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Review

Massive Sequencing: A New Tool for the Control of Alcoholic Fermentation in Wine?

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Abstract: In wine industry, there is a prevalent use of starter cultures to promote a controlled and efficient alcoholic fermentation preventing the growth of spoilage microbes. However, current trends in enology aim to combine the guaranteed success of monitored process and the complexity of fermentations either by inoculating autochthonous starters or by performing spontaneously to produce distinctive wines. To understand the complex roles of microorganisms on wine fermentation, we must understand their population dynamics and their relationships with wine quality and metabolome. Current metagenomics techniques based on massive sequencing are gaining relevance to study the diversity and evolution of microbial population on every stage of the wine making process. This new tool and technique increases the throughput and sensitivity to study microbial communities. This review focuses on the current knowledge about wine alcoholic fermentation, the contribution of massive sequencing techniques and the possibility of using this tool for microbial control.

Keywords: next-generation-sequencing; alcoholic fermentation; bioinformatics; high-throughput sequencing; wine; microbiota; metagenomics

1. Introduction

Wine is an alcoholic beverage with great cultural and economic importance, which results from the alcoholic fermentation process. During this process, yeasts derive energy by consuming sugars that occur naturally in the grapes and at the same time produce ethanol and carbon dioxide as byproducts [1]. From the yeasts genera the most widely used, due to its fermentation capacities, is the yeast *Saccharomyces* [1], whereas non-*Saccharomyces* yeasts contribute to wine flavor, although they can also spoil wines [2–4].

In addition to the different fermentation techniques used currently by the industry, the characteristics of the wine also depend upon other factors such as climate, soil and grape variety where variation of these factors attribute to the distinctiveness of the wine [5]. Moreover, the interplay between the wine microbiota and the microbiota of the fermentation facilities has been verified but not completely understood [6]. Due to this complexity of interactions between microorganisms in the wine itself, during fermentation, but also between wine microbiota and environment, the wine industry has adopted the use of starter cultures as a mean of control and quality improvement [7]. Nevertheless, more in-depth knowledge in needed in order to understand how microbial interactions may affect the wine quality.

The diversity of the vineyard and grape microbiota has been long ago investigated via traditional microbiological techniques involving agar plate cultivation, microscopy and biochemical characterization, focusing primarily on identifying pathogenic microorganisms or microorganisms that have been associated with wine spoilage. Nevertheless, these techniques fail to identify unculturable microorganisms that comprise a considerable fraction of the wine microbiota [8,9].

Nowadays, molecular techniques such as qPCR (quantitative polymerase chain reaction) and PCR-DGEE (polymerase chain reaction denaturing gradient gel electrophoresis) are widely used for detection and monitoring of microbial communities in wine. The former technique is more appropriate for detection and monitoring of a desired microorganism, whereas the latter for microbial community profiling. Although both techniques are supplemented with culture-dependent methods, however PCR-DGEE fails to detect species in low abudance, and qPCR suffers from scalability problems when many strains should be targeted [10,11].

These drawbacks of the aforementioned culture-dependent and molecular techniques come to solve recent novel techniques that are based on massive sequencing, and which in recent years have been regarded as the tool of choice for studying microbial communities during the various stages of alcoholic fermentation. Although there have been encouraging findings demonstrating the superiority of the massive sequencing over the classical methods concerning speed, sensitivity and accuracy, however most of the research has been confined to describing the constituents microorganisms and their abundance fluctuation over time. Therefore, the aim of this review, apart from exhibiting the contribution of massive sequencing to monitoring alcoholic fermentation, is to demonstrate the possibility of using this method as a tool for microbial control.

2. Sequencing Methods

The metagenomic analysis of wine samples is mainly performed via amplicon-based sequencing which through marker-genes amplification facilitates the taxonomic and phylogenetic profiling of the microbiome [12]. After the Sanger sequencing, that was developed back in 1977 by Sanger et al. [13] and was considered the first generation sequencing, second (SGS) and third (TGS) generation sequencing, collectivelly referred as next-generation sequencing (NGS), have been introduced in research as fast and cost-effective solutions.

