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CORSO DI DOTTORATO DI RICERCA IN: SCIENZE ANIMALI E AGROALIMENTARI
CURRICOLO: PRODUZIONI AGROALIMENTARI
CICLO XXIX

EVALUATION OF THE NON-CONVENTIONAL YEAST STARMERELLA BACILLARIS AS BIOCONTROL AGENT AND SELECTED STARTER FOR ALCOHOLIC BEVERAGES PRODUCTION

SCUOLA DI DOTTORATO DI RICERCA IN : SCIENZE ANIMALI E AGROALIMENTARI
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CICLO XXIX

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“Keep your thoughts positive because
your thoughts become your words.
Keep your words positive
because your words become your behavior.
Keep your behavior positive
because your behavior becomes your habits.
Keep your habits positive
because your habits become your values.
Keep your values positive
because your values become your destiny.”
-Mohandas Karamchand Gandhi

“Nunca deixe que lhe digam que não vale a pena
acreditar no sonho que se tem
ou que seus planos nunca vão dar certo
ou que você nunca vai ser alguém
tem gente que machuca os outros
tem gente que não sabe amar
mas eu sei que um dia a gente aprende
se você tiver alguém em quem confiar
confie em si mesmo
quem acredita sempre alcança. ”
-Renato Russo

“La magia è un ponte
che ti permette di passare
dal mondo visibile
in quello invisibile.
E imparare le lezioni
di entrambi i mondi.”
-Paulo Coelho

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ABSTRACT

Yeasts are the main protagonists of the alcoholic fermentation and strongly influence the final characteristics of wine and other alcoholic beverages. In winemaking, during spontaneous fermentation, this process is carried out by indigenous yeasts present on the grape berry and there is a progressive growth pattern of different yeast species, with the final stages invariably being dominated by the alcohol-tolerant strains of *Saccharomyces cerevisiae*. This species is universally known as the 'wine yeast' and is widely preferred for initiating wine fermentations.

From a technological point of view spontaneous grape juice fermentations sometime become stuck or sluggish. This lack of reproducibility and predictability has favored, in the past, the use of yeast starters, generally composed of single strains of *S. cerevisiae*. Selected *S. cerevisiae* strains predominate during must fermentation, ensure rapid and reliable grape juice fermentation and, as consequence, consistent and predictable wine quality. However, there has been increasing recognition that wines made with *Saccharomyces* starter cultures are less complex, producing standardized wines.

In fact, during spontaneous fermentation many other species (the so call non–*Saccharomyces* yeasts) are present in the grape must and traditionally they were considered deleterious as responsible for some wine off-flavors. Recently, the role of non-*Saccharomyces* species has been re-evaluated as some of them has been proposed as co-starter to simulate natural must fermentation and to confer greater complexity and specificity to the wine.

In this context, the thesis project was developed in order to select new non-*Saccharomyces* yeast strains from fermenting musts of overripe grape berries. This stage of grape ripening is characterized to be more susceptible to infection of fungal pathogens such as *Botrytis cinerea*, whom yeasts present on the berry surface have to compete with. Thirty-six isolates were identified as *Starmerella bacillaris* isolated from destemmed dried grape of Raboso Piave variety, grown on Bagnoli DOC (Appellation of Origin) area (North-East of Italy). This species is recently proven to be of oenological interest mainly for its high glycerol production. Among them, 14 strains were phenotypically characterized and, for the first time, the antifungal activity of *S. bacillaris* species against *B. cinerea* and *P. expansum* were demonstrated. Moreover, fermentation performances of *S. bacillaris* strains were evaluated in synthetic, grape and apple musts (for the first time in cider production).

When tested in sequential fermentations with *S. cerevisiae* this yeast improved the quality of wine and cider. The obtained results indicate that *S. bacillaris* can be proposed as biocontrol agent on grape in vineyard and during postharvest on stored apples and its presence on fruit surface positively influences the following juice fermentation. Therefore the released of this yeast in the vineyard can increase the sustainability of the production process and the final wine quality. The obtained results provide a solid basis for a new management of yeast used for winemaking and other alcoholic beverage production and open new prospects for a more integrated strategy to increase wine quality.

Then, in this thesis, new *S. bacillaris* characteristics were evaluated in order to improve alcoholic beverages quality, such as mannoproteins and glutathione production. The quantification of these compounds was determined at the end of fermentation in synthetic must. *S. bacillaris* was proved to produce different mannoproteins with functional characteristics related to wine stability. Mannoproteins extracted from *S.*

bacillaris cultures presented great potential in increasing the protein stabilization, while if they were produced during sequential fermentation with *S. cerevisiae*, enhanced the tartaric stabilization. Moreover, in this thesis, a very high glutathione content was demonstrated in *S. bacillaris* cells after fermentation. Therefore, yeast lees obtained from *S. bacillaris* fermentations can be proposed as as glutathione source.

In the last part of this thesis the aim was to understand the genomic determinants of *S. bacillaris* technological traits described in the previous parts. Therefore. the whole-genome sequence of two relevant *S. bacillaris* strains was performed. By means of genome comparisons between the two *S. bacillaris* strains and between *S. bacillaris*, *S. cerevisiae* and other oenological yeasts, specific genes and metabolic pathways involved in technologically-relevant traits were identified and studied.

RIASSUNTO

I lieviti sono i principali protagonisti della fermentazione alcolica e influenzano fortemente le caratteristiche finali del vino e di altre bevande alcoliche. Nella vinificazione, durante una fermentazione spontanea,questo processo viene effettuato da lieviti autoctoni presenti sulla bacca di uva. Alla fine della fermentazione la specie dominate tra i ceppi è *Saccharomyces cerevisiae*. Questa specie è universalmente conosciuta come il 'lievito di vino' ed è ampiamente preferita per iniziare fermentazioni del vino.

Da un punto di vista tecnologico, le fermentazioni spontanee di succo d'uva si sono spesso bloccate. In passato, questa mancanza di riproducibilità e prevedibilità non era favorevole per una produzione di vino di qualità. L'uso di ceppo di lievito, generalmente composti da singoli ceppi come ad esempio *S. cerevisiae* aiuta a controllare questo problema. I ceppi selezionati di *S. cerevisiae* predominano durante la fermentazione del mosto, assicurando una rapida e affidabile fermentazione e, di conseguenza, una qualità del vino coerente e prevedibile. Tuttavia, si è accresciuto la convinzione che i vini ottenuti con culture di starter *Saccharomyces* siano meno complesse, producendo vini standardizzati.

Infatti, durante la fermentazione spontanea, nel mosto d'uva sono presenti molte altre specie (i cosiddetti lieviti non *Saccharomyces*) e tradizionalmente sono stati considerati come responsabili di alcuni sapori no aggradevoli all'assaggio del vino. Recentemente, il ruolo delle specie non *Saccharomyces* è stato rivalutato poiché alcune di esse sono state proposte come co-avviatore per simulare la fermentazione dei mosti naturali e per conferire maggiore complessità e specificità al vino.

In questo contesto, il progetto di tesi è stato sviluppato per selezionare nuovi ceppi di lievito non *Saccharomyces* da mosti fermentativi di uva passito. Questa fase di

maturazione dell'uva è caratterizzata per essere più suscettibile all'infezione di patogeni fungini come *Botrytis cinerea*. I trentasei isolati sono stati identificati come *Starmerella bacillaris* , isolati da uve secche della varietà Raboso Piave, coltivate in zona Bagnoli DOC (denominazione d'origine) (nord-est dell'Italia). Questa specie è recentemente dimostrata di interesse enologico soprattutto per la sua elevata produzione di glicerolo.

Tra questi, 14 ceppi sono stati caratterizzati fenotipicamente e, per la prima volta, sono stati dimostrati l'attività antifungina delle specie *S. bacillaris* contro *B. cinerea* e *P. expansum*. Inoltre, le prestazioni di fermentazione dei ceppi di *S. bacillaris* sono state valutate nei mosti sintetici, uve e mele (per la prima volta nella produzione di sidro).

Durante la sperimentazione, questo lievito ha migliorato la qualità del vino e del sidro in fermentazioni sequenziali con *S. cerevisiae*. I risultati ottenuti indicano che *S. bacillaris* può essere proposto come agente biocontrollo sull'uva nel vigneto e durante la post-raccolta sulle mele conservate: la sua presenza sulla superficie di frutta influenza positivamente la seguente fermentazione dei succhi. Pertanto, il rilascio di questo lievito nel vigneto può aumentare la sostenibilità del processo produttivo e la qualità finale del vino. I risultati ottenuti forniscono una solida base per una nuova gestione del lievito utilizzato per la produzione di vino e altre bevande alcoliche e aprire nuove prospettive per una strategia più integrata per aumentare la qualità del vino.

Sono state valutate le nuove caratteristiche di *S. bacillaris* al fine di migliorare la qualità delle bevande alcoliche, come le manoproteine e la produzione di glutatione. La quantificazione di questi composti è stata determinata alla fine della fermentazione in mosto sintetico. *S. bacillaris* ha dimostrato di poter produrre mannoproteine diverse, con caratteristiche funzionali legate alla stabilità del vino. Le manoproteine estratte dalle colture di *S. bacillaris* presentavano un grande potenziale per aumentare la stabilizzazione delle proteine, mentre se venivano prodotte durante la fermentazione

sequenziale con *S. cerevisiae*, aumentavano la stabilizzazione tartarica. Inoltre, in questa tesi, è stato rilevato un contenuto di glutatione molto elevato in cellule *S. bacillaris* dopo la fermentazione. Pertanto, le fecce ottenute dalle fermentazioni di *S. bacillaris* possono essere proposte come fonte di glutatione.

Nell'ultima parte di questa tesi si intendeva comprendere le determinanti genomiche delle caratteristiche tecnologiche di *S. bacillaris* descritte nelle precedenti parti. Per questo è stata eseguita la sequenza intero-genoma di due importanti ceppi *S. bacillaris*. Con i confronti genomici tra i due ceppi di *S. bacillaris* e tra *S*. *bacillaris*, *S. cerevisiae* e altri lieviti enologici, sono stati identificati e studiati geni specifici e i percorsi metabolici coinvolti in tratti tecnologicamente rilevanti.

CHAPTER 1

Introduction

1.1 Technological potential of *Starmerella bacillaris*

Starmerella bacillaris (synonym of *Candida zemplinina*) is a non-*Saccharomyces* yeast, isolated for the first time in Napa Valley,California, United Statesin 2002, under the name of EJ1 (Mills et al., 2002; Duarte et al., 2012). Interesting, this specie showed an ability of fermentation by exploiting mainly the fructose contained in must of Chardonnay grape variety, without affecting significantly the initial concentration of glucose.

Then, one years later, Sipiczki (2003) recognised that this Candida yeast belonged to a new species and it gave the name of C. zemplinina. This decision was taken based on the significant difference observed between the ribosomal RNA sequences of this species compared to the species which it was originally associated: Candida starid (Sipiczki, 2004).

S. bacillaris has been noted for its potential in the wine industry mainly due to its high fructose consumption and its relatively low ethanol production relative to the consumption of sugars (Magyar and Tóth, 2011). Many studies have shown that *S. bacillaris* is present naturally in spontaneous fermentation of must on several continents (Alessandria et al., 2013; Bokulich et al., 2014; Mills et al., 2002; Rantsiou et al. Al., 2013), suggesting a possible implication of this species in the fermentative phenomenon. *S. bacillaris* possesses very interesting characteristics from an oenological point of view because its high capacity to develop at elevated concentrations of sugars and low temperatures (Sipiczki, 2003; Tofalo et al., 2012).

In addition, single *S. bacillaris* is able to produce low levels of acetic acid, acetaldehyde and ethanol and a good glycerol production in relation to the amount of sugars consumed at the end of fermentation (Magyar and Tóth, 2011).

S. bacillaris differs from other non-*Saccharomyces* yeasts by its ability to survive until the end of alcoholic fermentation, due to its remarkable ability to withstand high ethanol concentration conditions normally present in the wine. The particular characteristics of *S. bacillaris* are also the reducing of production of acetic acid linked to stress due to high concentration of sugars typical of sweet wines compared to fermentations of single *S. cerevisiae* strain (Rantsiou et al., 2012).

These phenotypic features support a possible use of *Starmerella* in wine production. In any case, it will be necessary to thoroughly investigate the new characteristics that can distinguish individual strains within the species in order to understand the different behaviours within the fermentation of wine (Lemos Junior et al., 2016).

1.2 Biocontrol of post-collection diseases and the use of fungi and yeast

Fruits that need a pre-sale storage can present problems from post-harvest diseases. It is estimated that around 20-25% of the fruit and vegetable productions stored in the post-harvest are lost. Besides that, some studies about contaminations of fruit have been showed that simple surface washing can be enough to remove the population of bacteria and yeasts normally present (Janisiewicz, 1987; Sobiczewski et al., 1996; Chalutz and Wilson, 1990). Then, it is a concern for some of the countries in the world where fruits are kept for long periods after harvesting (El-Ghaouth et al., 2004; Droby, 2006; Singh and Sharma, 2007).

The most commonly used technique to avoid the onset of post-harvest diseases is the use of synthetic fungicides (El-Ghaouth et al., 2004; Singh and Sharma, 2007). However, greater focus on health and environment areas is pushing public opinion to prefer alternatives to the use of synthetic substances and this is leading to greater researchers in seeking effective chemistry alternatives (Mari et al., 2007).

Several pathogenic biological control systems were studied and the most promising is antagonistic organism such as fungi, yeasts and bacteria (Eckert and Ogawa, 1988; Droby et al., 1991; Wisniewski and Wilson, 1992; Korsten, 2006).

Therefore, some strategies can be applied in the use of antagonists aiming to favor the microorganisms already present on fruits or vegetables, such as the use of artificially microorganisms from laboratory or others ecological system.

Biological control of diseases with the artificial introduction of antagonists was evaluated in 1977 using Trichoderma genus mushrooms to control *Botrytis cinerea* on strawberry as described by Tronsmo et al, 1977.

In recent decades, several investigations have been conducted to discover new microorganisms which can be used. There are several varieties of yeasts with interesting characteristics for biocontrol, among them the principal are: *Pichia*, *Candida*, *Debaryomyces*, *Aureobasidium, Pantoea*, *Saccharomyces* and *Metschnikowia* (El-Ghaouth et al., 1998, Wilson and Chalutz, 1989; Chalutz and Wilson, 1990; Droby et al., 1991; Arras, 1996; Ippolito et al., 2000. Nunes et al. Et al., 2001; Karabulut et al., 2003; Spadaro et al., 2004; Lahlali et al., 2005; Droby; 2006; Long et al., 2006, 2007; Torres et al., 2008).

However, some mechanism used by antagonistic microorganisms to limit the development of pathogenic fungi have not been fully understood yet. Moreover, different theories have been suggested to explain these systems in order to explam

the antagonistic effect in pathogenic yeast (Wilson and Wisniewski, 1989; Wisniewski and Wilson, 1992).

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CHAPTER 2

Isolation, genetic characterization, growth kinetics and fermentation performances of *Starmerella bacillaris* strains isolated from dried grape must

2.1 Introduction

Wine is an alcoholic beverage that is the object of a strong social debate related to a responsible and moderate consumption. Among the alcoholic beverages consumed in the world (fermented, distilled, etc.), the wine is in the third position, with 8% of the total volume. However, wine consumption worldwide has registered great reduction, determining a deficit between production and purchase (Pendurthi et al., 1999; Novello & Palma, 2013; World Health Organization, 2014).

Regarding to production process, researchers are seeking a way to balance the effects of the global average temperature increases that influence the vine cultivation, as responsible for the acidity decrease, the modification of phenolic compounds, tannins and sugar levels, therefore influencing directly the winemaking process. In addition, researchers are trying to respond to the consumers's demand, who tend to prefer low alcohol content with harmful effect mitigations and a better sensory quality (Quiróz et al., 2014).

The modern approach to the enological research offers possibilities for the development of products that are focused on risk reduction by alcohol intake (illness, death and social problems) and increases benefits associated directly to wine consumption. One of the possibilities is the reduction of the alcohol content (Chambers & Pretorius, 2010; Jolly et al., 2013; Quiróz et al., 2014; Duren, 2015).

The reduction of the alcohol content in wine can be pursuit through the addition of selected non-conventional yeasts during fermentation traditionally conducted by

Saccharomyces cerevisae species. These yeasts naturally present in grape must have been considered for a long time just as contaminants such as those of the following genera *Starmarella*, *Candida*, *Hanseniaspora*, *Pichia*, *Metschinokowia*, *Issacenkia*, *Torulaspora*, *Zygosaccharomyces*, *Saccharomycodes*, *Dekkera* and *Schizosaccharomyce* (Rojas et al, 2001; Contreras et al., 2014; Carrau et al, 2015; Maturano et al, 2015; Lemos Junior et al., 2016).

Among them, *Starmerella bacillaris* (synonym *Candida zemplinina*) contributes to flavor development without increase alcohol level, even if it isn't the main yeast involved in alcoholic ferementation. The technological properties of *S. bacillaris* strains evidenced a low ethanol yield from sugar (glucose and fructose) consumed, and a high glycerol and a moderate volatile acid production. However, when *S. bacillaris* is used in combination with *S. cerevisae*, it produces more complex wine with high glycerol and acetic acid concentration, it can decrease the pH and ethanol contents (Englezos et al., 2015; Lemos Junior et al., 2016).

Studies focused on *non-Saccharomyces* yeast have demonstrated the positive contributions of these species in wine during spontaneous,fermentation, since they are able to produce and secrete enzymes, such as proteases, β-glucosidades, pectinase, lipase, which interact with components present in the grape must, thus contributing to better varietal aromas, flavors and wine body, positively influencing the sensory characteristics of the final product (Pretorius, 2000; Mills et al., 2002; Mateo and Maicas, 2016; Fleet, 200).

Increasing the knowledge about the non-conventional yeasts opens new possibility of applications to better perform alcoholic fermentations. In this study we focus on *S. bacillaris* growth kinetic at different condition in oder to obtain data that

allow a correlation with the winemaking process. This type of analysis provides an objective view on the behaviour of the microorganism studied, in relation to nutrient assimilation and adaptation to a specific medium (Van Dijken et al., 1993; Gobbi, 2011).

Among the growth kinetics parameters, the lag phase duration (ʎ) allows to infer the adaptation time needed by the yeast in a specific medium. The lowest the values found, the best the adaptation achieved. The second parameter is the specific growth rate value (μ_{max}), which indicates the change in the number of cells or biomass formed over a period of time, in relation with the assimilation of nutrients from the medium. Finally, the OD_{max}, expresses the highest Optical Density level reached by the culture, this value is proportional to the cell number or biomass produced by yeasts. (Ciani & Picciotti, 1995; Phisalaphong et al., 2006).

Understanding non-conventional yeasts influences on the fermentation process by means of the kinetic parameters is of great relevance, as these parameters allow to predict a possible fermentation performance. Thus, extrapolating the use of non-conventional yeasts in wine-making, it is possible to direct the production of desirable compounds and optimize the operating conditions on large scale (Van Dijken et al., 1993; Zott et al., 2008).

In this context, the aim of this study was to genetically identify and to evaluate the growth kinetics of *Starmerella bacillaris* strains isolated from musts made of dried grapes collected from Veneto region. Moreover we compared different glucose and nitrogen concentration in order to explore *S. bacillaris* growth aptitudes finally, fermentation performances with different sugar concentrations in synthetic musts were evaluated in order to explore the possibility to apply *S. bacillars* for low-alchool wine production.

2.2 Materials and methods

2.2.1 Isolation of yeast strains

Yeast isolates were collected from fermenting dried grape of Raboso Piave variety (table 1). The samples were collected during two years in two wineries (A and B) located in the area of Bagnoli DOC (Guaranteed Origin Name) wine production (North-Eastern Italy). The strains were obtained by plating (surface) of the musts in selective agar WL (Wallerstein Laboratory, Oxoid). After incubation at 28 °C for 48 hours, 360 yeast colonies were selected at random on the boards and transferred to YPD agar (Yeast Extract Peptone Dextrose).

Table1: Strains used in this work

Strain	Species	Origin	Strain	Species	Origin	Strain	Species	Origin
FRI728	*S. bacillaris*	A	PAS106	*S. bacillaris*	B	R104.2	*S. cerevisiae*	C
FRI749	*S. bacillaris*	A	PAS13	*S. bacillaris*	B	R104.5	*S. cerevisiae*	C
FRI779	*S. bacillaris*	A	PAS130	*S. bacillaris*	B	R133.5	*S. cerevisiae*	C
FRI7100	*S. bacillaris*	A	PAS151	*S. bacillaris*	B	R150.1	*S. cerevisiae*	C
FRI719	*S. bacillaris*	A	PAS152	*S. bacillaris*	B	R150.4	*S. cerevisiae*	C
FRI720	*S. bacillaris*	A	PAS157	*S. bacillaris*	B	R151.1	*S. cerevisiae*	C
FRI729	*S. bacillaris*	A	PAS158	*S. bacillaris*	B	B125.5	*S. cerevisiae*	C
FRI730	*S. bacillaris*	A	PAS173	*S. bacillaris*	B	P138.1	*S. cerevisiae*	D
FRI745	*S. bacillaris*	A	PAS25	*S. bacillaris*	B	P283.4	*S. cerevisiae*	D
FRI751	*S. bacillaris*	A	PAS32	*S. bacillaris*	B	P293.8	*S. cerevisiae*	D
FRI754	*S. bacillaris*	A	PAS37	*S. bacillaris*	B	P301.4	*S. cerevisiae*	D
FRI757	*S. bacillaris*	A	PAS55	*S. bacillaris*	B	P301.Z	*S. cerevisiae*	D
FRI759	*S. bacillaris*	A	PAS66	*S. bacillaris*	B	P304.1	*S. cerevisiae*	D
FRI794	*S. bacillaris*	A	PAS86	*S. bacillaris*	B	P304.3	*S. cerevisiae*	D
FRI795	*S. bacillaris*	A	PAS89	*S. bacillaris*	B	B173.16	*S. cerevisiae*	D
FRI797	*S. bacillaris*	A	PAS92	*S. bacillaris*	B	B173.2	*S. cerevisiae*	D
FRI798	*S. bacillaris*	A	R8.3	*S. cerevisiae*	C	71B	*S. cerevisiae*	E
FRI799	*S. bacillaris*	A	R15.2	*S. cerevisiae*	C			
PAS102	*S. bacillaris*	B	R17.1	*S. cerevisiae*	C			
PAS103	*S. bacillaris*	B	R103.1	*S. cerevisiae*	C			

(A) - Bagnoli (North-Eastern Italy) Winery (B) - Bagnoli (North-Eastern Italy) Winery B, (C) - Conegliano (North-East of Italy), (D) - PAO Piave (North-East of Italy) and (E) - Industrial wine strain

2.2.2 Genetic identification of yeast strains

A total of 360 yeast colonies were isolated on WL agar medium (Oxoid) plates. All yeasts were identified at species level by PCR-RFLP analysis of the ITS1-5.8S-ITS2 rDNA region and D1/D2 region sequence analyses as described by Bovo et al. (2011). A BLAST search on sequence results gave the most probable species identification. Genetic diversity of 36 *S. bacillaris* were analyzed by Sau-PCR technique, according to the methodology described by Corich et al. (2005).

