes enhances cell viability and velum formation in Sherry wine yeasts. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 6867–6881. [[CrossRef](#)] [[PubMed](#)]
52. Muñoz, D.; Peinado, R.A.; Medina, M.; Moreno, J. Biological aging of sherry wines using pure cultures of two flor yeast strain under controlled microaeration. *J. Agric. Food Chem.* **2005**, *53*, 5258–5264. [[CrossRef](#)] [[PubMed](#)]
53. Mauricio, J.C.; Moreno, J.; Ortega, J.M. Aceleración de la crianza biológica mediante aireaciones periódicas. In *VII Jornadas Científicas de los Grupos de Investigación Enológica*; Gobierno de la Rioja: Logroño, Spain, 2003; pp. 117–119.
54. Muñoz, D.; Peinado, R.A.; Medina, M.; Moreno, J. Biological aging of sherry wines under periodic and controlled microaerations with *Saccharomyces cerevisiae* var. *capensis*: Effect on odorant series. *Food Chem.* **2007**, *100*, 1188–1195. [[CrossRef](#)]
55. Moreno-García, J.; Mauricio, J.C.; Moreno, J.; García-Martínez, T. Differential proteome analysis of a flor yeast strain under biofilm formation. *Int. J. Mol. Sci.* **2017**, *18*. [[CrossRef](#)] [[PubMed](#)]
56. Morata, A.; González, C.; Suárez-Lepe, J.A. Formation of vinylphenolic pyranoanthocyanins by selected yeasts fermenting red grape musts supplemented with hydroxycinnamic acids. *Int. J. Food Microbiol.* **2007**, *116*, 144–152. [[CrossRef](#)] [[PubMed](#)]
57. Moreno, J.; Moreno-García, J.; López-Muñoz, B.; Mauricio, J.C.; García-Martínez, T. Use of a flor velum yeast for modulating colour, ethanol and major aroma compound contents in red wine. *Food Chem.* **2016**, *213*, 90–97. [[CrossRef](#)] [[PubMed](#)]
58. Aguilera, F.; Peinado, R.A.; Millán, C.; Ortega, J.M.; Mauricio, J.C. Relationship between ethanol tolerance, H⁺-ATPase activity and the lipid composition of the plasma membrane in different wine yeast strains. *Int. J. Food Microbiol.* **2006**, *110*, 34–42. [[CrossRef](#)] [[PubMed](#)]
59. Roldán, A.M.; Lloret, I.; Palacios, V. Use of a submerged yeast culture and lysozyme for the treatment of bacterial contamination during biological aging of sherry wines. *Food Control* **2017**, *71*, 42–49. [[CrossRef](#)]
60. Nakagawa, Y.; Arai, Y.; Toda, Y.; Yamamura, H.; Okuda, T.; Hayakawa, M.; Iimura, Y. Glucose repression of *FLO11* gene expression regulates pellicle formation by a wild pellicle-forming yeast strain isolated from contaminated wine. *Biotechnol. Biotechnol. Equip.* **2017**, *31*, 120–127. [[CrossRef](#)]
61. García-Martínez, T.; Moreno, J.; Mauricio, J.C.; Peinado, R. Natural sweet wine production by repeated use of yeast cells immobilized on penicillium chrysogenum. *LWT Food Sci. Technol.* **2015**, *61*, 503–509. [[CrossRef](#)]
62. Peinado, R.A.; Moreno, J.J.; Villalba, J.M.; González-Reyes, J.A.; Ortega, J.M.; Mauricio, J.C. Yeast biocapsules: A new immobilization method and their applications. *Enzym. Microb. Technol.* **2006**, *40*, 79–84. [[CrossRef](#)]