Despite the fact that TGS solves many of the disadvantages of the SGS, still is under development and not widely applied in research. From the area of SGS, which is based on "sequencing by synthesis" method, the most popular platforms will be presented.

2.1. Ion Torrent

Introduced back in 2010 [14], Ion Torrent sequences the template DNA strand by detecting hydrogen ions that are released during the polymerization process. As a technology, with an error rate of 1.71% [15], it does not require modified nucleotides and it generates reads of around 200 bp in length allowing for multiple runs and more data generation [16].

2.2. Pyrosequencing

The most recent variant of pyrosequencing, 454 pyrosequencing, was introduced back in 2005 [17], and was the first affordable platform allowing whole genome sequencing. As a technology, it relies on the light signal detection that is emitted after the release of phosphate during the incorporation of a nucleotide by the DNA polymerase. With an error rate below 1% [18] 454 pyrosequencing is capable of generating reads of over 400 bp in length [19].

2.3. Illumina

With the first Illumina sequencer being available back in 2006, Illumina technology is based on the usage of fluorescently labeled dNTP terminators and the detection of light signal upon incorporation. Recent Illumina machines, HiSeq and MiSeq, have decreased the error rate below 1% and are capable of generating reads of around 300 bp in length [20].

From the aforementioned platforms, Illumina is the most widely used, with 52% of the published research citing it, followed by pyrosequencing that holds 48% of the total citations [21]. However, pyrosequencing technology has been discontinued, and currently Illumina is being considered as the largest contributor to SGS.

3. Amplified Genomic Regions

Apart from choosing the most appropriate sequencing platform, researchers have to decide the genomic region that is going to be used for the taxonomic classification of the metagenomic wine sample. As far as bacteria are concerned, the 16S ribosomal RNA (rRNA) gene is the common target that is used in research for taxonomic assignment.

The 16S rRNA gene contains nine hypervariable regions (V1–V9), which all have been used as potential classification targets generating different results. For instance, Bokulich et al. [22] used the V4 and V5 domain so as to ascertain which one is the most taxonomically informative for profiling bacterial communities. Based on the results, the V4 domain was regarded as more suitable for profiling lactic acid bacteria (LAB), as it gave more taxonomic depth comparing to the V5 domain.

Campisano et al. [23] used a 700 bp region that includes the domains from V5 up to V9 in order to assess the impact of pest management on bacterial endophytic communities of Merlot and Chardonnay grapevines, with the results indicating abundance differences of operational taxonomic units (OTUs) between organic and intergrated pest management (IPM) grapevines. The same genomic region was also targeted by Perazzolli et al. [24] in a study of leaf microbiota, that resulted in identification of beneficial microbial communities that could be used as a tool for crop protection. In the same manner, in the past years other researchers have been focusing on other domains for classification purposes. For instance, Sundquist et al. [25] favored the domains V1,V2 and V4, Liu et al. [26] the domains V2, V3 and V4, and Chakravorty et al. [27] the domains V2 and V3.

Regarding fungal classification, researchers have also displayed variability concerning genomic region preference. For instance, David et al. [28] sequenced the 18S rRNA gene to show that 454 pyrosequencing is much more reliable than classical techniques for studying yeast communities in alcoholic fermentation. Holland et al. [29] pyrosequenced the D1–D2 regions of the 26S rRNA, demonstrating that changes in arbuscular mycorrhizal fungal communities do not depend on irrigation frequency. Bokulich and Mills [30] targeted the IT1, ITS2 and the whole ITS in order to compare their classification efficiency by utilizing a mock community. Although they favored the IT1 region, nevertheless they urged for caution as none of these regions reconstructed reliably the whole mock community. Encouraging results targeting the ITS region have been yielded also from the researches of Pinto et al. [31] and Stefanini et al. [32] indicating this region as a suitable target for yeast classification.

4. Bioinformatic Tools

Regardless the NGS platform a researcher decides to utilize, the sequencing of wine metagenomic samples generates a significant amount of data that necessitate the use of bioinformatic pipelines. Despite the plethora of bioinformatic tools available, the most widely used will be presented.