For DNA extraction, the strains were ridged YPD agar and incubated at 28 °C for 48 hours. Subsequently, eight handles containing cells of each strain were transferred to eppendorfs containing 1 mL of sterile water. The eppendorfs were centrifuged for 3 minutes to 14000 rpm, the supernatant removed and 0.5 mL of distillate H_20 added. The content was homogenized in vortex and transferred to another eppendorf containing "beads" sterile glass, which was subjected to unrest in Vortex for 3 minutes. Then, 50 µL of SDS (Sodium Dodecyl Sulfate) were added and the mixture kept at 65° C for 30 minutes. After this period, 200 µL of potassium acetate 5M were added and the mixture held in ice bath for 30 min, being subsequently subjected to centrifugation at 14000 rpm for 3 min, for removal of DNA present in the supernatant.

Sau3AI restriction enzyme (Amersham Biosciences AB, Uppsala, Sweden) was used to digest genomic DNA. To this end, we used 10 µL of DNA, 1.25 µL Sau3AI, 6.75 µ sterile water and 2 µL of buffer 10XH, totaling a mix of 20 µL who remained overnight at 37 °C.

The digested genomic DNA was amplified by means of the technique the polymerase chain reaction (PCR) using primer SAG (MWGBiotech, Ebersberg, Germany), with 5 ' CCGCCGCGATCAG 3 ' sequence. The amplification reaction

occurred in thermal cycler Bio-Rad iCycler (Bio-Rad, Hercules, California) in three steps. At first, the material was maintained at 60° C for 30 seconds, to action of Taq polymerase. On second step, called the low-accuracy, the amplification occurred in three cycles, being them 94 °C for 60 seconds, 50 °C for 15 seconds and 50 °C per 30 seconds. In the third and final stage of amplification (thoroughness), the temperature was raised to 94 °C for 15 seconds, reduced the temperature to 48 °C and extended for another 2 minutes to 65 °C. This step was repeated 35 times. At the end of the repetitions, the temperature was maintained at 65 °C for 5 minutes and then reduced to 4 °C. The restriction fragments were separated by electrophoresis in agarose gel at 1% (p/v), using TBE buffer (Tris/Borate/EDTA-44.5 mM Tris-HCl, 44.5 mM boric acid, 1 mM EDTA, pH 8.3) to 0.5%. We used molecular weight marker Extended Range DNA Ladder (Lonza Rockland, USA) with 100 BP extended. Gel was visualized under UV light and photographed. The resulting amplification profiles were analyzed by standard analysis software GelCompar II v. 3.5 (Applied Maths).

The yeast growth kinetics in each condition tested was evaluated by measuring the following parameters: lag phase duration (λ), specific growth rate value (μ_{max}) and maximum OD (DO_{max} 600 nm) reached by the yeast culture. All the parameters were calculated using R software (www.rstudio.com), through the grofit data library.

2.2.3 Preparation of the inoculum

Strains were grown on YPD agar at 28 °C for 24 hours and subsequently, a colony was individually transferred into a tube containing 10 mL of YPD. After incubation under the previously described conditions, the number of cells present in the medium was assessed. The determination was performed using a standard curve by means of optical density (OD) measurements at 600 nm performed with a

spectrophotometer (Shimadzu UV-Visible UV model-1280). The OD data were confirmed through direct cell counts using a Neubauer Chamber (cfu/mL). After the determination of the cell concentration value the culture was diluted to reach the correct cell concentration in stationary phase cultures to be inoculated the growth kinetics test.

2.2.4 Growth in YPD medium

YPD growth tests were performed in microplates from 96 wells, around 2 - 4 x 10^5 CFU/mL of previously prepared with inoculum in stationary phase cultures, with approximately 10^7 - 10^8 CFU/mL, the hole inoculated with total of 200 µL YPD stock. The plates were incubated at 28 °C for 25 hours.

Selective growth in YPD was carried out following a completely randomized design (DIC), with three repetitions for each strain analysed. Being the strains as the only source of variation in the experiment and the growing medium kept default. The assay was performed using three replicates for each yeast strain.

2.2.5 Growth in modified YPD medium

This set of trials had as purpose the evaluation of ability of *S. bacillaris* strains to develop at different concentration of the two basic nutrients to development, peptone as nitrogen source and glucose as carbon source. The effects of these components have been studied by means of a binary mixture design, conducted from the base formulation variation of YPD medium (0.1% yeast extract, 0.2% peptone, 0.2% glucose), in which was kept the concentration of yeast extract (precursor source of amino acids and vitamins), peptone and glucose. The three defined concentrations of glucose and peptone were 30, 20 and 10 g L^{-1}, according to studies by Cruz et al. (2003) and Govindaswamy & Vane (2007).

The design of mixture for two components included five assays, being assay 5 the central point. All the assays were repeated three times, except for 5 assays with 9 repeated three times as shown in Table 2.

Table 2: Experimental design of binary mixture for glucose and peptone trials.

Assays	Coded		Concentrations ($g.L^{-1}$)	
	Peptone	**Glucose**	**Peptone**	**Glucose**
1	0,333	0,333	10	10
2	1	0,333	30	10
3	0,333	1	10	30
4	1	1	30	30
5	0,666	0,666	20	20

2.2.6 Evaluation of strain growth kinetics in YPD

All the yeast growth kinetics obtained in microplates test were evaluated by measuring the following parameters: lag phase duration (λ), specific growth rate value (μ_{max}) and maximum OD (DO_{max} 600 nm) reached by the yeast culture. All the parameters were calculated using R software (www.rstudio.com), through the grofit data library.

2.2.7 Fermentation trials

A loopful of a 3-day-old culture from YPD agar plate (yeast extract 10 g/L, peptone 10 g/L, dextrose 20 g/L) was used to inoculate 10 mL of YPD broth in 50 ml tubes. A stationary phase culture with approximately 10^7-10^8 cells/mL, determined by OD measurements, was obtained after 24 hours of incubation at 30 °C. A suitable aliquot of each yeasts culture, corresponding to a final cell concentration of 1.5×10^6 cells/mL, was used to inoculate 120 mL-capacity bottles fitted with closures that

enabled the carbon dioxide to escape, containing 100 mL of synthetic must and incubated at 20 °C and the fermentation performance of 21 *S. cerevisiae* was carried as describe above but at 25 ºC.

Two synthetic musts differing in sugar and malic acid concentration were prepared to all strains: the standard synthetic must with 100 g/L of glucose, 100 g/L fructose and 2 g/L of malic acid (200-2), as described by Delfini and Formica (2001) and one modified synthetic musts with 40 g/L glucose, 40 g/L fructose and 12 g/L malic acid (80-12). Nitrogen was supplied as casein hydrolysate (0.2 g/L), $(NH_4)_2SO_4$ (0.3 g/L) and $(NH_4)_2HPO_4$ (0.3 g/L) and pH adjusted to 3 with KOH. All the experiments were performed in triplicate. CO_2 production was monitored by weighing twice a day the bottles and calculating weight loss of each culture. The fermentations were stopped when the weight loss was lower than 0.05 g in 24 hours.

2.2.8 HPLC analysis

Concentrations of residual glucose and fructose, glycerol, acetic acid and ethanol were detected by means of HPLC analysis. Fifty microlitres of filtered (0.22 µm) samples were injected. For the separation of the components a Waters 1525 HPLC binary pump with a 300×7.8 mm stainless steel column packed with Aminex HPX_87H 300×7.8 mm was used. Waters 2414 Refractive Index Detector, set at 600 nm wavelength for the determination of glucose, fructose glycerol and ethanol was used, while for the acetic acid a UV detector was used. The concentrations, expressed as g/L, were calculated by using calibration curves of the individual compounds.

2.2.9 Statistical Analyses

The parameters obtained with the software RStudio by growth kinetic results were submitted to analysis of variance (ANOVA). Regarding the growth in YPD, the results showed significant differences ($p \leq 0.05$) in ANOVA were analyzed by Duncan and Fisher Test at 5% probability.

The significant effect ($p \leq 0.05$) of glucose, peptone and variation and effect the addition of amino acids were used multiple linear regression equations, quadratic, cubic and quartic special special, to analyze the effects of independent variables (x1, x2) process the replies (yi).

Student t-tests and Mann-Whitney-U tests (a non-parametric test) were employed to compare the anthropometric data related to glycerol production between 2 groups (1) Satamerella strans; (2) *S. cerevesae* strains.

The statistical analyses were performed on the program SAS (Statistical Analysis System-SAS Institute Inc., North Carolina, USA, 1989) 9.3, version licensed for the Padova of University.

2.3 Results and discussion

2.3.1 Isolation and genetic identification of yeast strains

A total of 360 colonies of yeasts were isolated ami the YPD from the fermentation by grape must of wine "Friularo Bagnoli" Passito. All colonies were identified at species level by PCR-RFLP analysis of the ITS1-5.8S-ITS2 rDNA region and D1/D2 region sequence analyses.

A total of 36 isolate were identified as *Starmarella bacillaris* and Sau-PCR technique was used to analyze the genetic diversity by GelCompar II v. 3.5 software. The dendrograms reveal that the 36 *S. bacillaris* isolates, were clustered into seven

different genetic profiles, (Figure 1). These results demonstrate the appropriateness of the methodology used for the definition of the genetic relationship between isolates.

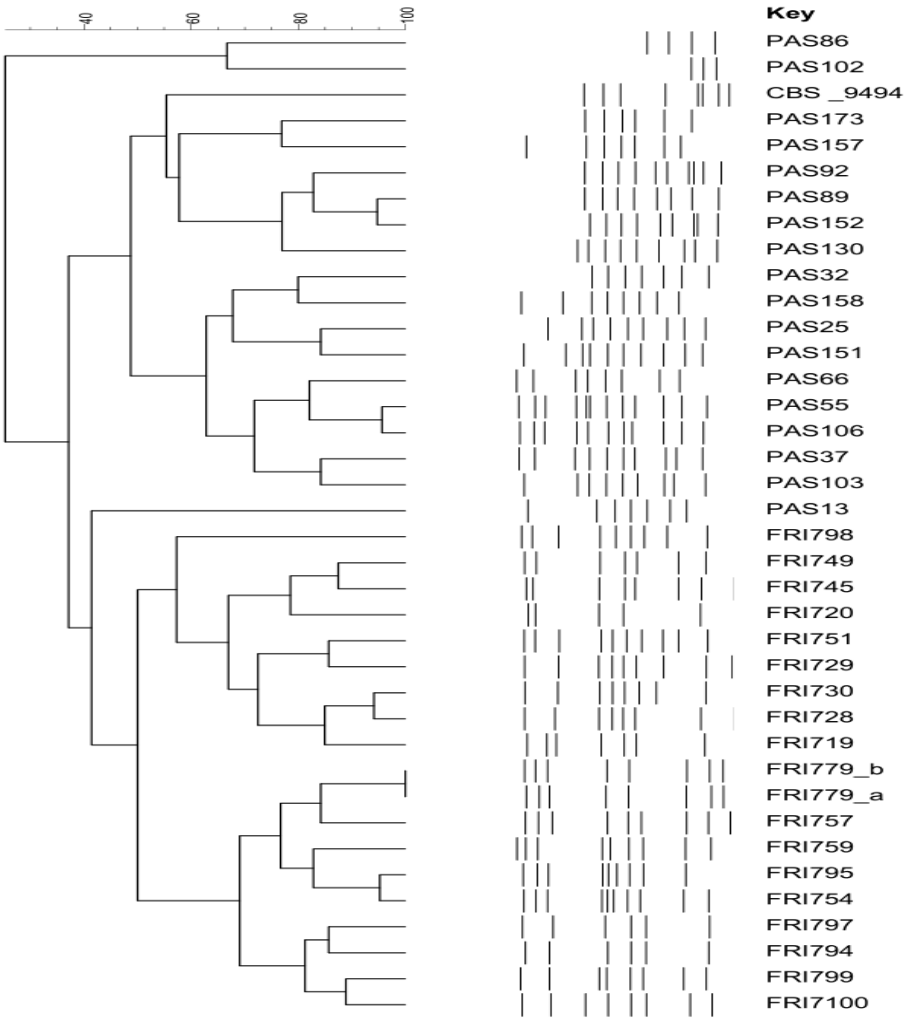

Figure 1: SAU-PCR analysis of the *S. bacillaris* strains. The strains named FRI were isolated from winery A, and PAS from winery B. CBS_9494: *S. bacillaris* type strain.

According to the phylogenetic tree (Figure 1) at least 70% similarity among the isolated from the two wineries was observed. The strain origin seem to be very relevant as in the dendrogram a clear separation between the isolated collected from the two winery was observed. DNA from isolate FR179 was extracted twice and two

independent amplifications were carried out (named FR179_ta and FRI179_b) to test the reproducibility of the method. The result showed 100% of similarity between the two replicates evidencing an excellent level of reproducibility. Notwithstanding 70% of similarity was chosen to cluster the genotypes in order to select representatives with clear genetic differences. Table 3 presents group of strains.

Table 3: Strains of *S. bacillaris* identified according the genetic similarity

Genetic profile	Strains
1	PAS86, PAS102
2	PAS173, PAS157
3	PAS89, PAS92, PAS152, PAS130
4	PAS32, PAS158
5	PAS25, PAS151,
6	PAS66,
7	PAS55, PAS106
8	PAS37, PAS103,
9	PAS13
10	FRI798
11	FRI745, FRI749, FRI720
12	FRI751
13	FRI729
14	FRI719,
15	FRI730, FRI728
16	FRI757, FRI779a, FRI779b
17	FRI754, FRI759, FRI795
18	FRI794, FRI797, FRI799, FRI7100

2.3.2 Growth in YPD medium

Among the strains isolated from the must of the winery B, seven strains according to the genetic distribution in the SAU-PCR dendrogram were selected for further evaluation (Table 4).

Table 4: Yeast Strains selected for the evaluation of the growth kinetics

Strain	Species	Origin
FRI719	*S. bacillaris*	Winery A
FRI728	*S. bacillaris*	Winery A
FRI729	*S. bacillaris*	Winery A
FRI751	*S. bacillaris*	Winery A
FRI754	*S. bacillaris*	Winery A
FRI779	*S. bacillaris*	Winery A
FRI7100	*S. bacillaris*	Winery A

The selected *S. bacillaris* strains were grown in YPD medium at 28 ºC and the growth kinetics are showed in figure 2.

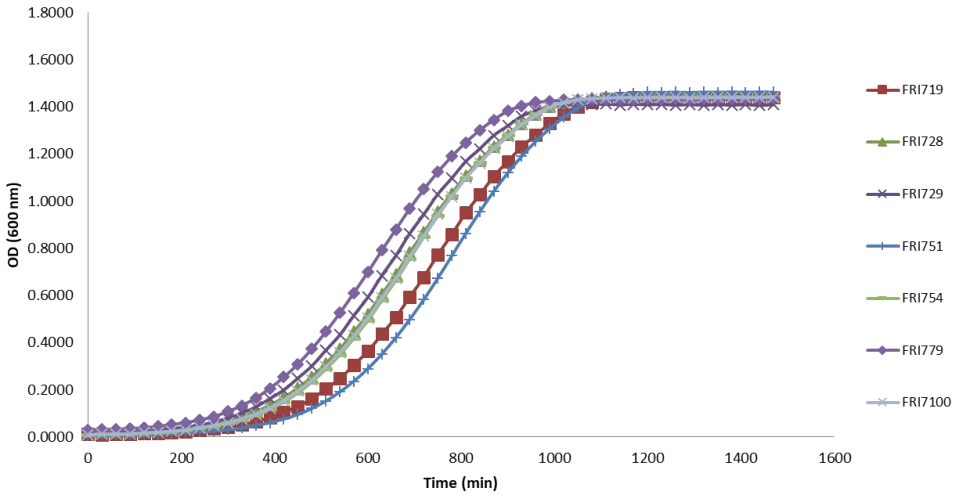

Figure 2: Growth kinetics of *S. bacillaris* strains

The growth parameters were obtained by the curves and analysed by ANOVA. Results are reported in Table 5.

Table 5: ANOVA analysis of μ_{max}, lag phase (λ) and OD_{max} in YPD medium

Parameters	df	MS	P(F)
μ_{max} (h^{-1})	6	5.096×10^{-7}	<0.0001*
λ (h)	6	1.431×10^{6}	<0.0001*
DO_{max} (600nm)	6	0.0017	0.0224*

μ_{max}: maximum specific growth rate; λ: lag phase; DO_{max}: maximum amount of dissolved oxygen. * Significant at the 5% probability.

The analysis of variance indicated significant differences ($p \leq 0.05$) between all parameters,Table 6 shows the groupings the averages and standard deviation of each parameter evaluated for each strain.

According the data shown in Table 6 specific growth rate (μ_{max}) gathered the tested strains in 3 clusters. The first included FRI751 FRI754 and FRI779 strains that showed the highest values (from 0.189 to 0.187 h^{-1}), followed by the strains FRI729 and FRI7100 (0.184 h^{-1}). Finally the group with lowest specific growth rate values included FRI719 and FRI728 strains (0.181 h^{-1}). The high growth rate found for each strain indicated that the medium is suitable for the growth of the *S. bacillaris* strains .

Table 6: Evaluation of the growth kinetics parameters

Strain	Mean ± Standard deviation		
	μ_{max} (h^{-1})	λ (h)	DO_{max} (600nm)
FRI719	0.181 ± 0.001^{c}	7.95 ± 0.313^{b}	1.508 ± 0.025^{ab}
FRI728	0.181 ± 0.001^{c}	6.88 ± 0.301^{cd}	1.501 ± 0.001^{ab}
FRI729	0.184 ± 0.001^{b}	6.49 ± 0.212^{de}	1.459 ± 0.024^{c}
FRI751	0.189 ± 0.001^{a}	8.64 ± 0.214^{a}	1.535 ± 0.022^{a}
FRI754	0187 ± 0.001^{a}	7.15 ± 0.311^{c}	1.496 ± 0.012^{abc}
FRI779	0.188 ± 0.002^{a}	5.97 ± 0.261^{e}	1.478 ± 0.021^{bc}
FRI7100	0.184 ± 0.002^{b}	7.05 ± 0.412^{c}	1.495 ± 0.031^{abc}

μ_{max}: maximum specific growth rate; λ: lag phase; DO_{max}: maximum amount of dissolved oxygen. Distinct letters in the same column differ the 5% probability by Duncan Test.

Lag phase is the time during microorganisms are adapting to the specific medim activating the proper enzyme set. During this period no cellular growth is detected. According to Table 5 it turns out that the strains FRI728 FRI729 FRI754 FRI779 and FRI7100 presented minors values ($p \leq 0.05$).

The assessment of maximal cell concentration DO_{max} values were considered The strain that showed the lowest OD_{max}. was FRI729. However no statistical difference ($p > 0.05$) were present with the group composed of FRI754, FRI779, FRI7100.

On the contrary FRI754 and FRI779 showed the highest values.

2.3.3 Effects glucose and peptone concentrations on the cell growth

Figure 3 shown strains growth at different glucose and nitrogen concentrations. Comparing the growth kinetics curves a strain-specific effect related to the different glucose and peptone was observed. Table 6 presents the regression models for testing

if different concentration of peptone and glucose could affect the cell growth parameters lag phase (λ), specific growth speed (μ_{max}) and ODmax .

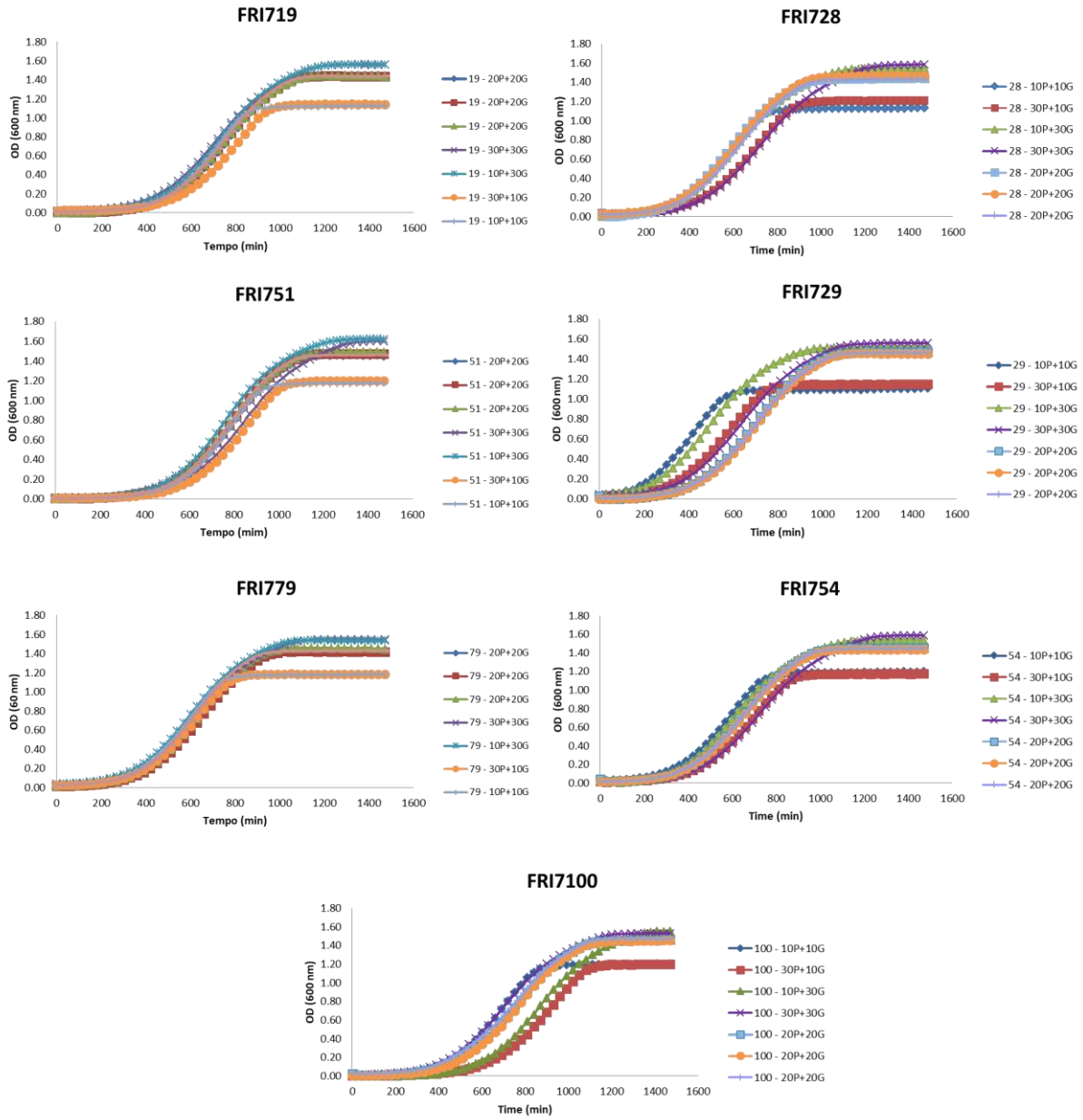

Figure 3: Growth kinetics of the *S. bacillaris* strains at different glucose and peptone concentrations. Conditions: 10P+10G, 10P+30G, 30P+10G, 30P+30G, 20G+20P (G= glucose (g/L), P peptone (g/L)).

For strain FRI719 μ_{max} significant variation was obtained when the concentration of peptone was modified and the lower the concentration of peptone, the greater

growth rate value. The same results were obtained with strain FRI729 that showed significant differences when glucose concentration is modified, as well. The results obtained with FRI728, FRI751, FRI754, and FRI7100 demonstrated that the best growth parameters were obtained when the concentration of glucose and peptone were identical and at the lowest values.

The strains FRI719, FRI751, FRI779 and FRI7100 showed higher values of DO_{max}. regardless the amount of peptone present in the medium.

The lag phase (λ) of the strains FRI728 and FRI754 significantly varied in function of the peptone concentration. The lowest λ values were obtained when the lowest peptone concentration was present in the medium. The strain FRI751 seems to prefer to a higher of glucose concentrations (table 7).

Table 7: Regression Models of the effect the concentration of glucose and peptone on the growth kinetics of *S. bacillaris*

Strain	Parameters	Regression model or Mean ± SD	R^2	P(F)
FRI719	μ_{max} (h^{-1})	0.0168 + 0.006*P – 0.006*P²	0.9139	0.0003*
	OD_{max} (600nm)	1.264 + 0.430*G²	0.8338	<0.0001*
	λ (h)	78 ± 6.36	---	0.0073*
FRI728	μ_{max} (h^{-1})	0.016 + 0.008*G – 0.007*G² - 0.001*P	0.9999	<0.0001*
	OD_{max} (600nm)	0.663 + 1.679*G - 0.732*G² + 0.087*P	0.9980	<0.0001*
	λ (h)	64.26 – 51.9*P + 58.2*P²	0.9987	<0.0001*
FRI729	μ_{max} (h^{-1})	0.016 + 0.008*P – 0.006*P² - 0.0006*P*G	0.9787	<0.0001*
	OD_{max} (600nm)	0.603 + 2.150*G – 1.175*G² + 0.054*P	0.9999	<0.0001*
	λ (h)	53.82 ± 18.3	---	<0.0001*
FRI751	μ_{max} (h^{-1})	0,019 + 0.004*G – 0.004*G² - 0.002*P	0.9923	<0.0001*
	OD_{max} (600nm)	0.688 + 1.964*G – 0.972*G²	0.9836	<0.0001*
	λ (h)	96 – 55.14*G + 35.16*G² + 16.74*P	0.9502	<0.0001*
FRI754	μ_{max} (h^{-1})	0,017 + 0.007*G – 0.0006*G² - 0.002*P	0.9985	<0.0001*
	OD_{max} (600nm)	0.7833 + 1.4813*G – 0.6550*G² + 0.0780*G*P	0.9995	<0.0001*
	λ (h)	47.7 + 23.88*P	0.9839	<0.0001*
FRI779	μ_{max} (h^{-1})	0,019 + 0.005 *G - 0.005*G² - 0.001*P	0.9911	<0.0001*
	OD_{max} (600nm)	0.823 + 1.350*G – 0.555*G²	0.9943	<0.0001*
	λ (h)	39.24 + 68.04*G – 55.2*G² + 4.2*P	0.9961	<0.0001*
FRI7100	μ_{max} (h^{-1})	0,017 ± 0.001	---	0.0003*
	OD_{max} (600nm)	1.2584 + 0.4307*G²	0.8880	<0.0001*
	λ (h)	81.9 ±12	---	<0.0001*

Kinetic parameters μ_{max}. λ (lag phase) and OD_{max} 600nm. obtained by 600 nm absorbance. * Significant at the 5% probability, P = peptone. G = glucose and SD = standard deviation.

In the case of FRI719, FRI729 and FRI7100 parameters it was not possible to fit a regression model for the lag phase (λ) and the specifics growth rate (μ_{max}), therefore we analyzed the behavior trends according to the tested (Figure 4).

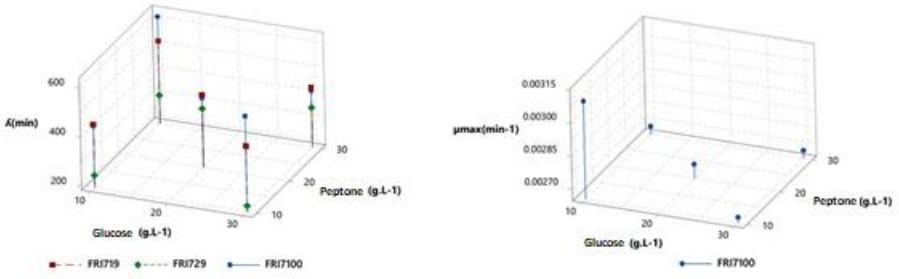

Figure 4 - Tendency of the kinetic parameters lag phase (λ) and μ_{max} for the strains FRI719, FRI729 and FRI7100

The graphics allow to see clearly the negative effect caused by peptone high concentration on the strain FRI7100 rate of growth. Interestingly, the lag phase of the strains FRI719 and FRI729 was not influenced by glucose concentration, but showed high values when high peptone concentration was present.

Some studies report the influence of different nitrogen sources on cell growth during brewing production (Albers et al.. 1996). The peptone, despite it is composed of relatively long amino-acid chains, in *S. cerevisiae* increase fermentation performances. Comparing the consumption of isolated amino acids generally yeasts tended to use compounds with less structural complexity (Cruz et al., 2003).

2.3.4 Fermentation performances at different glucose and malic acid concentrations

In order to understand how the level of sugar influences the fermentation of unripe grape musts all the 14 *S. bacillaris* strains collected from dried grape must were tested in two synthetic must conditions: musts with usual sugar and malic acid concentrations (200 g/L and 2 g/L, respectively) mimicking standard fermentation condition and low sugar musts containing low sugar and high malic acid concentration (80 g/L and 12 g/L, respectively) mimicking unripen grape juice.

The CO_2 production was followed during all the fermentation process. To assess strain fermentation performances the fermentation vigor in terms of CO_2 production after 48h of incubation was considered in order to evaluate the adaptation ability to the must conditions. CO_2 production after 312h was considered as at these fermentations step the widest range of CO_2 production was found between strains. As regards the single fermentation of *S. bacillaris* strains in 200g/L (table 8) Strains FRI719. FRI728. FRI729. FRI751 and FRI754 showed high fermentation vigor (CO_2 production after 48 h).

On the contrary after 321 and 624 hours (when the CO_2 production was very low, indicating the fermentations were competed) only small differences were detected. As expected glucose residue (from 80.28 to 100.44) was higher than fructose residue (from 18.37 to 1.94).

At high glucose concentration, the fermentation was not completed after 26 days. Residue sugar (from 116.85 to 85.02) can be related to a limited ethanol production (from 4.04 to 6.53% v/v) and acetic acid production (from 0.86 to 0.429). Glycerol production was very high (from 4.92 to 7.69 g/L) and acetic acid production showed high differences between strains. These results are in agreement with Englezos et al. (2015) and Lemos Junior et al. (2016).

Table 8. CO_2 production during fermentation. Residual glucose and fructose concentrations and concentrations of the main fermentation products at the end of the fermentation of *S. bacillaris* strains in must with high sugar content (200 g/l). Data are expressed as the average of three replicates ± standard deviations. Different letters indicate significant differences between values (Fisher's test. p=0.05).

Strain	CO2/100mL			Glucose (g/L)	Fructose (g/L)	Residual sugar (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Ethanol (%v/v)
	48 h	336h	624h						
FRI719	0.03±0.00D	2.24±0.40D	4.09±0.56A	91.84±0.53D	6.91±0.07E	98.75±0.60C	7.69±0.04A	0.80±0.00BAC	6.53±0.17A
FRI728	0.02±0.00D	2.42±0.16DC	4.21±0.34A	92.17±0.48D	13.64±0.16B	105.82±0.64B	7.08±0.01BA	0.82±0.00BA	6.07±0.23BA
FRI729	0.09±0.01C	2.33±0.23D	3.99±0.46A	98.52±3.11BC	13.05±0.31B	111.57±3.41BA	7.56±0.28A	0.86±0.03A	6.05±1.44BA
FRI751	0.08±0.00C	2.70±0.17BDC	4.41±0.21A	98.47±1.92BC	18.37±0.24A	116.85±2.14A	6.36±0.15BC	0.73±0.00BC	5.99±1.12BA
FRI754	0.08±0.01C	3.10±0.05BA	4.39±0.02A	94.91±3.02DC	11.92±0.38C	106.83±3.35B	7.46±0.24A	0.84±0.03A	6.02±1.55BA
FRI779	0.32±0.01BA	3.31±0.25A	4.44±0.10A	90.26±2.20D	1.94±0.02H	92.21±2.22C	7.29±0.17A	0.71±0.01C	6.00±1.02BA
FRI7100	0.31±0.05BA	3.33±0.23A	4.38±0.40A	80.28±6.804E	4.74±0.29F	85.02±7.14D	7.58±0.62A	0.74±0.06BC	5.74±3.58B
PAS13	0.38±0.05A	2.97±0.11ABC	4.46±0.11A	91.81±6.44D	2.97±1.12G	94.79±7.35C	4.93±0.79E	0.51±0.08ED	4.02±4.63D
PAS55	0.25±0.02BA	2.64±0.55BDC	4.05±0.76A	100.44±0.11A	8.34±0.38D	113.78±1.97A	6.00±0.34DC	0.58±0.04D	5.92±2.72BA
PAS66	0.26±0.07BA	2.78±0.49ABDC	4.22±0.56A	99.96±2.32AB	11.28±0.19C	113.64±2.18A	4.95±0.24E	0.42±0.05E	4.69±1.37C
PAS92	0.28±0.07BA	2.62±0.19BDC	4.09±0.19A	99.86±2.67AB	8.21±0.18D	110.48±2.84BA	5.26±0.27DE	0.47±0.02E	4.79±1.96C
PAS103	0.21±0.01B	2.72±0.61BDC	3.99±0.68A	99.37±3.41ABC	6.94±0.83E	106.32±4.19B	4.92±0.67E	0.45±0.06E	4.75±4.93C
PAS151	0.33±0.04BA	2.94±0.27BAC	4.27±0.16A	99.82±1.79ABC	8.86±1.21D	110.69±2.48BA	4.98±0.83E	0.42±0.11E	4.51±6.10C
PAS173	0.30 ± 0.06^{BA}	2.93 ± 0.06^{BA}	4.23 ± 0.06^{A}	99.08 ± 6.0511^{ABC}	5.08 ± 0.48^{F}	106.16 ± 6.49^{B}	5.19±0.62E	0.47±0.07E	4.79±4.12C

In synthetic must mimicking unripen grape juice (80 g/L sugar and 12 g/L malic acid) fermentations were performed adding *S. bacillaris* strain to evaluated the response to low osmotic stress (table 9). In general the strains of *S. bacillaris* showed low fermentation

vigors (CO_2 production after 48 h) if compared with *S. cerevisie* with the exception FRI719 FRI728 and FRI751 that showed higher values. After at 312 and 624 hours no significant differences were found among the strains.

For all the 14 strains very low limited sugar residues were found (from 0.38 to 0.56 g/L). The average glycerol concentration produced by yeasts was 4.33 g/L whereas PAS103 produced 5.07 g/L. Acetic acid concentration ranged from 0.56 to 0.38. Ethanol concentration ranged between 3.29 and 4.7% (v/v). At low glucose concentration yeasts generally improved the fermentation performances and very small differences in fermentation rate were observed. At high malic acid concentration, all *S. bacillaris* strains consuming higher quantity of sugar than in 200-2 condition, although *S. bacillaris* strains fermented slower than *S. cerevisiae* strains as discribed by Bovo et al. (2016). Our results suggest that the fermentative ability of the tested *S. bacillaris* strains is not sufficient to transform all sugar must, but it could be enough to complete the fermentation of must with low sugar content.

Table 9. CO_2 production during fermentation. residual glucose concentrations and concentrations of the main fermentation products at the end of the fermentation of *S. bacillaris* strains Delfine (80-12). Data are expressed as the average of three replicates ± standard deviations. Different letters indicate significant differences between values (Fisher's test. p=0.05).

Strain	CO_2/100mL			Glucose (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Ethanol (%v/v)
	48 h	336h	624h				
FRI719	0.03±0.00ED	2.32±0.17BAC	4.40±0.64BDAC	0.50±0.09BAC	3.99±4.92BAC	17.59±1.91DC	4.40±0.64BDAC
FRI728	0.02±0.00E	2.01±0.32E	3.53±0.90D	0.46±0.12BAC	3.33±6.52C	24.67±5.52A	3.53±0.90D
FRI729	0.12±0.02D	2.21±0.13EBDAC	4.43±0.50BDAC	0.55±0.06A	3.89±3.52BAC	23.40±2.19BA	4.43±0.50BDAC
FRI751	0.07±0.04ED	2.06±0.09ED	3.64±0.38DC	0.48±0.02BAC	3.93±1.91BAC	21.98±1.29BAC	3.64±0.38DC
FRI754	0.11±0.01ED	2.42±0.00A	4.45±0.074BDAC	0.52±0.03BA	4.06±0.90BAC	20.99±0.27BAC	4.45±0.074BDAC
FRI779	0.34±0.06BAC	2.09±0.04EDC	3.98±0.47BDC	0.38±0.05C	3.29±3.35C	14.44±0.84EDF	3.98±0.47BDC
FRI7100	0.35±0.07BA	2.27±0.10BDAC	4.82±0.21BA	0.46±0.019BAC	3.95±1.19BAC	10.60±0.36F	4.82±0.21BA
PAS13	0.40±0.07A	2.21±0.11EBDAC	4.65±0.18BAC	0.46±0.03BAC	3.71±1.52BC	14.65±0.45EDF	4.65±0.18BAC
PAS55	0.37±0.10A	2.32±0.15BAC	4.23±1.32BDAC	0.42±0.14BC	3.60±8.30BC	17.22±4.87EDC	4.23±1.32BDAC
PAS66	0.26±0.03BC	2.39±0.07A	4.14±0.07BDAC	0.44±0.02BAC	4.09±0.78BAC	14.30±0.17EDF	4.14±0.07BDAC
PAS92	0.27±0.06BC	2.34±0.20BA	4.25±1.11BDAC	0.43±0.12BC	4.34±7.76BA	18.33±8.89BDC	4.25±1.11BDAC
PAS103	0.27±0.05BC	2.28±0.13BDAC	5.07±0.06A	0.56±0.02A	4.76±0.00A	12.63±4.31EDF	5.07±0.06A
PAS151	0.25±0.02C	2.13±0.03EBDC	4.21±0.64BDAC	0.46±0.03BAC	3.75±3.36BC	14.07±0.92EDF	4.21±0.64BDAC
PAS173	0.32±0.07BAC	2.25±0.11BDAC	4.87±0.66BA	0.52±0.02BA	4.02±6.57BAC	11.83±1.01EF	4.87±0.66BA

In order to evaluate glycerol content produced by *S. bacillaris* showing values that can really change the characteristic of the wine, twenty *S. cerevisae* strains were grown in the same synthetic low sugar must and in standard condition. In this way it is possible to confirm if glycerol content produced by this non-*Saccharomyces* species is generally higher of that produced by the conventional one.

All *S. cerevisiae* strains completed the fermentation in two conditions used. As expected *S. bacillaris* single-strain fermentation showed higher glycerol production than *S. cerevisiae* strains. Moreover, this fermentation trial confirmed the fructofilic character, and a fermentation rate slower than that of *S. cerevisiae* (Magyar and Tóth. 2011; Englezos et al. 2015). Overall, the amount of glycerol produced by *S. bacillaris* and *S. cerevisiae* was

significantly different at the two conditions (200 g/L sugar p<.0001) and (80 g/L sugar p<.0001) as demonstrated by Mann-Whitney-U test.

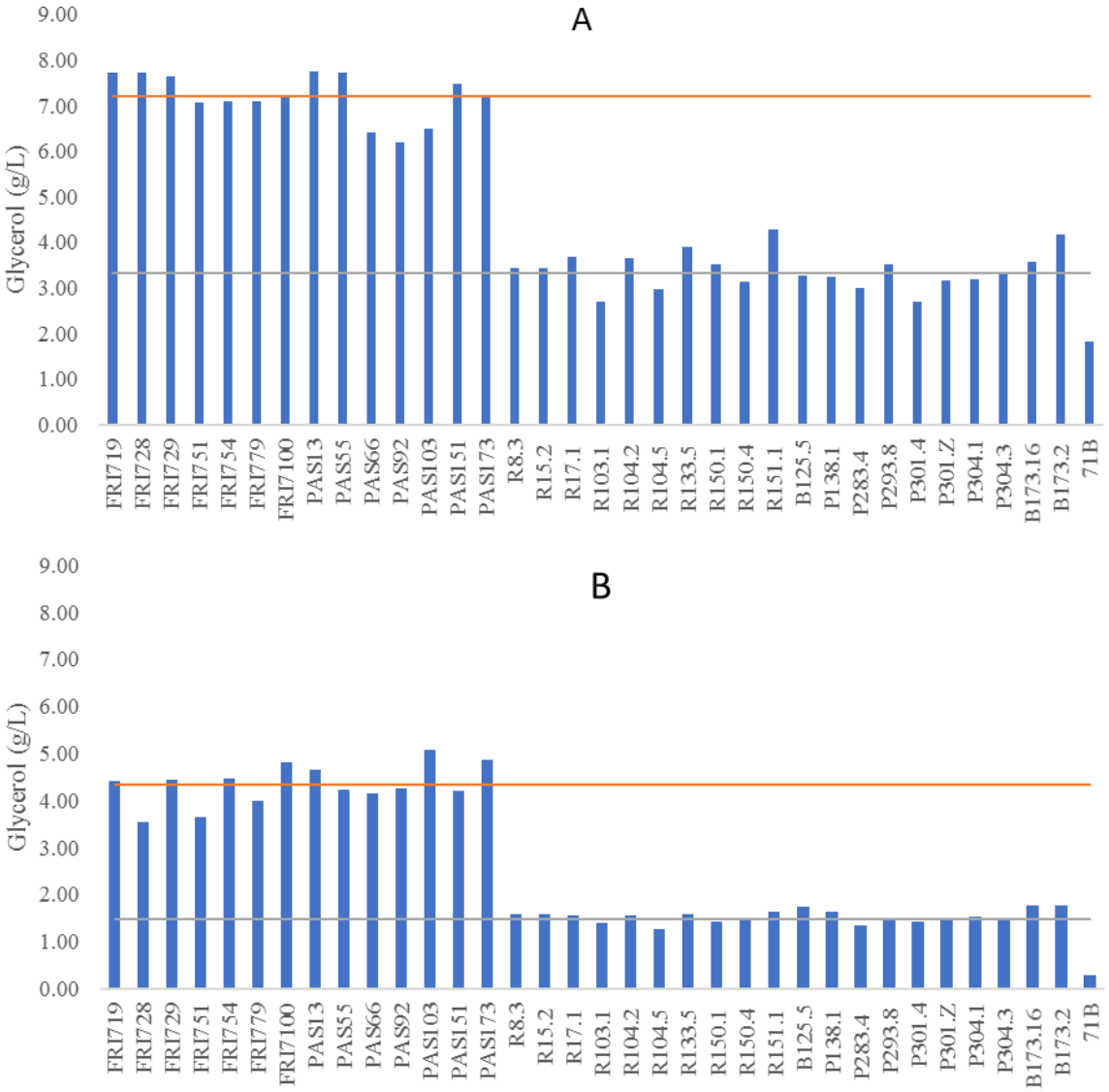

Figure 5 Glycerol production from *S. bacillaris* and *S. cerevisiae* strains (A). 200-2 condition (B). 80-12 condition. (--) mean glycerol production of *S. bacillaris* strains. (--) mean glycerol production of *S. cerevisae* strains.