4.1. QIIME

QIIME, which stands for Quantitative Insights Into Microbial Ecology, is a bioinformatic package, offering a variety of microbial community analyses and visualizations, that wraps other software packages with python code [33]. Some of the most frequent wrapped applications include mothur [34], blast [35], PyNAST (Python Nearest Alignment Space Termination) [36], RDP (Ribosomal Database Project) Classifier [37], FastTree [38] and USEARCH (unique word count search) [39].

4.2. MOTHUR

Mothur is a bioinformatic package that re-implements in C and C^{++} code other software packages removing that way any external dependecies during installation. Some of the re-implemented algorithms include DOTUR (Distance-Based OTU and Richness), SONS (Shared OTUs and Similarity), TreeClimber, LIBSHUFF, and UniFrac, and additioanlly the mothur team has incorporated its own analytical features to the platform [34].

4.3. MG-RAST

MG-RAST, which stands for Metagenomics Rapid Annotation using Subsystem Technology, is a server based platform with initial aim the annotation of complete or draft microbial genomes [40]. Currently, MG-RAST offers an automated solution for phylogenetic classification and functional classification of metagenomic samples.

A comparison of these three bioinformatic pipelines has been conducted by Plummer et al. [41] using 16S rRNA gut microbial data. The study concluded that all of the three pipelines were able to generate similar and reliable results with common limitation the ability to classify at the species level due to the type of data. The main differences between the pipelines concerned the usability and duration of analysis. MG-RAST is a more user friendly pipeline compared to the command-line based QIIME and MOTHUR, whereas QIIME required approximately 1 h to complete the analysis with MOTHUR and MG-RAST 10 h and 2 days respectively.

5. Databases

One of the most crucial steps of metagenomic analysis is the taxonomic classification of the microbial community. Apart from other factors, such as the sequence length, the parameters used for quality filtering and the implemented algorithm, this step can be greatly influenced by the chosen database. Currently, there are a number of highly curated databases available, such as Greengenes for 16S rRNA [42], SILVA for small (16S/18S, SSU) and large (23S/28S, LSU) subunit rRNA [43], UNITE for ITS region [44] and RDP for 16S and 28S rRNA classification [45]. However, classification based on these databases should be regarded as a rough estimation of the microbial composition as genera abundances or even taxonomic assignments can be greatly influenced by the chosen percentage of homology.

6. Analysis of Alcoholic Fermentation

There are numerous studies dedicated to the microbial analysis of wine alcoholic fermentation, but until now great focus has been given on describing microbial abundance succession during the various stages of alcoholic fermentation. These studies have attested the superiority of NGS over classical methods [28] and offered novel insights into the microbial communities.

Although bacteria are not directly connected to wine quality, acetic acid bacteria (AAB) and lactic acid bacteria (LAB) play a significant role to the final wine product. Portillo and Mas [46], in a Grenache variety wine fermentation study, showed that AAB and LAB are more abundant than previously thought, with a dominance of *Gluconobacter* during the mid fermentation. The latter finding contradicts the previous notion that *Gluconobacter*, being alcohol sensitive, usually declines during the alcoholic fermentation [47–49]. Similar results have also been yielded in other studies of low-sulfited or unsulfited wine fermentations [50].

Additionally, NGS analysis has created the notion that apart from AAB, other bacteria, not previously described, may be present during the process. Support to this hypothesis came from Godálová et al. [51] in a study of Blaufränkisch and Grüner Veltliner vines, where in addition to genera already found in other studies, such as *Sphingomonas*, *Variovorax*, *Pantoea*, *Enterobacter* and *Tatumella*, new genera were detected, namely *Amycolatopsis*, *Hydrogenophilus*, *Snodgrassella*, *Telluria*, *Gilliamella*, *Lelliottia*, and *Lonsdale quercina*. However, the possible impact of these newly described genera is still to be demonstrated.