The *S. bacillaris* occurs in various regions and usually is isolated on the surface of dried grape. Therefore it presents characteristics closely related to such environment: to be

highly fructophilic (high affinity for fructose), and high levels of glucose tolerance. Moreover acetic acid low production and low ethanol tolercance was detected when compared to *S. cerevisaie*. The results confirm the osmotolerant profile and the fructose affinity (Sipiczki. 2003; Tofalo et al. 2012; Rantsiou et al. 2012; Quiròz et al. 2014; Englezos et al. 2015).

Moreover the results demonstrate the importance of using non-conventional yeasts in grape must fermentation where high concentration of sugar (glucose and fructose) are present since non-*Saccharoyces* yeast can consume part of the sugar reducing the osmotic stress suffered by *S. cerevisiae* (that is involved in high acetic acid production by *S. cerevisae*).

2.4 Conclusion

This work presented an evaluation of factors intimately affecting metabolic pathways of *Starmerella bacillaris*. As preliminary work the strains identification of *S. bacillaris* isolates was performed by SAU-PCR. This technique showed to be suitable to genetically differentiate the yeast isolates. The growth of *S. bacillaris* in the presence of high concentrations of glucose offers a great opportunity to verify if this species could be used as co-inoculant with *S. cerevisiae* in harsh winemaking condition such as high sugar must. The positive results obtained confirmed this hypothesis. Nitrogen is confirmed to be limiting factor in *S. bacillaris* fermentation, as well. In fact it directly interfered with the yeast growth, as growth rate is influenced by Nitrogen concentration.

The strain FRI779 showed the best aptitude for must fermentation followed by FRI754. The results indicate those strains as the best candidate to be used in winemaking. These results were confirmed in synthetic grape must.

Moreover, the condition reproducing unripe grape must were tested and compared to standard one. In this condition *S. bacillaris* enhanced its ability to degrading sugars, although no effect was evident on malic acid degradation. The increase in sugar consumption was associated to a very high glycerol production that contributes to the “body” of wine. These results are certainly promising although wine physical-chemical treatments will be still required to reduce wine acidity. Moreover, the use of *S. bacillaris* can be recommended as sequential fermentation with *Saccharomyces cerevisae* starter culture to improve high glycerol wines from unripe and mature grape.

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CHAPTER 3

Biocontrol ability and action mechanism of *Starmerella bacillaris* (synonym *Candida zemplinina*) isolated from wine musts against grey mold disease agent *Botrytis cinerea* on grape and their effects on alcoholic fermentation

3.1 Introduction

Botrytis cinerea is one of the most important fungal plant pathogen that causes serious grey mold disease in more than 200 economically relevant plant species during preharvest (especially when plants are grown under protection) and postharvest (Agrios, 2005). Grapes, vegetables, berries and stone fruits cultivated worldwide are the most susceptible to this fungal disease (Rosslenbroich & Stuebler, 2000).

The fungal agent infects leaves, stems, flowers and fruits of plants, either by direct penetration or through wounds caused by cultivation practices. This fungus kills host cells through the production of cell wall degrading enzymes, phytotoxic metabolites and reactive oxygen species accumulating after the induction of a plant-produced oxidative burst. Thanks to degrading enzymes, *B. cinerea* succeeds in the decomposition and consumption of different plant tissues (van Kan, 2006). Infestation is stimulated by high humidity, particularly if free moisture is present on the plant surface and low temperatures (Williamson et al., 2007).

Generally the control of the disease is achieved by the use of synthetic fungicides (Elad & Evensen, 1995). From the middle of the 1990s, fungicides with excellent activity against *B. cinerea* came to the market and more recently the control of the disease was mainly achieved by integrating several cultural methods with the use of these fungicides (Rosslenbroich & Stuebler, 2000). Although synthetic fungicides are effective, their

continued or repeated application has disrupted biological control by natural enemies of the fungus and stimulated the development of resistant pathogen populations, leading to widespread outbreaks of the disease (Elad et al., 1992).

The increasing concern over the adverse agronomical and environmental effects of synthetic fungicides brought to search new types of crop protection methods without or with reduced use of conventional fungicides. The salts of weak acids, such as sodium benzoate and potassium sorbate, can inhibit growth of several postharvest fungal pathogens. These compounds present several benefits as they possess low toxicity towards mammalians, a wide spectrum of activity and are relatively cheap.

However, these compounds need to be used at concentrations that can determine potential organoleptic changes of the products. For example, calcium propionate completely inhibited mycelial growth of *B. cinerea* at a concentration of 5% (w/v) (Droby et al., 2002). Essential oils obtained from aromatic and medicinal plant species have been proposed as new classes of possibly disease control agents, since they are a rich source of bioactive chemicals. These chemicals are often active against a limited number of species, are biodegradable to nontoxic products and are potentially suitable for integrated use (da Cruz Cabral et al., 2013).

Specific activity against *B. cinerea* was found in essential oils obtained from the aerial parts of aromatic plants, which belong to the *Lamiacea* family, such as origanum (*Origanum syriacum L*. var. *bevanii*), lavender (*Lavandula stoechas L*. var. *stoechas*) and rosemary (*Rosmarinus officinalis* L.) (Soylu et al., 2010). Traditional medical plants from Africa and Asia were found to be a source of essential oils proposed for postharvest control of gray mold (Tripathi et al., 2008).

Among the alternatives to synthetic fungicides, the use of plant resistance inducers demonstrated the potential for large-scale application. The induced resistance can be defined as an increased expression of natural defense mechanisms of plants against different pathogens provoked by external factors of various type: elicitors of pathogenic origin (glucans, proteins, lipids, etc.); abiotic elicitors, including synthetic harmless chemical products (Edreva, 2004). Some molecules, that act as inducers, also present antimicrobial activity. That is the case of chitosan that decreases gray mold and other fungal deseases through the reduction of mycelial growth and spore germination and induction of morphological alterations in the fungal pathogen. Moreover, chitosan acts as a potent elicitor to enhance plant resistance (Amborabé et al., 2008; El Hadrami et al., 2010).

An alternative strategy to reduce grey mold disease is based on the selection and application of biocontrol agents. Among the microorganisms used as biocontrol agents, yeasts have been targeted by many surveys as potential mold antagonists, focusing mainly on postharvest diseases, since they are naturally occurring on fruits and vegetables, and have shown great ability to colonize wound sites (Bai et al., 2008). Some have been present on the market for a long time and showed specific activity against *B. cinerea*. *Candida oleophila*, the base of the commercial product “Aspire”, is recommended for the control of postharvest decay in citrus and pome fruits. Its modes of action include nutrient competition, site exclusion, and direct mycoparasitism (Droby et al., 2002).

The yeast *Cryptococcus albidus,* included in the commercial product “Yield Plus”, is an antagonist isolated from peach fruit and effective against the pathogen *B.cinerea* in apple (Fan and Tian, 2001). As regards other yeast species the investigations as biocontrol agents are still ongoing. Recently, the ascosporic yeast *Metschnikowia fructicola* AL27 was tested

on several apple varieties and found to be as competitive as the chemical fungicides used as control (Spadaro et al., 2013).

Focusing on viticulture, gray mold is one of the most important diseases of grapevine in temperate climates worldwide and can cause extensive economic losses through grape desiccation, rot and biochemical changes that reduce wine quality.

Biological control of *B. cinerea* is a successful strategy that has been introduced as an alternative to synthetic fungicides in grapevine cultivation. Filamentous fungi from the genera *Trichoderma*, *Ulocladium* and *Gliocladium*, bacteria from the genera *Bacillus* and *Pseudomonas* and, lately, yeasts from the genera *Pichia* and *Candida* have been used as biocontrol agents (Jacometti et al., 2010).Recently, an integrated approach that combined low dosage of fungicides and antifungal yeasts has been tested in order to reduce chemicals concentration and enhance biocontrol efficacy. *Hanseniaspora uvarum* was tested under laboratory conditions in combined treatment with NH_4–Mo, showing inhibitory effects on spore germination and mycelial growth of *B. cinerea* in vitro and induced defense reactions in grape berries (Liu et al., 2010).

Although several yeasts with antifungal property have been successfully identified, yeast selection to find out new biocontrol agents remains challenging and species – to - species interaction studies are of great interest to understand native and introduced fungal population dynamics in both vineyard and cellar. Indeed, after grape harvest, antifungal yeasts become part of the must microbiota and, if well adapted to must condition, they could have a role during the fermentation process and therefore directly influence wine quality. At the moment, no information are available about the fate of selected yeasts proposed as biocontrol agents during must fermentation and winemaking, although they can be found on the grape surface at high level due to repeated treatments. Moreover, the possibility to select

yeasts that are simultaneously antifungal agents in vineyards and wine starters for grape must fermentation is completely unexplored.

Non-*Saccharomyces* yeasts are a group of wine-related yeast species once defined spoilage microorganism. Generally, they are well adapted to vineyard condition and are predominant in grape musts during the early stages of fermentation. Recently, there has been a re-evaluation of the role of these yeasts, as some of them were found to enhance the analytical composition and aroma profile of the wine (Ciani & Comitini, 2015).

Starmerella bacillaris (synonym *Candida zemplinina*) is a non-*Saccharomyces* yeast, commonly found on grapes and particularly associated with botrytized grapes and wines fermented from these grapes (Csoma and Sipiczki, 2008; Duarte et al., 2012; Magyar and Tóth, 2011; Masneuf-pomarede et al., 2015; Wang et al., 2015). Magyar and Tóth (2011) investigated the technological properties of *C. zemplinina* strains evidencing an extremely poor ethanol yield from sugar consumption, high glycerol and moderate volatile acids production. High glycerol production contributes to palate fullness (“body”) of wine, whereas high acetic acid content confers an unpleasant vinegar aroma.

Therefore, with the aim of improving wine quality, *S. bacillaris* was recently tested, together with *Saccharomyces cerevisiae*, in sequential and mixed yeast inoculations during grape must fermentation to balance the glucophilic character of the *Saccharomyces* species, to increase glycerol concentration in wine and, due to the low ethanol yield, to reduce ethanol content (Ciani et al., 2015; Wang et al. 2014; Bely et al., 2013; Rantsiou et al., 2012). With the aim to investigate the double role of *S. bacillaris* as both potential biocontrol agent and unconventional enological starter, 14 strains belonging to this species were studied in this work to evaluate their antifungal activity against *B. cinerea,* both *in vitro* and *in vivo*. Moreover, the technological properties of these non-*Saccharomyces* strains were evidenced

at lab-scale, both in single-strain fermentation and in sequential fermentation together with *Saccharomyces cerevisiae*.

3.2 Materials and methods

3.2.1 Isolation and characterization of yeast isolates

The yeast strains used in this work were isolated from fermenting musts obtained from dried grape of Raboso piave variety. They were collected during two harvests in two wineries located in the winemaking area of Appellation of Origin Bagnoli (North-East of Italy) where the production of Friularo Bagnoli Passito wine is performed. A total of 360 yeast colonies were isolated on WL agar medium (Oxoid) plates. All yeasts were identified at species level by PCR-RFLP analysis of the ITS1-5.8S-ITS2 rDNA region and D1/D2 region sequence analyses as described by Bovo et al. (2011). A BLAST search on sequence results gave the most probable species identification. Thirty-six isolates identified as *S. bacillaris* were characterized at molecular level using SAU-PCR method, as described by Corich et al. (2005).SAU-PCR amplification patterns were analysed using the software GelComparII V. 3.5 (AppliedMaths).

3.2.2 Extracellular lytic enzymes activity

S. bacillaris strains were screened for the production of extracellular cellulase, xylanase, lipase, pectinase and proteinase using plate tests as described by Favaro et al. 2013. The presence of extracellular chitinolytic activity was tested on glycol chitin agar medium (yeast nitrogen base, 6.7 g/L, glycol chitin, 5 g/L, agar 16 g/L). After the growth of the yeast colonies a solution containing 500 mMTris-HCl pH 8.9 with 0.01% w / v of Calcofluor white MR2 was poured on the plates. The plates were incubated for 10 minutes.

Subsequently, the solution was discarded and replaced with water overnight. The presence of chitinolytic activity was evidenced by the observation of dark lytic plaques, where the colonies were present, on a light background under UV exposure. Extracellular β-glucosidase activity was evaluated using the esculin (esculetin 6-O-glucoside) agar hydrolysis test described by Njokweni et al. (2012) on Esculin agar (esculin 1 g/L, YNB 1.7 g/L , 0.5 g/L ferric citrate, agar 16g/L) plates. Extracellular β-glucosidase activity were also tested by evaluating yeast growth on Cellobiose agar (cellobiose 5 g/L, YNB 6.7 g/L, agar 16g/L) plates after incubation at 30 °C for 72 h.

3.2.3 In vitro antagonistic activity

The antagonistic activity on agar plates and volatile organic compounds (VOCs) assay was performed as described by Parafati et al. (2015) and modified as follows. The *B. cinerea* strain used was BC0510.

3.2.3.1 Antagonistic activity on agar plates

The yeast and mold strains to be tested were respectively growth on YPD for 24 hours and on PDA for 5 days at 25 °C. Each yeast strain was streaked orthogonally from the center of a plate, containing PDA (Potato Dextrose Agar) medium at two different pH (5.5 and 3.5). Simultaneously, for each plate 2 mycelial discs (6 mm square plug) of *B. cinerea* were placed on agar plates 3 cm away from the yeast streak. A control plate was prepared inoculating only *B.cinerea*. At the end of the incubation period (5 days at 25 °C) the radial growth reduction was calculated in relation to the growth of the control as follows: %I=(C-T/C)*100, where %I represented the inhibition of the radial mycelial growth, C was the radial growth

measurement in control and T was the radial growth of the pathogen in the presence of yeast strains. The assay was performed using four replicates for each yeast strain and pH.

3.2.3.2 Effects of volatile organic compounds (VOCs)

A dual culture method was used to evaluate the efficacy of volatile compounds produced by yeasts against *B. cinerea.* Aliquots of 20 µL of yeast suspensions (10^7cells/mL) were seeded on plates with PDA at two pH values, 5.5 and 3.5, and incubated 4 days at 25 ºC. Aliquots (10 µl) of the conidial suspension of *B. cinerea* (10^6 conidia/mL) were inoculated on PDA and dried at room temperature. The plates with *B. cinerea* conidia were individually covered face to face under the plates containing the yeast strains. The controls were prepared facing the plates containing *B. cinerea* suspension with un-seeded PDA plates. Each plate pair was wrapped with two layers of Parafilm around the edges to prevent air leakage, and incubated a 25 ºC. The radial growth reduction of *B. cinerea* was calculated after 5 days of incubation as previously described.

3.2.3.3 In vivo antagonistic activity

In order to assess the efficiency of yeasts as biocontrol agents, the method described by Parafati et al. (2015), with slight modifications, was used. Table grape fruits derived from orchards located in Padova, Italy. Healthy and homogeneous grape berries were selected, washed and surface-disinfected. Artificial wounds were performed and inoculated with 10 µL drop of 10^6 conidia/mL of *B. cinerea*. After air drying (2 h), a 10µL drop of 10^8 cells/mL of yeast were added to each wound. The same amount of 0.09% NaCl buffer (20 µL) was used in the control. For each strain 10 grape berries were used. The grape berries were placed on plastic packaging trays. To create a humid environment, a wet paper was placed on cavity trays coated with a plastic bag. The trays were incubated at 25 ºC and 95% relative

humidity (RH) for 5 days after inoculation to provide favorable conditions for the disease development. The disease severity (DS) was evaluated by using an empirical 1-to-4 rating scale evaluating both soft rot size and mycelium growth: + barely visible symptoms, ++ small, +++ intermediate, ++++ large (comparable to control).

Data concerning the disease reduction incidence (DRI) was calculated as follow (DRI)=(C-T/C)*100, where C was the average radial growth measurement in control (10 berries) and T was the radial growth of the pathogen in the presence of yeast strain in each berry.

The lesion diameter (LD) was evaluated by measuring the average diameter of the damaged area 5 days after *Botrytis* inoculation. Each yeast strain was tested on ten berries.

3. 2.4 Fermentation trials in synthetic and natural must

3.2.4.1 Inoculum preparation

A loopful of a 3-days-old culture of each yeast strains from YPD agar plate (yeast extract 10 g/L, peptone 10 g/L, dextrose 20 g/L) was used to inoculate 10 mL of YPD broth in 50 ml tubes. A stationary phase culture with approximately 10^7-10^8 cells/mL, determined by OD measurements and confirmed by means of plate counts analysis (CFU/ml), was obtained after 24 hours of incubation at 30 °C. In single-strain fermentation the inoculum concentration was 2×10^6 cells/ml. In sequential fermentation the same inoculum size for both *S. bacillaris* strain and *S. cerevisiae* EC1118 ($1\text{-}1{,}5\times10^6$ cells/ml) was used. EC1118 was added 48h after the inoculum of *S. bacillaris*.

3.2.4.2 Fermentation conditions

Fermentations were run in synthetic and natural musts. The synthetic must MS300 was prepared as described by Bely et al. (1990) with the addition of 100 g/L of glucose, 100 g/L of fructose and 6 g/L of malic acid, pH 3.2. Incrocio Manzoni grape must, containing 160 g/L of reducing sugars (pH 3.5) was used. In the fermentation trials 120-ml capacity bottles fitted with closures that enabled the carbon dioxide to escape and containing 100mL of must were used. After yeast inoculation the bottles were incubated at 20 °C. The fermentation process was followed by measuring twice a day the weight loss of each culture. When the weight loss was lower than 0.05 g per day the fermentations were considered concluded. All the fermentation trials were performed in triplicate.

3.2.5. HPLC analysis

Ethanol, glycerol, fructose and glucose concentrations were quantified with HPLC (Shimadzu, Japan) equipped with a refractive index detector, set at 600nm wavelength, while for the acetic acid quantification a UV detector was used.

The concentrations, expressed as g/L, were calculated by using calibration curves of the individual compounds. The chromatographic conditions were realized with the ROA-Organic Acid H+ column (Phenomenex, USA), which was run at 65°C with 5mM H_2SO_4 as the mobile phase, with a flow rate of 0.5mL/min.

3.2.6 Statistical analysis

The statistical data analysis was performed with XLSTAT software, vers.7.5.2 (Addinsoft, Paris, France) using the principal component analysis (PCA) and the one-way

analysis of variance (ANOVA) at 95 % accuracy level. Fisher's test was used as comparison test when samples were significantly different after ANOVA ($p < 0.05$).

3.3 Results

3.3.1 Isolation, identification and evaluation of extracellular activity of yeast isolates

A total of 360 yeast colonies were isolated from fermenting musts for the production of Friularopassito wine. By means of PCR-RFLP analysis of the ITS1-5.8S-ITS2 rDNA region and D1/D2 rDNAregion sequencing (Kurtzman and Robnett, 1998) each isolate was identified at species level. A total of 36 isolates were identified as *Starmerella bacillaris.* The characterization at strain level, by means of Sau-PCR (Corich et al., 2005) and the cluster analysis of the amplification pattern, allowed the selection of fourteen different strains listed in Table 1. All the strains were tested for the production of extra-cellular enzymes using specific plate assays.

Table 1. Yeast strains used in this work.

Strain	Species	Origin
FRI719	*S. bacillaris*	Winery A
FRI728	*S. bacillaris*	Winery A
FRI729	*S. bacillaris*	Winery A
FRI751	*S. bacillaris*	Winery A
FRI754	*S. bacillaris*	Winery A
FRI779	*S. bacillaris*	Winery A
FRI7100	*S. bacillaris*	Winery A
PAS13	*S. bacillaris*	Winery B
PAS55	*S. bacillaris*	Winery B
PAS66	*S. bacillaris*	Winery B
PAS92	*S. bacillaris*	Winery B
PAS103	*S. bacillaris*	Winery B
PAS151	*S. bacillaris*	Winery B
PAS173	*S. bacillaris*	Winery B
EC1118	*S. cerevisiae*	Industrial strain

Some of the activities are of industrial interest: beta-glucosidase, cellulase, lipase and xylanase. Pectinase, protease, chitinase are involved in degrading mold cell wall. The results of the screening are reported in Supplementary material Table 2. All the strains showed chitinase activity, although at low level. Only strains FRI719 and FRI751 produced proteolytic enzymes as they were able to grow on skin milk. None of the other activities tested was found in any strain.

Table 2. Extracellular enzymatic activities of the 14 *S. bacillaris* strains isolated from Raboso piave musts.