Other studies come to supplement existing ones. For instance, Bokulich et al. [22] showed that *Acetobacter*, *Gluconobacter*, and *Gluconoacetobacter* are dominant in winemaking processes, whereas Campanaro et al. [52] in a grape marc study added that *Gluconobacter* and *Gluconoacetobacter* do not survive a prolonged grape marc storage period.

Moreover, the empirically based concept of the coined term "terroir", that is distinction of wine quality due to regional features, has been put under the prism of NGS analysis and verified recently.

Results from Zarraonaindia et al. [53] suggested that the soil serves as a bacterial reservoir for the vines and subsequently Bokulich et al. [54], in a 200 commercial wine fermentations study, demonstrated the correlation of wine microbiota, wine performance and wine metabolome. These authors even predicted the metabolome of the wine from the microbial composition by using machine learnig techniques [54]. Similar results have also been generated from other studies [55,56].

Besides bacteria, NGS analysis has also given significant insights into the yeast population during fermentation. The most frequent fungi described by NGS analysis are *Saccharomyces*, *Hanseniaspora*, *Issatchenkia*, *Rhodotorula*, *Penicillium*, *Cladosporium*, *Botrytis*, *Sporobolomyces*, *Aspergillus*, *Cryptococcus* and *Pichia* [56–58], with most studies reporting high abundance of *Hanseniaspora* and *Saccharomyces* during the mid and end of the fermentation respectively. Stefanini et al. [32] in a Vino Santo study, found that fungal species composition undergoes a dynamic change with a declining tendency overtime, and that small changes in fermentation procedures may result in significant differences in microbial communities. As advocates to these findings come older studies that have demonstrated that aerobic yeasts are the first to decrease in abundance, and that the mid fermentation yeast genera, such as *Hanseniaspora*, *Candida*, *Metschnikowia* and *Torulaspora*, cannot be not recovered on plates at high ethanol concentration in presence of *Saccharomyces* [59,60]. Interestingly, *S. cerevisiae*, found in very low abundance at the beginning of the fermentation, manages to rise in dominance at the end of the it. In accordance to this, Lleixà et al. [61] drew a comparison between the dynamics of *Saccharomyces cerevisiae* and *Hanseniaspora vineae* after inoculation in Macabeo and Merlot grape varieties. The results indicated that fermentation of *S. cerevisiae* inoculated must was faster than the one with *H. vineae* inoculation, and that inoculation with *S. cerevisiae* is necessary as *H. vineae* alone leads to incomplete alcoholic fermentation. However *H. vineae* was able to dominate the microbiota in Macabeo must but not the Merlot perhaps due to high exhibited yeast diversity of Merlot must.

Another important question that NGS analysis has been called to answer, is whether grapes are the source of spoilage microorganisms [62], or the wine-making equipment [63]. Even though there is no clear answer to this debate, studies from Suárez et al. [64] and Pinto et al. [56] seem to support the latter hypothesis.

7. Control of Alcoholic Fermentation

Controlling the alcoholic fermentation of wine-making is a very complex process. Unlike fed-batch alcoholic fermentation in bioreactors, where algorithms have been developed for the estimation of parameters that may lead to higher biomass concentrations and yield of a specific compound [65], wine alcoholic fermentation incorporates higher order of complexity, as it concerns (i) the determination of all the microbial composition throughout the fermentation process; (ii) the comprehension of the interplay between different microbial communities; (iii) the definition of a series of metabolites that contribute to the wine quality, and (iv) the integration of all these information into a predictive machine-learning model.

In the past, a series of studies have set the ground for controlling alcoholic fermentation by monitoring or modifying certain fermentation parameters, but most of the results were empirical and their interpretation was not an easy task. Various studies have shown that yeasts increase their production of volatile compounds at low fermentation temperatures [66–68]. Therefore, wine-makers that aim at enhancing wine aroma could take advantage of this factor. Another popular method, is choosing a specific yeast strain for improving specific aspects of the wine, with studies having used this technique so as to improve wine characteristics of Sauvignon [69] and Chardonnay [70]. Furthermore, addition of certain nutrients that will prevent the fermentation from stucking, is a common practice. For instance, Cramer et al. [71] developed a fermentation kinetic model which showed that fermentation rate can be increased upon addition of ammonium salts, whereas Birch et al. [72] supported that yeast growth rate and sugar degradation could be influenced by magnesium concentrations. On the other hand, adaptive evolution approaches are aiming towards the creation of non-recombinant yeast strains that could modify wine characteristics, as for instance in