Strain	PrA	CellA	XylA	LipA	*β*-gluA	*β*-gluA	PectA	ChitA
	skinmilk	CMC	Xylan	tributim	cellobiose	esculin	polygalacturonic acid	glycolchitin
FRI719	+	-	-	-	-	-	-	+
FRI728	-	-	-	-	-	-	-	+
FRI729	-	-	-	-	-	-	-	+
FRI751	+	-	-	-	-	-	-	+++
FRI754	-	-	-	-	-	-	-	++
FRI779	-	-	-	-	-	-	-	++
FRI7100	-	-	-	-	-	-	-	+
PAS13	-	-	-	-	-	-	-	+
PAS55	-	-	-	-	-	-	-	+
PAS66	-	-	-	-	-	-	-	+
PAS92	-	-	-	-	-	-	-	+
PAS103	-	-	-	-	-	-	-	++
PAS151	-	-	-	-	-	-	-	++
PAS173	-	-	-	-	-	-	-	++

PrA proteolytic activity, CellA cellulolytic activity, XylA xylan-degrading activity, LipA lipolytic activity, *β*-gluA *β*-glucosidase activity, PectA pectinolytic activity, ChitA chitinolytic activity; + very faint, ++ low, +++ medium, ++++ high

3.3.2 In vitro antagonistic activity

Data from dual culture assays are reported in figure 1. All the *S. bacillaris* strains were able to inhibit the growth of *B. cinerea* mycelium both at pH 5.5 and 3.5 when co-cultivated with *B. cinerea* (Fig.1A). The percentage of the inhibition of the radial mycelial growth ranged from 12 to 33. Strains FRI719, FRI779, PAS13, PAS66 showed higher antagonistic activity at pH 3.5 than at pH 5.5. On the contrary FRI100, PAS92 and PAS173 showed higher antagonistic activity at pH 5.5. In the other cases no significant differences were detected. In these conditions, for all the strains, the inhibition level found was limited, although comparable with that found in literature for some other yeast species (Parafati et al. 2015).

This could be due to the different growth rate of *S. bacillaris* and *B. cinerea* on PDA medium where *S. bacillaris* can not found the optimal growth conditions. As the two microorganisms were inoculated simultaneously, *S. bacillaris* inhibited, only partially, the fungal growth. The inhibition of *B. cinerea* mycelium growth due to the production of volatile compounds by *S. bacillaris* strains was tested, as well (Fig 1B).

To overcome the different growth rate between the two microorganisms, plates inoculated with *S. bacillaris* strains were incubated 4 days at 25 ºC before covering face to face each plate with that containing *B. cinerea*. Generally, results showed notably higher inhibition percentages than those found when *B. cinerea* and each yeast strains were co-cultivated on the same plate: the values ranged from 44 up to 79%. Comparing the results

with those of the previous growth inhibition assay, only PAS13 and FRI100 confirmed their inhibition ability in relation to the pH of the growing medium.

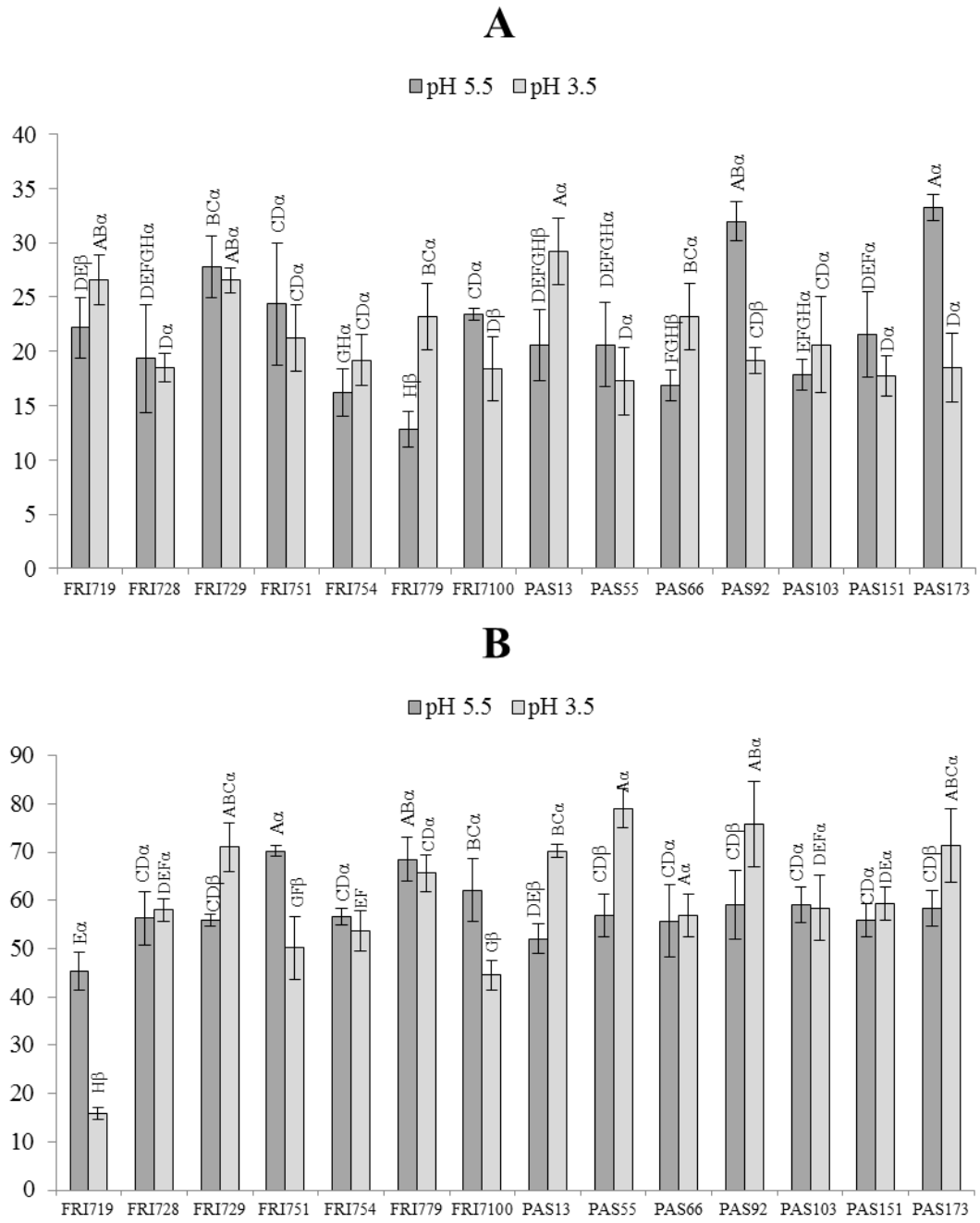

Figure 1. In *vitro* antagonistic activity of *S. bacillaris* strains against *B. cinerea* on PDA plate at pH 5.5 and 3.5. Growth inhibition, measured as inhibition percentage of the radial mycelial growth, induced by yeast cells (A) and by volatile organic compounds (VOCs) (B). Vertical bars indicate the standard error of the mean.

Statistical analysis: one-factor ANOVA (p value < 0.05). At the same pH, mean values followed by the same Roman letter are not significantly different according to Fisher's test (P≤0.05). For each strain mean values obtained at pH 5.5 and 3.5, and followed by the same Greek letter are not significantly different according to Fisher's test (P≤0.05).

3.3.3 In vivo Antagonistic activity

A qualitative evaluation of the efficacy of the tested yeasts in reducing grey mold growth on grape berries is reported in Table 2. Although at different levels, all yeasts decreased the size of decay (soft-rot developed area) and the mycelium growth. Eight strains out of 14 showed remarkable effects on the developing of the *Botrytis* infection. In details (Fig 2), the disease reduction incidence (DRI) values ranged from 39 up to 85%. Strains FRI751, FRI754, PAS173 showed the highest gray mold decay as their values were significantly higher ($p < 0.05$) than those found for the other strains. The lesion diameter (LD) evaluation confirmed the remarkable ability of *S. bacillaris* strains to reduce the infection size, although only small differences were found between strains.When *S. bacillaris* strains were present in the wound the LD was always lower than 1cm, while for the control the LD size was 1.8 cm.

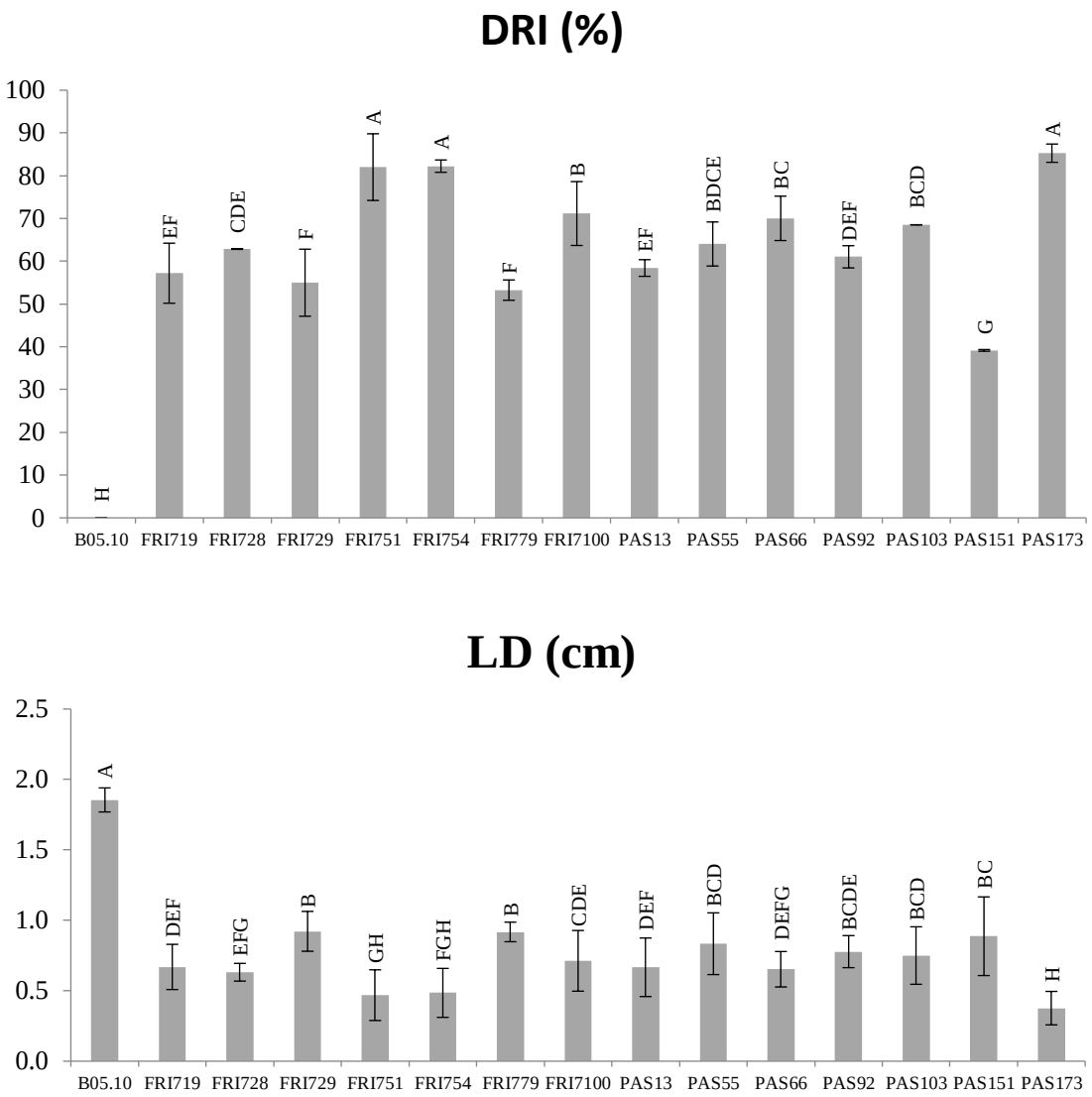

Figure 2. In *vivo* antagonistic activity of *S. bacillaris* yeast strains in inhibiting gray mold decay on grape berries. Effect of yeasts is referred to disease reduction inhibition percentage (DRI%) and lesion diameter (LD) caused by *Botrytis cinerea* 5 days after incubation at 25 ºC. Vertical bars indicate the standard error of the mean. Mean values followed by the same letter are not significantly different according to Fisher's test (P≤0.05).

Strain PAS173 showed the highest level of LD reduction that was significantly

different ($p< 0.05$) from those found for the other strains. At the end of the incubation time (5 days) each grape berry was squeezed and homogenized and *S. bacillaris* concentration was determined by plate counts (Table 3). The yeast concentration was very similar for all the strains ranging from 1.83×10^6 up to 2.35×10^6 CFU/ml.

Table 3. Qualitative evaluation of the *botrytis* infection severity on grape berries and yeast cell concentration in the grape juice obtained by berry squeeze after 5 days from yeast inoculation.

Strain	**soft rot**	**mycelium**	**10^6 CFU/mL**
Control	++++	++++	-
FRI719	+	+	1.98±0.07
FRI728	+	+	2.07±0.09
FRI729	++	+	1.83±0.08
FRI751	+	+	1.89±0.10
FRI754	+	+	2.35±0.01
FRI779	++	+	2.10±0.07
FRI7100	+	+	2.20±0.04
PAS13	++	+	2.25±0.08
PAS55	++	++	2.05±0.05
PAS66	++	++	2.08±0.08
PAS103	++	++	2.38±0.11
PAS151	+	++	2.04±0.07
PAS92	+	+	2.07±0.10
PAS173	+	+	2.34±0.06

3.3.4 Fermentation activity in synthetic must

The fermentation activity of the 14 strains of *S. bacillaris* were evaluated in synthetic must MS300 at 20 °C using an inoculum of 2×10^6 cells/ml. The CO_2 production was followed

during all the fermentation process. To assess strain fermentation performances, the fermentation vigor, in terms of CO_2 production after 48h of incubation, was considered in order to evaluate the adaptation ability to the must conditions. CO_2 production after 312h was considered, as at these fermentation step the widest range of CO_2 production was found between strains. The industrial wine strain *S. cerevisiae* EC1118 was used as control. The fermentations of *S. bacillaris* strains were stopped after 624h when the fermentation of *S. cerevisiae* EC1118 was completed. As expected, *S. bacillaris* strains showed a very low CO_2 production if compared to that of EC1118 (table 4).

Fermentation performances were very similar between strains as no significant differences were found after 312 and 624h of incubation. Regarding fermentation vigor, strains FRI719, FRI728 and PAS92 showed a significant delay in the fermentation start (0,03, 0,01, 0,07g/100mL CO_2 after 48h, respectively). Strain PAS173 showed the highest CO_2 production after 48h (0.33g/100mL CO_2).

Concerning residual sugars, as expected, *S. bacillaris* consumed more fructose than glucose due to its fructophilic aptitude (Englezos et al., 2015). The sugars residues were very high (from 100.41 to 135.87g/L), this was related to a limited ethanol production (from 4.12 to 6.19% v/v). Regarding secondary metabolites, their production was strongly strain dependent. As expected glycerol production was very high (from 5.58 to 7.81g/L), while acetic acid concentration was generally limited (from 0.28 to 0.45g/L).

Table 4. CO_2 production during fermentation, residual glucose and fructose concentrations and concentrations of the main fermentation products at the end of the fermentation of *S. bacillaris* strains in MS300. Data are expressed as the average of three replicates ± standard deviations. Within the column, mean values followed by the same letter are not significantly different according to Fisher's test ($p \leq 0.05$).

Strain	CO_2/100mL			Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Ethanol (%v/v)
	48 h	312h	624h					
FRI719	0.03 ± 0.00D	1.58 ± 0.25A	3.63 ± 0.58A	85.32 ± 1.12EF	26.53 ± 1.14EF	5.58 ± 0.02E	0.39±0.09ABCDE	4.58 ± 0.01E
FRI728	0.01 ± 0.00D	1.06 ± 0.02A	2.97 ± 0.10A	84.92 ± 3.10EFG	32.93 ± 1.19C	4.77 ± 0.30F	0.28±0.03G	4.15 ± 0.01E
FRI729	0.22 ± 0.03BC	1.57 ± 0.12A	3.12 ± 0.23A	90.39 ± 0.35CD	29.07 ± 1.16DE	6.03 ± 0.15E	0.42±0.03ABCD	4.45 ± 0.02E
FRI751	0.26 ± 0.04AB	1.81 ± 0.29A	3.28 ± 0.50A	78.22 ± 2.41H	24.49 ± 1.84FG	5.72 ± 0.20E	0.38±0.05ABCDEF	4.52 ± 0.024E
FRI754	0.27 ± 0.02BC	1.64 ± 0.06A	3.10 ± 0.07A	91.5 ± 0.01BCD	31.51 ± 0.56CD	5.58 ± 0.60E	0.29±0.01GF	4.48 ± 0.01E
FRI779	0.24 ± 0.02BC	1.8 ± 0.07A	3.60 ± 0.11A	89.75 ± 1.81D	21.01 ± 0.38I	7.05 ± 0.12BC	0.34±0.02DEFG	5.12 ± 0.02E
FRI7100	0.27 ± 0.04AB	2.00 ± 0.27A	3.74 ± 0.50A	82.87 ± 0.64FG	25.07 ± 0.19FG	7.05 ± 0.04BC	0.32±0.03GF	4.86 ± 0.01C
PAS13	0.28 ± 0.04AB	1.93 ± 0.19A	3.54 ± 0.33A	85.46 ± 2.10E	23.87 ± 1.31GH	7.81 ± 0.36A	0.40±0,02ABCD	4.94 ± 0.01CD
PAS55	0.28 ± 0.04AB	1.99 ± 0.22A	3.82 ± 0.44A	86.33 ± 1.31E	21.25 ± 0.84HI	6.86 ± 0.53BCD	0.37±0.08ABCDEF	5.52 ± 0.03B
PAS66	0.21 ± 0.02BC	1.46 ± 0.20A	2.93 ± 0.41A	93.26 ± 1.39AB	39.15 ± 2.14B	6.54 ± 0.31D	0.44±0.05AB	4.53 ± 0.02E
PAS92	0.07 ± 0.02D	1.39 ± 0.26A	2.80 ± 0.43A	92.67 ± 0.28ABC	43.20 ± 0.05A	5.70 ± 0.01E	0.45±0.11A	4.12 ± 0.01E
PAS103	0.26 ± 0.01AB	1.75 ± 0.01A	3.99 ± 0.95A	82.44 ± 0.27G	28.43 ± 1.26GH	6.63 ± 0.25E	0.35±0.05BCDEFG	4.55 ± 0.01E
PAS151	0.16 ± 0.08BC	1.64 ± 0.11A	3.15 ± 0.20A	94.41 ± 1.63A	33.84 ± 4.01C	7.22 ± 0.19B	0.43±0.04ABC	4.49 ± 0.02E
PAS173	0.33 ± 0.12A	2.05 ± 0.44A	3.77 ± 0.64A	78.63 ± 1.06H	21.78 ± 1.59HI	6.95 ± 0.16BCD	0.35±0.05CDEFG	6.19 ± 0.03A

In order to evaluate differences in fermentation performances among the strains all the collected data (CO_2 production after 48, 312 and 624h, and the concentration of glucose and fructose residues, glycerol, acetic acid and ethanol) were analyzed by principal component analysis (PCA) (Fig 3). Function (F1) accounted for 58.81% of the total variance and significantly correlated ($\alpha<0.001$) with CO_2 production after 312h, 624h and ($\alpha<0.01$) 48h, with fructose residue and ($\alpha<0.01$) ethanol concentration. The second function (F2) explained 20.62% of the total variance and was correlated ($\alpha<0.01$) with acetic acid concentration. No significant correlations were found with glucose residue and glycerol production. The analysis confirmed the high level of similarity between the fermentation

performances of the different strains when they are tested as single starter, irrespectively of the strain origin. FRI728 and PAS92 confirmed to be the strains with the worst fermentation aptitudes in terms of fermentation rate, and ethanol production. On the contrary PAS 173 showed the best fermentation performances.

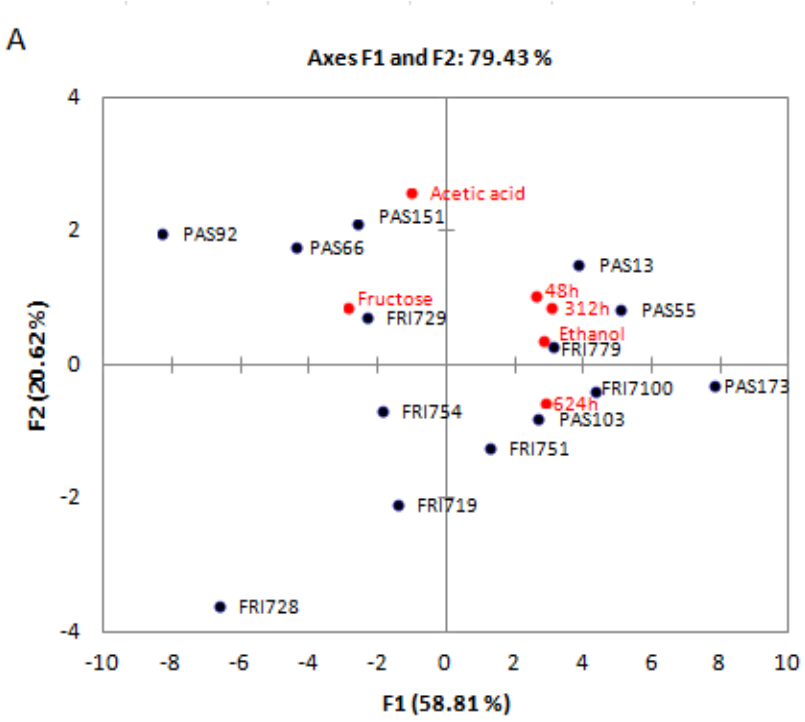

Figure 3. Principal component analysis (PCA) biplot showing fermentation performances (CO_2 production after 48h, 312h and 624, glucose and fructose residues, ethanol, glycerol and acetic acid production) of *S. bacillaris* strains in sequential fermentation with EC1118 in MS300.

All the strains were tested in co-fermentation with strain EC1118 in synthetic must MS300 at 20°C (Table 5). Each time sequential inoculations were performed adding *S. bacillaris* strain at first, followed, after 48h, by *S.cerevisiae* EC1118. Both strains were added at the same concentration (1-1.5×10^6cells/ml). Strains PAS55, PAS66, PAS92, PAS103, PAS151 and PAS173 that showed high fermentation vigor (CO_2 production after 48h), evidenced a significant lower CO_2 production after 312h than that of the other strains.

This means that after the addition of EC1118 a lower fermentation rate than that of EC1118 single-strain fermentation occurred. These strains, together with PAS13, showed the presence of sugar residues, although at low concentrations (from 1.92 to 7.9 g/L), confirming a lower fermentation rate than that of the other strains.

The alcohol content was significantly higher in EC1118 single-strain fermentation than in sequential fermentations. Ethanol concentration in EC1118 single-strain fermentation was 13.16% (v/v), whereas sequential fermentations, where the reducing sugar were completely consumed, produced an average of 12,15% (v/v) ethanol, reducing 1% the alcohol content. These results confirmed the well-known ability of *S. bacillaris* species to reduce alcohol content in wine (Bely et al., 2013).

Glycerol concentration was significantly lower in EC1118 single-strain fermentation (5,77 g/L) than in co-fermentations (average value 7,05g/L). An average increase of 1.28 g/L was found. Strain glycerol production seems not to be related to sugar consumption, as strain PAS 13, that left 3.66 g/L of sugars, showed one of the highest levels of glycerol production. Only small differences were found in acetic acid production.

Table 5. CO_2 production during fermentation, residual glucose and fructose concentrations and concentrations of the main fermentation products at the end of the sequential fermentation of *S. bacillaris* strains with EC1118 in MS300. Data are expressed as the average of three replicates ± standard deviations. Within the column, mean values followed by the same letter are not significantly different according to Fisher's test ($p \leq 0.05$).

Strain	CO_2/100mL			Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Ethanol (%v/v)
	48 h	312h	624h					
EC1118	1.10 ± 0.14A	7.63 ± 0.34B	9.57 ± 0.20A	-	-	5.77±0.14E	0.51±0.02A	13.16±0.02A
FRI719	0.01 ± 0.00E	8.66 ± 0.16A	9.38 ± 0.10AB	-	-	6.79±0.06C	0.52±0.01A	11.67±0.18I
FRI728	0.01 ± 0.00E	7.21 ± 0.26BC	9.13 ± 0.06BC	-	-	6.69±0.01C	0.48±0.01BC	12.21±0.04FG
FRI729	0.15 ± 0.03DC	7.57 ± 0.01BC	8.73 ± 0.11DE	-	-	7.47±0.19B	0.46±0.01CDE	12.19±0.06FGH
FRI751	0.01 ± 0.01E	8.59 ± 0.21A	9.29 ± 0.18AB	-	-	6.57±0.29C	0.47±0.03CD	12.39±0.06CDE
FRI754	0.26 ± 0.01CB	8.56 ± 0.04A	8.72 ± 0.12DE	-	-	8.26±0.30A	0.45±0.04DEF	12.22±0.10EFG
FRI779	0.01 ± 0.00E	8.60 ± 0.33A	9.38 ± 0.07AB	-	-	6.82±0.13C	0.51±0.03AB	12.07±0.05GH
FRI7100	0.13 ± 0.02D	6.94 ± 0.16C	8.73 ± 0.06DE	-	-	7.40±0.07B	0.44±0.01EF	12.28±0.02DEF
PAS13	0.33 ± 0.06B	6.90 ± 0.97C	8.95 ± 0.05CD	1.36±0.68B	2.30±1.03B	7.59±0.14B	0.42±0.01GF	12.20±0.08FG
PAS55	0.31 ± 0.07B	5.83 ± 0.63D	8.69 ± 0.17DE	0.12±0.22C	1.80±0.43B	6.85±0.23C	0.36±0.06I	12.02±0.28H
PAS66	0.33 ± 0.09B	5.98 ± 0.91D	8.78 ± 0.39DE	3.14±0.35A	4.76±0.46A	6.67±0.11C	0.36±0.01I	11.42±0.01J
PAS92	0.34 ± 0.04B	5.62 ± 0.27DE	8.64 ± 0.04EF	0.67±0.67C	2.58±1.25B	6.7±0.09C	0.36±0.01I	12.03±0.02H
PAS103	0.28 ± 0.10B	5.01 ± 0.04E	8.38 ± 0.05F	-	2.49±0.23B	6.64±0.25C	0.38±0.02HI	12.55±0.02C
PAS151	0.33 ± 0.06B	6.05 ± 0.19D	8.79 ± 0.06DE	-	0.08±0.14C	7.43±0.17B	0.40±0.02GH	12.75±0.04B
PAS173	0.23 ± 0.01BCD	5.36 ± 0.42DE	8.60 ± 0.29EF	-	2.62±0.37B	6.85±0.03C	0.38±0.06HI	12.43±0.06DC

All the data obtained were analyzed by PCA (Fig 4). Function (F1) accounted for 44.67% of the total variance and significantly correlated ($\alpha<0.001$) CO_2 production after 312h and ($\alpha<0.05$) 624h, with fructose residue and acetic acid production concentration. The second function (F2) explained 24.20% of the total variance and was correlated ($\alpha<0.01$) with fermentation vigor and ethanol production.

No significant correlations were found with glucose residue and glycerol production. In these conditions, strain origin seems to be the explanation of the strain clustering. "PAS" strains isolated from grape must B showed the worst fermentation performances with the presence of fructose residues, whereas "FRI" strains showed good fermentation

performances, producing the highest level of acetic acid. EC1118 single strain fermentation clustered separately due to higher fermentation vigor and ethanol production than the sequential fermentations.

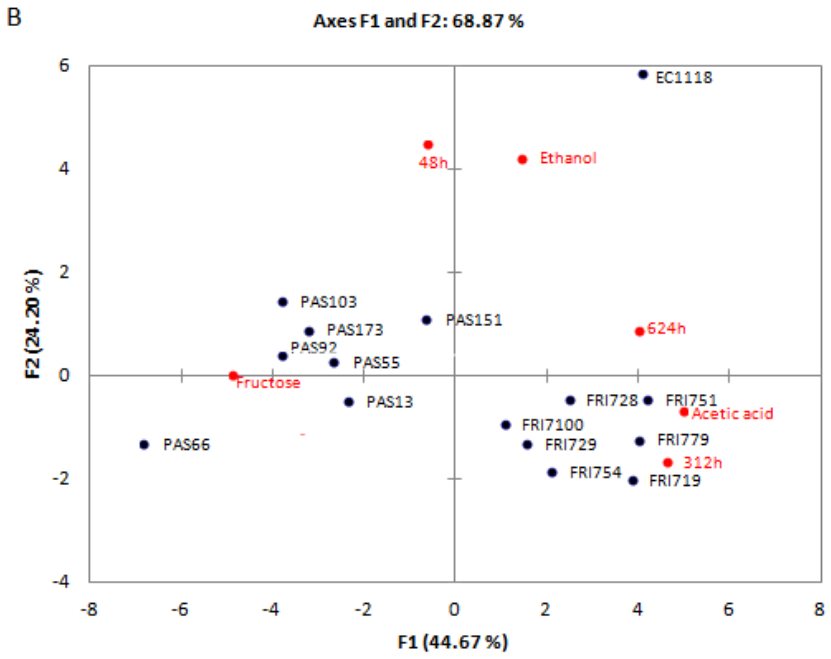

Figure 4. Principal component analysis (PCA) biplot showing fermentation performances (CO_2 production after 48h, 312h and 624, glucose and fructose residues, ethanol, glycerol and acetic acid production) of *S. bacillaris* strains in MS300 (A); in sequential fermentation with EC1118 in MS300 (B) and in sequential fermentation with EC1118 in natural must (C). Only variables that showed significant correlations are reported.

3.3.5 Fermentation activity in natural must

Sequential fermentations of *S. bacillaris* and *S. cerevisiae* EC1118 were run in natural must in the same condition used for the synthetic must (Table 5). In this case the widest range of CO_2 production was found after 288h. EC1118 completed the fermentation within 12 days, sequential fermentations in 16 days. Only when FRI754, FRI779 and PAS103 were tested a limited fructose residue was found (from 1,47 to 2,40 g/L).

Glycerol was significantly higher in sequential fermentations than in EC1118 single strain fermentation. Glycerol concentration in EC1118 single strain fermentation was 4,86 g/L, where as sequential fermentations produced an average of 5,84 g/L glycerol, with an average increase of 0,98 g/L. Ethanol concentration ranged between 11,19 and 11,61% (v/v). No significant differences were found between EC1118 fermentation and sequential fermentations for 5 out of 14 strains tested. This could be due to the lower sugar concentration present in the natural must than in the synthetic must of the previous trial. Indeed, glycerol production is directly proportional to the sugar content: the higher the sugar content, the higher the glycerol concentration, therefore the lower the ethanol concentration (Tilloy et al., 2014). Acetic acid concentrations were very limited and lower than those found during synthetic must fermentations (ranging from 0,28 to 0,36 g/L).

Table 6. CO2 production during fermentation, residual glucose and fructose concentrations and concentrations of the main fermentation products at the end of the sequential fermentation of *S. bacillaris* strains with EC1118 in natural must. Data are expressed as the average of three replicates ± standard deviations. Within the column, mean values followed by the same letter are not significantly different according to Fisher's test ($p \leq 0.05$).

Strain	CO2/100mL			Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Ethanol (%v/v)
	48 h	288h	384h					
EC1118	2.17±0.07A	8.26±0.05A	-	-	-	4.86±0.06D	0.36±0.01A	11.61±011A
FRI719	0.12±0.02D	6.29±0.21DE	7.78±0.11CDE	-	-	5.76±0.18BC	0.31±0.01B	11.34±0.21BCDE
FRI728	0.14±0.02CD	5.70±0.10GH	7.39±0.02GH	-	-	5.82±0.17BC	0.32±0.01B	11.33±0.19BCDE
FRI729	0.15±0.06CD	6.22±0.13DEF	7.77±0.16CDE	-	-	6.06±0.57B	0.29±0.02B	11.43±0.15ABCD
FRI751	0.11±0.01D	6.96±0.09C	7.59±0.26EFG	-	-	5.79±0.47BC	0.31±0.02B	11.21±0.30DE
FRI754	0.13±0.02D	5.66±0.15H	7.28±0.010H	-	2.05±0.42B	5.66±0.17BC	0.31±0.03B	11.19±0.14E
FRI779	0.14±0.03D	6.1±0.47DEFG	7.68±0.28DEF	-	2.40±0.27A	5.77±0.25BC	0.31±0.05B	11.30±0.06CDE
FRI7100	0.21±0.02C	7.02±0.16C	8.03±0.03BC	-	-	6.05±0.13BC	0.32±0.03AB	11.55±0.08AB
PAS13	0.11±0.01D	7.56±0.08B	8.66±0.25A	-	-	5.80±0.20BC	0.32±0.02B	11.29±0.11CDE
PAS55	0.35±0.04B	6.27±0.26DEF	7.92±0.10BCD	-	-	6.64±0.54A	0.28±0.03B	11.48±0.05ABC
PAS66	0.13±0.01D	6.90±0.20C	7.49±0.3FGH	-	-	5.86±0.18BC	0.30±0.00B	11.53±0.04AB
PAS92	0.15±0.06CD	6.33±0.16D	7.99±0.16BC	-	-	5.79±0.25BC	0.35±0.02AB	11.44±0.03ABCD
PAS103	0.13±0.06D	5.88±0.11FGH	7.51±0.02EFGH	-	1.47±0.35C	5.46±0.07C	0.33±0.03B	11.25±0.07DE
PAS151	0.13±0.06D	5.96±0.61DEFG	8.20±0.05B	-	-	5.69±0.25BC	0.32±0.01B	11.39±0.03BCDE
PAS173	0.11±0.04D	5.91±0.04EFGH	8.12±0.11B	-	-	5.58±0.17BC	0.33±0.03B	11.35±0.14BCDE

All the data obtained were analyzed by PCA (Fig 5). Function (F1) accounted for 54.01% of the total variance and significantly correlated ($\alpha<0.001$) CO_2 production after 48h, ($\alpha<0.05$) 312h and 624h, and ($\alpha<0.05$) glycerol production. The second function (F2) explained 23.45% of the total variance and was correlated ($\alpha<0.001$) glucose and ($\alpha<0.05$) fructose residue. No significant correlations were found with acetic acid and ethanol content. In these conditions differences between sequential fermentations and EC1118 single-strain fermentation were more evident than in synthetic must in term of fermentation performances.

In all the sequential fermentations a slower fermentation rate than that of EC1118 single-fermentation was found. The main differences among sequential fermentations were due to the presence of different level of sugar residues (axe F2).

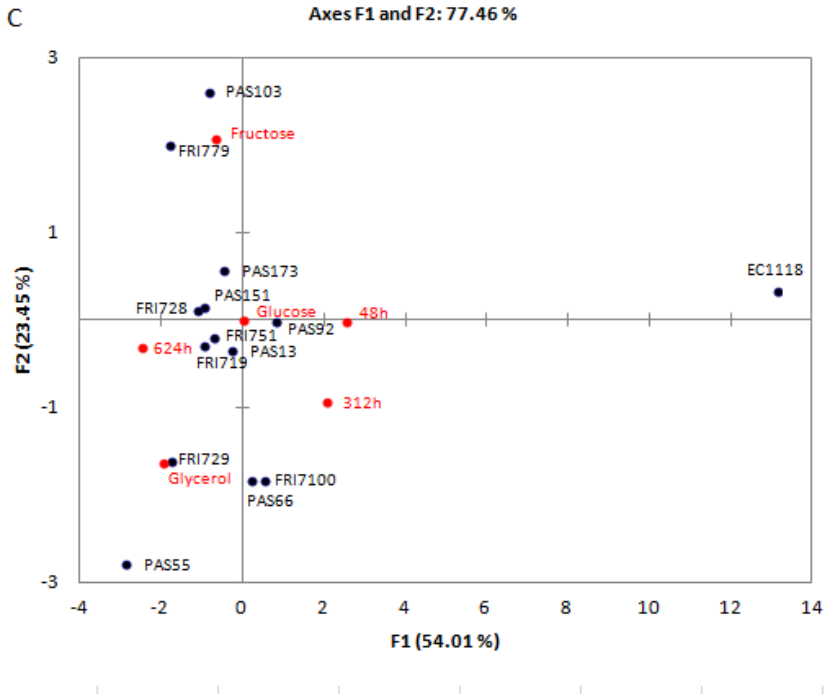

Figure 5. Principal component analysis (PCA) biplot showing fermentation performances (CO_2 production after 48h, 312h and 624, glucose and fructose residues, ethanol, glycerol and acetic acid production) of *S. bacillaris* strains in MS300 (A); in sequential fermentation with EC1118 in MS300 (B) and in sequential fermentation with EC1118 in natural must (C). Only variables that showed significant correlations are reported.

3.4 Discussion

With the aim of selecting wine yeasts carrying antifungal activity, fermenting musts obtained from late-harvest, overripe grape variety, naturally dried, were considered. The overripe grape berries show a very soft texture, due to the senescence or aging of fruit tissues. These physical features increase susceptibility to mechanical damage and infection by fungal pathogens (Genovese etal., 2007). Molds such as *Botrytis cinerea* are abundant

in this environment and yeasts must carry antifungal activity to compete. After yeast isolation and identification, 36 isolates were found to belong to the *Starmerella bacillaris* species. This yeast possesses a fructophilic character and a poor ethanol yield from sugar consumed (Magyar and Tóth, 2011). Several ecological studies evidenced the presence of this species on grape berry surface and during spontaneous fermentations of musts in several countries (Bokulich et al., 2013a, 2013b; Milanović et al., 2013; Wang et al. 2015), suggesting that this species has a specific role in the fermentation process. *S. bacillaris* carries some very interesting enological traits such as growth at high concentrations of sugars and low temperatures (Sipiczki, 2003; Tofalo et al., 2012) and production of low levels of acetic acid, acetaldehyde and significant amounts of glycerol from consumed sugars (Magyar and Tóth, 2011). Contrary to the most common non-*Saccharomyces* yeasts, it can survive until the end of the alcoholic fermentation due to its ability to tolerate high concentrations of ethanol present in the wine (Ciani et al., 2015; Englezos et al., 2015; Rantsiou et al., 2012).

By means of SAU-PCR analysis at least 14 genetically defined groups were found.

One isolate for each group was selected to test antagonistic activity to *Botrytis cinerea* both in vitro and in vivo. The results of the antagonistic activity in vitro assay, obtained growing simultaneously the yeast strains together with the fungal mycelium, demonstrated that yeast isolates were able to limit the causal agent of gray mold disease and this seems not to be related to the acidic condition of the environment (PDA medium at pH 3.5). The values of the inhibition of the radial mycelium growth were comparable with those previously found for other antagonistic yeasts (Parafati et al., 2015). Due to the different growth rate of *S. bacillaris* and *B. cinerea* on PDA medium, where *S. bacillaris* can not found the optimal

growth conditions, a 4 days pre-incubation of yeast strains was performed before testing the antifungal activity in the following *in vitro* assays.

Since several mechanisms have been reported to play a significant role in the biocontrol activity of antagonistic yeasts, in this study we evaluated the possible role of the main biocontrol modes of action, such as production of volatile organic compounds (VOCs) and cell wall-degrading enzymes, in controlling the in vitro growth of *B. cinerea*. Plate assays evaluating cell-wall degrading enzymes (pectinolitic, proteolytic and chitinolytic activities) indicated that pectinolitic activity was not present, chitinolytic activity was evident for all the strains although at low level, and only two strains FRI719 and FRI751 showed potential to produce proteolytic enzymes as they grew well on plates containing skin milk. The results regarding VOCs production were more promising. These compounds have been shown to have an antifungal effect and contribute to the biocontrol activity found in several yeast species such as *Wickerhamomyces anomalus*, *Candida intermedia* and *Sporidiobolus pararoseus* (Druvefors and Schnürer, 2005; Huang et al., 2011; Huang et al., 2012). In particular, more recently Hua and co-workers (2014) demonstrated that the biocontrol ability of *W. anomalus* can be attributed to the production of 2-phenylethanol, a secondary alcohol which affects spore germination, growth, toxin production, and gene expression in *Aspergillus flavus*.

It is well known that volatile molecules such as higher alcohols and esters are produced by non-*Saccharomyces* wine yeasts and their concentration is strain dependent (Rojas et al., 2001; Clemente-Jimenez et al., 2004; Jolly et. al. 2006; Jolly et al. 2014).

The inhibition percentage of the radial mycelial growth, during *in vitro* plate assay, was very high indicating a strong antifugal activity and suggesting VOCs as main

responsible for *S. bacillaris* antifungal effects. The inhibitory effect of the *S. bacillars* strains was further proven on wounded grape berries artificially inoculated with *B. cinerea*. All the strains were able in reducing *B. cinerea* gray mold decay. In particular the lesion diameter reduction was comparable with that found previously for other antifungal yeasts (Parafati et al., 2015).

Regarding the ability of the yeast strains to survive and multiply in artificial wounds made on grapes, results indicated that after 5 days from the inoculation, after squeezing the berries, the cell concentrations were very high (from 2 to 3 $x10^6$ CFU/ml). This finding suggested that *S. bacillaris* strains can easily growth in the wound environment on grape berries and have a considerable colonizing potential. Due to the promising *S. bacillaris* antifungal activity and the well-proved enological property of this species, fermentation ability of the *S. bacillaris* strains isolated in this study were tested using an inoculum carrying a cell concentration similar to that found in the infected berries. This concentration is interesting from an enological point of view as natural yeast population size in grape must after pressing, usually ranges from 10^4 to 10^6 cells/ml (Fleet at. al., 1984; Combina et al., 2005; Jolly et al., 2006.).

Moreover, in several studies where *S. bacillaris* was used in sequential fermentation together with *S. cerevisiae* the inoculum size was 10^6 cell/ml and at this concentration this yeast produced positive effect on wine (Andorrà et al., 2010; Rantsiou et al., 2012). *S. bacillaris* single-strain fermentation confirmed the fructofilic character, the high *glycerol* production and a fermentation rate slower than that of *S. cerevisiae* EC1118 (Magyar & Tóth, 2011; Englezos et al., 2015).

When sequential fermentations were performed in synthetic must *S. bacillaris* strains significantly increased glycerol content and reduced ethanol concentration. In sequential fermentations of natural must the mixed starters consumed all the reducing sugars (only in few cases a minimal sugar residues remained in the wine) and *S. bacillaris* significantly increased the glycerol content, although the fermentation rate was slower than that of EC1118 single-strain fermentation. In all the fermentation trial *S. bacillaris* strains produced very low acetic acid concentrations.

The level is lower than that found for other *S. bacillaris* strains isolated from another Italian winemaking region (Englezos et al., 2015). This finding is very interesting as one of the main concerns in the use of non-*Saccharomyces* strains in winemaking is their propensity to produce high level of volatile acidity (Jolly et al., 2006).

In this paper we demonstrated for the first time that strains of *S. bacillaris* carry antifungal activity and this property can be used to control the growth of the fungal pathogen *B. cinerea* on grape. Moreover, the interesting enological properties possessed by these strains have been proven to enhance wine quality.

The high wound colonization ability of *S. bacillaris* found in this work together with its propensity to colonize the grape berry surface (Wang et al., 2015) suggests that the use of this yeast as biocontrol agent on grape plant and berries could influence the following must fermentation, although the presence of *S. cerevisiae* is needed to complete the fermentation. Further studies will be needed to assess the efficacy of *S. bacillaris* as biocontrol agent directly in vineyard to couple the antifungal activity with the enological properties of these strains. In this sense our results provide a new insight in the management of non-

Saccharomyces yeast for winemaking and open new prospects to a more integrated strategy for increasing wine quality.

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CHAPTER 4

Antifungal activity of the non-conventional yeast *Starmerella bacillaris* (formerly *Candida zemplinina*) on apple fruits and its effect on apple cider fermentation

4.1 Introduction

Penicillium expansum, the pathogen responsible of blue mold rot (or soft rot), is the most important and common postharvest disease of fruits and vegetables, responsible for major economic losses in agriculture worldwide. Apples, pears, peaches, nectarines, apricots, plums and cherries, grapes, strawberries, raspberries, persimmons, mango, passion fruit, avocado, cucurbits, carrots, onions and tomato are susceptible to this fungal disease (Errampalli, 2014).