the study of McBryde et al. [73]. Additionally non-*S. cerevisiae* yeasts are known of adding distinct flavors to the wine but due to the fact that they can easily become replaced by *S. cerevisiae*, authors such as Soden et al. [74] have suggested the use of mixed cultures controlled by sequential inoculation.

Although all the above practices are means of manipulating specific aspects of wine fermentation towards a specific outcome, they treat alcoholic fermentation as a black-box without controlling the microbial composition of the wine and consequently the wine quality consistency they are aiming to provide may not be certain. NGS analysis is aiming to tackle these obstacles, but as a relatively new approach so far has yielded descriptive results on the bacteria and yeast genera abundances that have been encountered during the various fermentation stages. Until now, studies from the food industry have already evinced this type of analysis as a promising strategy for the detection of previously undescribed spoiler bacteria [75,76], underlying its suitability for controlling alcoholic fermentation. Nevertheless, NGS analysis has as an intrinsic difficulty the overwhelming amount of metagenomic analysis tools, machine-learning algorithms, databases and parameters that the researcher has to choose from. Because small changes of parameters may result in singificantly altered taxonomic assignment results [26], a possible solution may come from the use of mock communities datasets with known species compositions [77]. This strategy has already been implemented in studies such as the one by Bokulich et al. [78] in order to compare the performance of different classifiers. Even though mock communities datasets cannot lead to the development of a standardized NGS analysis with fixed parameters, as metagenomic samples are bound to laboratory protocols, NGS platforms, environmental and grape variety differences, they may nonetheless serve as a way to validate the robustness of a bioinformatic pipeline or as a starting point for the subsequent metagenomic analysis.

Setting a solid ground for metagenomic analysis is of paramount importance, so additional analyses such as metatranscriptomics and metabolomics can function as determinant factors for the development of system-biology networks aiming for the understanding of microbial communities interaction, and machine-learning prediction models focusing on the quality of the final wine product. With encouraging results coming from studies such as the one by Bokulich et al. [54] where it has been demonstrated that microbial composition of grape must can predict wine metabolome, the future of controlling alcoholic fermentation via NGS analysis seems nothing but promising.

8. Conclusions

The aim of this review is to cite contemporary contributions of massive sequencing techniques to wine alcoholic fermentation, and the possibility of being used as a tool for microbial control. Wine alcoholic fermentation is a complex process that encompasses an intricate and dynamic interaction between microbial populations that leads towards the composition of a wine metabolome that defines the final wine quality and characteristics.

As a way of controlling alcoholic fermentation, the industry has adopted various techniques, such as starter cultures and process monitoring and modification, but these approaches rely on empirical results as little is known about the relationships within the wine microbiome and its correlation to the final wine product.

High-throughput sequencing, based on NGS platforms, has been presented as a metagenomic analysis tool that offers higher speed, accuracy and taxonomic resolution compared to classical culture-dependent and molecular techniques. Till now, the implementation of this technology has yielded significant yet descriptive research results on microbial dynamics in connection to the fermentation stages. Although, NGS metagenomic analysis comprises a vast amount of bioinformatic tools, databases and machine-learning algorithms, however publicly available mock communities datasets may serve as ways of algorithm benchmarking, robustness check of bioinformatic pipelines, and parameters initialization.

These mock communities and highly curated taxonomic databases could set a solid foundation for the metagenomic analysis, upon which metatranscriptomics and metabolomics will be based and provide all the necessary knowledge for the development of system-biology networks and

prediction models for deciphering microbial population dynamics and prediction of final wine product, correspondingly. Regarding the latter, research has provided encouraging results highlighting the potential and benefits of massive sequencing as a tool for controlling alcoholic fermentation.

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