P. expansum can grow at low temperatures and conidia can germinate at 0 ºC (Rosenberger, 1990). The fungus survives in soil and bark trees; conidia are also present in the air and on fruit surface and spores are commonly present in the air and on the walls of storage and manufacturing rooms. Wounds caused by adverse weather conditions before harvest or by unsuitable harvesting and postharvest handling processes are an optimal locus for fungus infection (Vilanova et al., 2012; Tannous et al., 2016). Fruits infected in the orchard may be asymptomatic till storage (Amin et al., 2017). The maturity stage of fruit is an important factor in the defense response, in fact *P. expansum* infects mostly mature and overripe fruit than immature fruit (Vilanova et al., 2014; Norelli et al., 2017).

Blue mold symptoms appears as soft, watery lesions that are light brown in color, and, at the later stages of decay development, conidia turn blue green (Jurick et al., 2010). There is a very sharp margin between diseased and healthy tissues. Moreover, during postharvest decay *P. expansum* produces polygalacturonase enzyme that hydrolyzes pectins, softening

tissues, and facilitates the invasion and colonization of fruits (Yao et al., 1996; Jurick et al., 2010; Amin et al., 2017).

Moreover, *P. expansum* is the main producer of the mycotoxin patulin, a secondary metabolite that accumulates in fruits leading to serious health problems (Morales et al., 2008; 2010). *P. expansum* is believed to be the major fungal species that produces patulin in apples and apple products (Errampalli, 2014). Thus, *P. expansum* is a big economic problem to both fresh-fruit and fruit-processing industries.

Traditionally, synthetic chemical fungicides are used to control blue mold incidence in stored fruits (Vero et al., 2002; Amin et al., 2017). However, during the past few decades, increased use of these compounds often leads to the establishment of resistant pathogen populations which reduces their efficacy (Baraldi et al., 2003; Jurick et al., 2010). Moreover, public concerns for the environment and for human health and regulatory restrictions about the presence of fungicide residues on crops bring to the search and development of alternative control methods (Vero et al., 2002; Amin et al., 2017).

In recent years, low impact chemical preservative agents have been developed, such as salts of weak acids, that can inhibit the growth of postharvest fungal pathogens. These compounds present low toxicity, a wide spectrum of activity and relatively low cost, but to act as fungicides high concentrations are required, leading to potential organoleptic changes (da Cruz Cabral et al., 2013).

An interesting alternative to chemical fungicides is the use of natural compounds, such as plant extracts and essentials oils. They are considered relatively safe, they are easily decomposed, environmentally friendly and non phytotoxic (da Cruz Cabral et al., 2013; Errampalli, 2014). Moreover, the induction of resistance in fruit against pathogens infection is a promising approach for postharvest diseases control. The triggered resistance is due to

biological, chemical or physical elicitors, generally with salicylic acid, a phenolic compound that naturally exists in plants, as a signal molecular in signal transduction pathway for systemic-induced resistance (Errampalli, 2014). Chitosan, a derivative of the polysaccharide chitin, has the double effect of induction of host resistance to pathogens and inhibition of spore germination and mycelial growth of pathogens (Liu et al., 2007).

A promising alternative strategy is biological control, achieved by the use of microorganisms called biocontrol agents. Different strains of yeasts, bacteria and filamentous fungi have been identified and characterized for the control of blue mold caused by *P. expansum* in fruits and vegetables, even if the mode of action of these microorganisms has not been fully elucidated. Antagonistic yeasts have been selected mainly for their capability to rapidly colonize and grow in surface wounds, and subsequently to compete with the pathogen for nutrients and space (Droby et al., 2002). Several putative mechanisms have been proposed to play a role in biocontrol effectiveness of antagonistic yeasts: competition for space and nutrients, production of extracellular hydrolases, ability to maintain normal metabolism at high osmotic potential, resistance to oxidative stress and the induction of resistance responses at the wound site (Scherm et al., 2003).

Over the last 20 years a large number of researches on blue mold biocontrol has been published, but very few products have been patented and registered for commercial use against postharvest decay of citrus, apples and pears. Different strains of yeasts, from the genera *Candida*, *Cryptococcus*, *Metschnikowia, Kluyveromyces*, *Pichia*, *Rhodotorula*, *Rhodosporidium*, *Saccharomyces* and *Torulaspora* have been studied as biocontrol agents for blue mold (Errampalli, 2014). The yeast antagonist *Candida oleophila* Montrocher (strain I-182) was proved to be effective against a wide range of postharvest decay and

subsequently, it was developed by Ecogen Corp. (Langhorne, PA) as a commercial formulation and registered in 1995 under the name “Aspire” (Droby et al., 1998).

Competition for nutrients and space is believed to be the major mode of action. Moreover, it produces and secretes various cell wall-degrading enzymes, including exo-β-1,3-glucanase, chitinase and protease. (Bar-Shimon et al., 2004). Another yeast-based product commercially available is “Yield Plus” (Anchor Yeast, Cape Town, South Africa) containing the yeast *Cryptococcus albidus*, an antagonist isolated from peaches and registered for the control of post-harvest rots of pome fruits (Errampalli, 2014).

During storage, apples are treated several times with biocontrol yeasts and, after fruits pressing for fermentation, these yeasts become part of the juice microbiota. Thus, the selection of yeasts with both antifungal and fermentation properties could be of great interest for cider production.

There is a growing interest in isolating and characterising non-*Saccharomyces* yeasts for development of starter cultures that increase flavour diversity in beer, wine and spirits. Fermented beverages from the juice of several fruits have been studied from a microbiological, compositional and sensorial perspective and non-*Saccharomyces* yeasts have been also evaluated in the production of fermented beverages other than wines or beers, including beverages made from fermented apples (Varela, 2016).

Starmerella bacillaris (formerly *Candida zemplinina*) is a non-*Saccharomyces* yeast mainly used for wine fermentation because of its interesting technological properties: a strong fructophilic character (it consumes fructose in preference to glucose), reduction of acetic acid formation in sweet wines, high glycerol and low ethanol concentration, enhance wine flavour and mouthfeel (Rantsiou et al. 2012; Soden et al. 2000; Englezos et al. 2015; Domizio et al. 2014; Englezos et al. 2016; Varela and Borneman, 2016). During grape must

fermentation it has been tested both in sequential and mixed yeast inoculations with *Saccharomyces cerevisiae* (Rantsiou et al., 2012; Wang et al., 2014; Lemos Junior et al., 2016). Moreover, Lemos Junior et al. (2016) demonstrated the role of *S. bacillaris* strains as both potential biocontrol agent against *Botrytis cinerea* and unconventional starter in grape must fermentation.

As fruit wines, apple cider and grape wine fermentations share many similarities in microbiological flora and mechanism. Nevertheless, there is limited information in the research on the cider co-fermentation with *S. cerevisiae* and non-*Saccharomyces*, compared with grape wine (Ye et al., 2014). Several yeasts have been proposed for sequential mixed cultures with *S. cerevisiae* on apple cider fermentation, to improve the quality of cider, such as *Wickerhamomyces anomalus* (Ye et al., 2014), *Kloeckera apiculata* (Bilbao et al., 1997), *Hanseniaspora valbyensis* (Xu et al., 2006). To date, the use of *S. bacillaris* as a biocontrol agent against *P. expansum* and as a starter in apple cider fermentation has not been reported.

In this work 14 *S. bacillaris* strains have been studied to evaluate their postharvest antifungal activity against *P. expansum* on apples. Moreover, the fermentation performances in apple juice of these non-*Saccharomyces* strains were tested, both in single-strain fermentation and in sequential fermentation, together with *Saccharomyces cerevisiae*.

4.2 Materials and methods

4.2.1 Yeast strains

The yeast strains used in this work (Table 1) were isolated from fermenting must obtained from dried grape of Raboso piave variety, as described by (Lemos Junior et al., 2016).

Table 1. Yeast strains used in work.

Strain	Species	Origin
PAS13	*S. bacillaris*	Winery B
PAS55	*S. bacillaris*	Winery B
PAS66	*S. bacillaris*	Winery B
PAS92	*S. bacillaris*	Winery B
PAS103	*S. bacillaris*	Winery B
PAS151	*S. bacillaris*	Winery B
PAS173	*S. bacillaris*	Winery B
FRI719	*S. bacillaris*	Winery A
FRI728	*S. bacillaris*	Winery A
FRI729	*S. bacillaris*	Winery A
FRI751	*S. bacillaris*	Winery A
FRI754	*S. bacillaris*	Winery A
FRI779	*S. bacillaris*	Winery A
FRI7100	*S. bacillaris*	Winery A
EC1118	*S. cerevisiae*	Industrial strain

4.2.2 Biocontrol assays

In order to assess the efficiency of yeasts as biocontrol agents on apples, the method described by Vero et al. (2002) was used. Golden Delicious apples derived from biological orchards. Healthy and homogeneous apples were selected. The *P. expansum* strain used was PVPD 2016_3.

For each yeast strain, 2 apples were used in a first preliminary test. In the second test, 7 apples per strain were used. Apples were placed in a plastic box that was kept at 25 °C for 7 days. After this period, wounds were examined and the lesion diameters were measured.

Disease reduction incidence percentage was calculated as follow (DRI%) = (C − T/C)*100, where C was the average radial growth measurement in control, and T was the radial growth of the pathogen in the presence of yeast strain in each wound. The lesion diameter (LD) was evaluated by measuring the average diameter of the damaged area 7 days after *P. expansum* inoculation.

4.2.3 Colonization of wound site

Growth curves were done in fruit wounds at 25 °C as described by (Vero et al. (2002). The wounds were incubated for 11 days. Pieces of apple (approximately 0.8 g) bearing a wound were cut and placed in 15 ml parafilm-capped tubes. Controls were inoculated with saline (0.9%). At different times (0, 24, 96, 144, 192 e 264 hours), three tubes per treatment and three controls, were weighed and 7.2 ml of sterile saline (0.9%) was added to them. Samples were then homogenized in vortex for 2 min. Quantification of viable yeast cells in the resulting abstract was performed by plate count on malt agar.

4.2.4 Fermentation trials

Pre-cultures of each strain used in this work were prepared as described by Bovo et al. (2016). A suitable aliquot of each yeast culture, corresponding to a final cell concentration of 1.5×10^6 cells/ml was used to inoculate 120 ml-capacity bottles, fitted with closures that enabled the carbon dioxide to escape, containing 100 ml of sterile apple juice (75 g/l fructose and 40 g/l glucose, pH 3.4). Apple juice was extracted from fresh Golden Delicious apples. In single-strain fermentation the inoculum concentration was $1\text{-}1.5\text{x}10^6$ cells/ml. In sequential fermentation the same inoculum size for both *S. bacillaris* strain and *S. cerevisiae* EC1118 ($1\text{x}10^6$ cells/ml) has been used. EC1118 was added 48h after the inoculum of *S. bacillaris*. After yeast inoculation the bottles were incubated at 20°C. All experiments were performed in triplicate. CO_2 production was monitored by weighing the bottles twice a day and calculating weight loss of each culture. The fermentations were stopped when the weight loss was lower than 0.1 g in 24 hours.

4.2.5 HPLC analysis

HPLC analysis was performed to determine the concentration of residual sugars, glycerol, ethanol and acetic acid as described by Nadai et al. (2016).

4.2.6 Statistical analysis

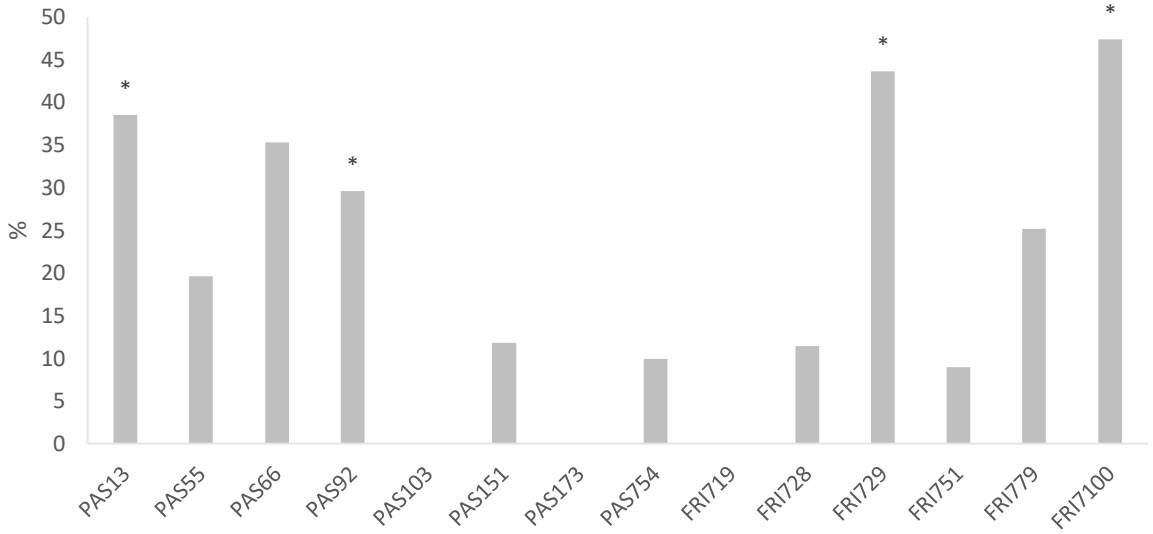
Statistical analysis was conducted using the software XLSTAT, vers.7.5.2 (Addinsoft, Paris, France), using simple analysis of variance (one-way ANOVA), followed by the Tukey test as "post-hoc" tests. Differences were considered statistically significant for p-value less than 0.05.

4.3. Results

4.3.1 Antifungal activity

A first preliminary test, aimed of identifying those yeast strains with the highest activity against the pathogen, was performed with all 14 strains of *S. bacillaris*. Each strain was tested in 4 different wounds. The efficacy of all tested yeasts in reducing *P. expansum* growth directly on apples is reported in Figure 1. Approximatly $4x10^6$ yeast cells/wound have been inoculated and, after 24 hours, approximatly $4x10^3$ conidia of the pathogen were inoculated in all wounds (this pathogen concentration had previously reported proved to produce 100% infection of wounds (Vero et al., 2002).

A



B

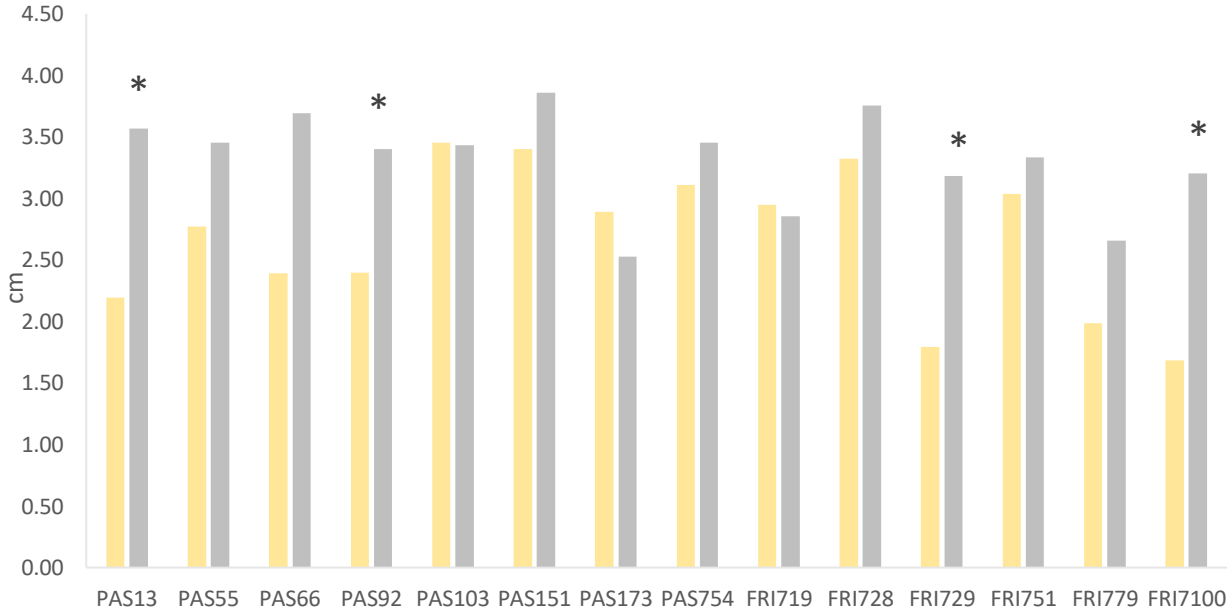
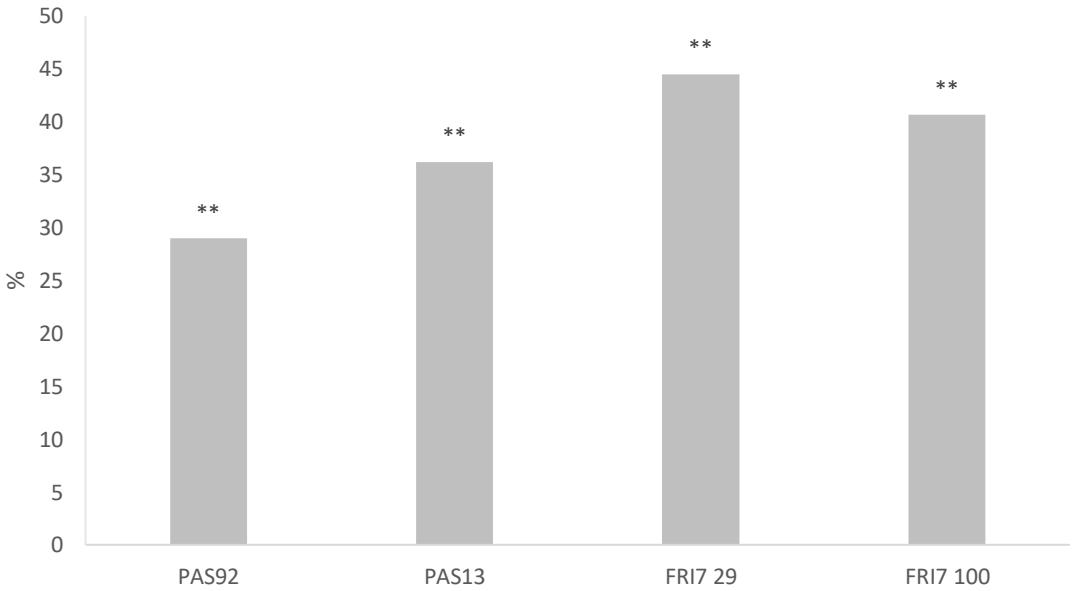


Figure 1. *In vivo* antagonistic activity of 14 *S. bacillaris* strains in inhibiting blue mold decay on apples. Effect of yeasts is referred to a) disease reduction inhibition percentage (DRI%) and b) lesion diameter (LD) caused by *Penicillium expansum* 7 days after incubation at 25°C in control (■) and tested (■) wounds. Significant differences are indicated with * (p-value < 0.05).

In details (Figure 1a), the disease reduction incidence (DRI) values, compared to the related controls, ranged from 0% to 47.4%. Strains FRI7100, FRI729, PAS13, PAS66 and PAS92 showed the highest DRI values, resulting respectively in 47.4%, 43.6%, 38.5%, 35.3% and 29.6% disease reduction, but only FRI7100, FRI729, PAS13 and PAS92 values were significantly higher ($p < 0.05$) than those found for the other strains. The lesion diameter (LD) evaluation (Figure 1b) confirmed the ability of 4 *S. bacillaris* strains to reduce the infection size. In fact, PAS13, PAS92, FRI729 and FRI7100, showed a significant ($p < 0.05$) reduction in the diameter of the lesion respect to the control.

The test was repeated for the 4 strains of *S. bacillaris* significantly limiting the pathogen growth, to confirm their antifungal activity. Each strain was tested in 14 different wounds. Their efficacy in reducing *P. expansum* growth on apples is reported in Figure 2.

A



B

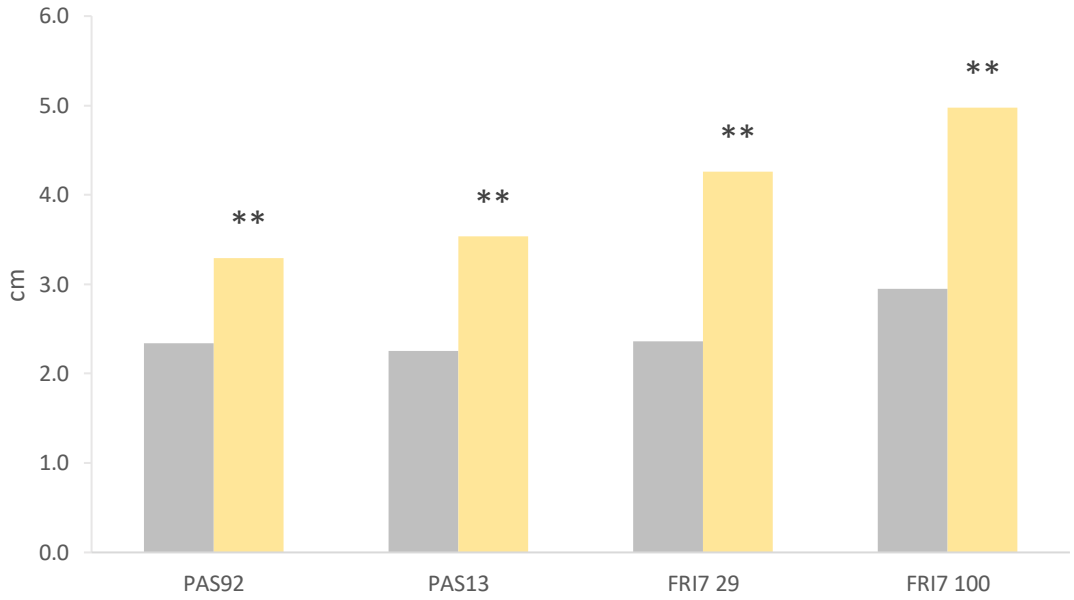


Figure 2. *In vivo* antagonistic activity of 4 *S. bacillaris* strains in inhibiting blue mold decay on apples. Effect of yeasts is referred to a) disease reduction inhibition percentage (DRI%) and b) lesion diameter (LD) caused by *Penicillium expansum* 7 days after incubation at 25°C in control (■) and tested (■) wounds. Significant differences are indicated with ** (*p*-value < 0.01).

The DRI values (Figure 2a), compared to the related controls, ranged from 29.0% to 44.5%. These results were in accordance with those obtained during the preliminary test. As regard LD evaluation (Figure 2b), all the 4 strains were able to assure a significant ($p < 0.01$) reduction of lesion diameter with respect to the control. These results confirmed those previously found.

4.3.2 Colonization of apples wounds

The population dynamics of the 4 selected strains of *S. bacillaris* on artificially wounded apples are reported in Figure 3.

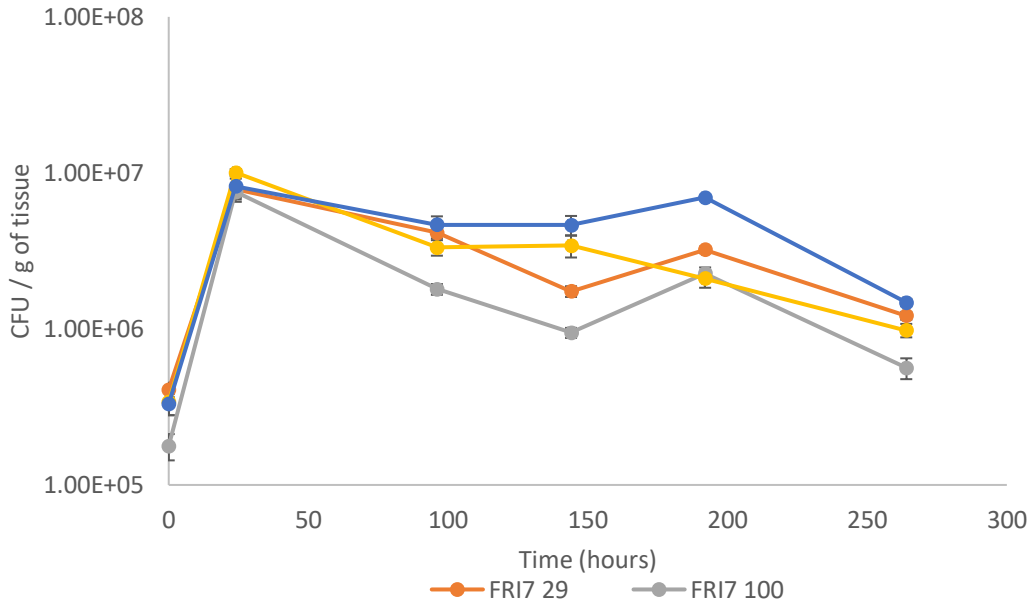


Figure 3. Population dynamics of the four selected *S. bacillaris* strains in apple wounds at 25 °C.

The population trend, starting from a similar concentration for all strains (around $3.0x10^5$ CFU/g of tissue), rapidly increase within 24 hours for all strains (around $8.1x10^6$ CFU/g of tissue). A first growth phase was then followed by a stable phase, with a progressive decline in the number of viable cells. However, this decline is limited and after 264 hours from the inoculum all the populations were at higher levels than the initial ones (around $1.3x10^6$ CFU/g of tissue).

4.3.3 Fermentation performances in apple juice

Fermentation performances of 14 strains of *S. bacillaris*, both in single-strain and in sequential fermentation together with *S. cerevisiae* EC1118, were evaluated in apple juice. The CO_2 production was followed during all the fermentation process. The fermented juices were analyzed to evaluate glucose and fructose residue and the concentrations of the main fermentation products.

As regards the single-strain fermentations (Table 2), to assess strains performances, CO_2 production after 72 h of incubation was considered in order to evaluate the adaptation ability to the juice conditions. Moreover CO_2 productions at the middle and at end of fermentation were considered.

Fermentation performances were very similar between *S. bacillaris* strains, and none of the tested strains was able to finish the fermentation after 672 hours. As expected, *S. bacillaris* strains produced low CO_2 amounts (3.10 - 4.94 g/100 ml). Strain FRI719 showed a delay in the fermentation start (0.12 g/100 ml of CO_2 after 72 h). Strain FRI779 showed the lowest CO_2 production after 672 hours (3.10 g/100 ml of CO_2).

Regarding sugars residues, *S. bacillaris* confirmed its fructophilic character (Englezos et al., 2015) consuming more fructose than glucose. The sugars residues were high (from 22.46 to 50.59 g/l), and these data were related to a limited ethanol production (from 4.09 to 6.07 % v/v). The production of secondary metabolites was strongly strain dependent. Glycerol production was generally very high (from 4.86 to 5.95 g/l), as expected, except for FRI779 (4.02 g/l). Unfortunately, also acetic acid concentrations were very high (from 1.16 to 1.86 g/l), maybe due to the long fermentation time.

Sequential fermentations were performed inoculating *S. bacillaris* strains at first and *S. cerevisiae* EC1118 after 48 hours. To assess strains performances, fermentation vigor,

in terms of CO_2 production after 48 h of incubation, was considered, together with CO_2 production after 360 hours and at the end of fermentation (528 hours).

S. bacillaris strains showed a low fermentation vigor, while EC1118 displayed a high fermentation vigor with CO_2 production values of 1.13 g/100 mL. After the addition of EC1118, *S. bacillaris* strains showed a higher fermentation rate than that of single-strains fermentations. After 360 hours from the inoculum, when EC1118 single-strain fermentation finished, sequential fermentations evidenced a lower CO_2 production compared to EC1118. This means that in sequential fermentations a lower fermentation rate than that of EC1118 single-strain fermentation occurred.

A limited fructose residue was detected (from 2.02 to 4.80 g/l), while no glucose residue was found. Ethanol concentration in EC1118 single-strain fermentation was 8.73 % (v/v) and in sequential fermentations ranged from 8.11 to 8.91 % (v/v). No significant differences were found between EC1118 fermentation and sequential fermentations. This could be due to the lower sugar concentration present in apple juice. Glycerol concentration in sequential fermentations was higher than that of EC1118, ranging from 3.76 to 5.11 g/l, whereas EC1118 single-strain fermentation produce only 3.22 g/l of glycerol. Acetic acid concentrations were very limited and lower than those found during *S. bacillaris* single-strains fermentations (ranging from 0.62 to 0.92 g/L), and also EC1118 produce only 0.50 g/l of acetic acid.

Table 2. CO_2 production during fermentation, glucose and fructose residue and concentrations of the main fermentation products at the end of single-strain fermentation of *S. bacillaris* in apple juice. Data are expressed as the average of three replicates ± standard deviations. Different letters indicate significant differences between values (Tukey's test, p=0.05).

Strain	CO_2/100ml			Glucose (g/l)	Fructose (g/l)	Glycerol (g/l)	Acetic acid (g/l)	Ethanol (%v/v)
	72 h	336 h	672 h					
PAS13	0.59±0.01^{AB}	3.29±0.19^{A}	4.91±0.69^{A}	16.03±7.82^{E}	6.43±0.81^{B}	5.43±0.25^{A}	1.86±0.19^{ABC}	6.07±0.15^{A}
PAS55	0.56±0.03^{BC}	3.34±0.10^{A}	4.92±0.20^{AB}	24.62±0.49^{BCD}	9.97±2.24^{B}	5.84±0.19^{A}	1.85±0.04^{A}	5.64±0.22^{AB}
PAS66	0.52±0.07^{BC}	2.85±0.34^{ABC}	4.58±0.59^{AB}	21.97±1.32^{DE}	16.80±6.41^{AB}	5.58±0.38^{A}	1.37±0.05^{A}	5.13±0.49^{ABC}
PAS92	0.47±0.07^{BC}	2.89±0.36^{ABC}	4.54±0.49^{AB}	21.70±2.78^{DE}	14.43±7.50^{AB}	5.50±0.55^{A}	1.42±0.13^{ABC}	4.63±0.88^{ABC}
PAS103	0.53±0.01^{BC}	3.10±0.20^{AB}	4.66±0.28^{AB}	21.65±1.86^{DE}	10.52±3.75^{B}	5.57±0.33^{A}	1.50±0.13^{ABC}	5.42±0.32^{ABC}
PAS151	0.57±0.14^{ABC}	3.20±0.48^{AB}	4.43±0.42^{AB}	27.89±2.36^{ABCD}	10.01±8.95^{B}	5.52±0.43^{A}	1.47±0.09^{ABC}	5.11±0.52^{ABC}
PAS173	0.55±0.01^{BC}	3.04±0.07^{ABC}	4.60±0.09^{AB}	24.29±0.93^{CD}	9.42±2.35^{B}	5.30±0.41^{AB}	1.46±0.14^{ABC}	5.30±0.26^{ABC}
FRI719	0.12±0.01^{D}	2.50±0.12^{BC}	3.99±0.14^{BC}	32.11±0.26^{ABC}	17.40±3.81^{AB}	5.23±0.24^{AB}	1.16±0.53^{C}	5.35±0.28^{ABC}
FRI728	0.54±0.06^{BC}	2.68±0.15^{ABC}	3.92±0.20^{BC}	32.60±0.40^{AB}	17.99±3.15^{AB}	4.86±0.29^{AB}	1.52±0.11^{BC}	4.79±0.40^{ABC}
FRI729	0.45±0.04^{BC}	2.73±0.16^{ABC}	4.20±0.21^{B}	28.63±0.51^{ABCD}	15.03±3.03^{AB}	5.48±0.04^{A}	1.74±0.18^{ABC}	5.42±0.18^{ABC}
FRI751	0.41±0.08^{C}	2.58±0.34^{BC}	4.13±0.42^{BC}	34.02±0.92^{A}	15.79±5.22^{AB}	5.95±0.91^{A}	1.78±0.20^{ABC}	4.13±0.68^{BC}
FRI754	0.44±0.03^{BC}	2.83±0.16^{ABC}	4.24±0.25^{B}	31.02±1.13^{ABC}	15.42±2.58^{AB}	5.91±0.97^{A}	1.35±0.09^{AB}	4.12±0.89^{BC}
FRI779	0.74±0.03^{A}	2.35±0.10^{C}	3.10±0.11^{C}	14.25±0.15^{E}	27.49±0.61^{A}	4.02±0.13^{B}	1.72±0.09^{ABC}	4.09±0.16^{C}
FRI7100	0.58±0.07^{ABC}	3.33±0.19^{A}	4.94±0.19^{AB}	25.53±4.65^{BCD}	10.30±2.56^{B}	5.92±0.16^{A}	1.71±0.25^{ABC}	4.26±0.80^{BC}

4.4. Discussion

Biological control is an interesting alternative to the use of synthetic fungicides against fruits postharvest molds. Yeasts have been extensively studied as promising biocontrol agents because of their simple nutritional requirements, their ability to colonize dry surfaces for long periods of time and grow rapidly in bioreactors. Moreover, they do not produce allergenic spores, mycotoxins or antibiotics as fungi or bacteria do (Parafati et al., 2015).

In this work, 14 strains belonging to the *S. bacillaris* species have been studied for their potential biocontrol efficacy against blue mold of apples caused by *P. expansum*. These

strains were previously reported to be antagonists of *B. cinerea* on grapes (Lemos Junior et al., 2016).

The antagonistic activity of the strains was tested on wounded apples artificially inoculated with *P. expansum*. Applying 10^5 yeast cells per wound, 4 out of the 14 strains were able to significantly ($p < 0.01$) reduce *P. expansum* growth and lesions at 25 °C. Disease reduction inhibition was from 29.0% to 44.5%. The ability of the 4 strains significantly inhibiting the pathogen to colonize artificial wounds on apples was also verified. The ability of biocontrol agents to survive and multiply colonizing the wounds is critical to compete effectively with pathogens infecting fruits surface.

After 24 hours the population trend rapidly increased (around 8.1×10^6 CFU/g of tissue). This growth phase was then followed by a progressive but limited decline in the number of viable cells, in fact, after 264 hours from the inoculum all the populations were at higher levels than the initial ones (around 1.3×10^6 CFU/g of tissue). This finding demonstrated that *S. bacillaris* strains can easily grow and develop in wound on apples and colonize this environment. This result is in line with the isolation from apples of other species belonging to *Candida* genus: *Candida sake*, *Candida lambica* and *Candida stellimalicola* (Viñas et al., 1998; Donaghy et al., 1999; Suzuki et al., 1994). Yeasts ability to grow and colonize wounds on fruits is fundamental to achieve its inhibition activity on pathogens development. In fact, growing and consuming surface nutrients, *S. bacillaris* compete with the development of other microorganisms on fruits surface.

Moreover, due to the well-known fermentation properties of *S. bacillaris*, the fermentation performances in apple juice of the 14 non-*Saccharomyces* strains were tested, both in single-strain fermentation and in sequential fermentation, together with *S. cerevisiae*, to evaluate the possible positive effects in cider production. A cell concentration of 1.5×10^6

cells/ml was used to inoculate apple juice, to reproduce a concentration similar to that found on colonized apple wounds. In several studies, sequential fermentations of *S. bacillaris* with *S. cerevisiae* at this inoculum concentration had a positive effect on wine (Rantsiou et al., 2012; Lemos Junior et al., 2016).

Single-strain fermentations confirmed *S. bacillaris* fructofilic character, together with a high glycerol production and a slow fermentation rate. In sequential fermentations the presence of *S. bacillaris* strains significantly increased glycerol content, compared to *S. cerevisiae* single-strain control, and produced low acetic acid concentrations. Fermentation vigour was significantly lower than that of EC1118 single-strain fermentation.

This work reported for the first time the potential of *S. bacillaris* as biocontrol agent of blue mold in apples caused by *P. expansum*. Moreover, the fermentation performances of this yeast in apple juice have been demonstrated and could improve cider quality. Due to *S. bacillaris* ability to colonize wounds on apples and to inhibit *P. expansum* growth, this yeast could be used as a postharvest biocontrol agent on stored apples and its presence on fruits surface could influence the following apple juice fermentation, in sequential inoculums with *S. cerevisiae*.

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CHAPTER 5

Effects of the mannoprotein of the non-conventional yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine stability

5.1 Introduction

Mannoproteins are macromolecules released by yeasts during alcoholic fermentation and during the autolysis of cells in wine ageing on lees (Chalier et al., 2007; Dupin et al., 2000). Their release depends on yeast strain (Gonzales-Ramos et al., 2008), must turbidity (Boivin et al., 1998), temperature (Llauberes et al., 1987), nutritional condition (Ribereau-Gayon et al., 2006) and the chemical composition of the wine (pH, ecc).

Mannoproteins are proteins with a high sugar content, mainly mannose, and due to this composition they behave like polysaccharides rather than like proteins. (Quiros et al., 2010). They are, together with fibrous β-1,3-glucan, one of the major component of the yeast cell wall, representing 35% of total polysaccharides in wine (Vidal et al., 2003).

The cell wall of yeast is mechanically strength and highly elastic. It functions as a protective coat against physical stress, it is involved in maintaining osmotic homeostasis and contributes to morphogenesis maintaining cell shape (Klis et al., 2006).

In the past years, mannoproteins, due to their oenological properties, become one of the most interesting yeast molecules for the improvement of wine quality.

Addition of mannoproteins obtained from hydrolysis of yeast cell wall was authorized by the European Community since 2005 (OIV, 2012).

One of the first enological properties described for yeast mannoproteins is the protection against protein precipitation in white wines. Ledoux et al. (1992) showed that wine aged on yeast lees had lower haze potential and lower bentonite requirements for stability

than wine aged without lees. The active component was identified as a 32 kDa fragment of yeast invertase (Moine-Ledoux & Dubourdieu, 1999).

Waters and colleagues (1993, 1994) isolated a high molecular weight mannoprotein from wine that is able to prevent visible wine protein haze formation. This molecule, called Haze Protective Factor (HPF) was characterized and a putative structural gene has been identified in the *Saccharomyces cerevisiae* genome (Dupin et al., 2000).

Another property attributed to mannoproteins is the protection against tartaric instability. Mannoproteins inhibit the crystallization of tartrate salts by lowering the crystallization temperature (Gerbaud et al., 1996; Moine-Ledoux & Dubourdieu, 2002) preventing the occurrence of precipitates in wine.

Furthermore mannoproteins improve the foam of sparkling wines. Their hydrophobicity, high glycosylation and high molecular mass allow mannoproteins to surround and thus stabilize the gas bubbles of the foam. It has been demonstrated that both the production and stabilization of foam also depend on other proteins, however the main contributors to the foam formations are mannoproteins (Coelho et al., 2011; Vincenzi et al., 2014).

Some authors proposed recombinant *S. cerevisiae* wine yeasts engineered to overproduce mannoproteins (Gonzales-Ramos & Gonzales, 2006; Gonzales-Ramos et al., 2008; Brown et al., 2007). To bypass countries regulation in the use of GMOs in food, Quiros et al. (2010) developed a non-recombinant method to select wine yeasts overproducing mannoproteins, consisting in a random mutagenesis using UV light as a physical agent, followed by a direct selection on YPD plates containing killer 9 toxin from *Williopsis saturnus.*

To avoid the use of these microorganisms, likely to be viewed as releasing components not normally present in wine, Domizio et al. (2014) proposed the use of non-*Saccharomyces*

wine yeasts, found in grape and winemaking environments, as novel sources of mannoprotein in wine. Giovani et al. (2012) firstly demonstrated that non-*Saccharomyces* yeasts can release polysaccharides from cell wall during alcoholic fermentation, like *S. cerevisiae.*

They found out that the amount released depends on yeast species and cells vitality. Non-*Saccharomyces* yeasts, once defined spoilage microorganism, predominate in the early stages of must fermentation, and recently their role has been reevaluated as some of them were found to enhance the analytical composition and aroma profile of the wine (Ciani and Comitini, 2015). Many studies indicate that the use of the non-*Saccharomyces* yeast *Starmerella bacillaris* (synonym *Candida zemplinina*), together with *S. cerevisiae*, enhance the glycerol content of wines, with moderate volatile acids production, and, due to the low ethanol yield, reduce ethanol content (Rantsiou et al., 2012; Bely et al., 2013; Wang et al., 2014; Lemos Junior et al., 2016).

In this study seven different strains of the non-conventional yeast *S. bacillaris* have been studied for their ability to produce and release mannoproteins during fermentation in synthetic must, both in single-strain and in sequential fermentation together with *S. cerevisiae*. These molecules have been quantified, characterized in terms of carbohydrate composition analyzed by HPLC, and used to assess their ability to reduce protein and tartrate instability.

5.2 Materials and methods

5.2.1 Yeast strain and fermentation trials in synthetic

The yeasts strains used in this work (Table 1) were isolated from fermenting must obtained from dried grape of Raboso piave variety as described by (Lemos Junior et al.,

2016). A loopful of a 3-day-old culture of each yeast strains from YPD agar plate (yeast extract 10 g/L, peptone 10 g/L, dextrose 20 g/L) was used to inoculate 10 mL of YPD broth in 50 ml tubes.

A stationary phase culture with approximately 10^7-10^8 cells/mL, determined by OD measurements and confirmed by means of plate counts analysis (CFU/ml), was obtained after 24 hours of incubation at 30 °C. In single-strain fermentation the inoculum concentration was 1-1.5x10^6 cells/ml. In sequential fermentation the same inoculum size for both *S. bacillaris* strain and *S. cerevisiae* EC1118 (1x10^6 cells/ml) has been used. EC1118 was added 48h after the inoculum of *S. bacillaris*.

Fermentations were run in synthetic must MS300, prepared as described by Bely et al. (1990), with 100 g/L of glucose, 100 g/L of fructose and 6 g/L of DL-malic acid, pH 3.3. Fermentation trials have been performed in triplicate in 120-ml capacity bottles as described by (Bovo et al., 2016). After yeast inoculation the bottles were incubated at 20°C. CO_2 production was followed by measuring twice a day the weight loss of each culture. When the weight loss was lower than 0.05 g per day the fermentations were considered concluded.

Table 1. Yeast strains used in work.

Strain	Species	Origin
FRI719	*S. bacillaris*	Winery A
FRI728	*S. bacillaris*	Winery A
FRI729	*S. bacillaris*	Winery A
FRI751	*S. bacillaris*	Winery A
FRI754	*S. bacillaris*	Winery A
FRI779	*S. bacillaris*	Winery A
FRI7100	*S. bacillaris*	Winery A
EC1118	*S. cerevisiae*	Industrial strain

5.2.2 Macromolecules isolation

The fermentation broth was filtered at 0.45 µm (cellulose acetate filter, Sartorius) and successively ultrafiltered using an Amicon 8400 apparatus (3000 Da cutoff, regenerated cellulose, Millipore) under a constant nitrogen pressure of 3.5 bar. The retentate was carefully recovered, dialysed against water using a regenerated cellulose membrane (3500 Da, Fisherbrand), then freeze dried. The obtained powder was weighted and resuspended in water at 10 mg/mL.

5.2.3 Characterize mannoprotein

To confirm that polysaccharides isolated were really mannoproteins, samples of FRI751 single and sequential fermentation and single EC1118 were used.

5.2.3.1 Spectroscopy

Spectroscopy was performed at the Brazilian Center for Physical Research (CBPF), with the infrared spectrophotometer (FT-IR Prestige - 21 / Shimadzu). Clear pellets of KBr were prepared with a mixture in a 1:10 ratio (sample / KBr), followed by a uniaxial pressure of the powder under vacuum. All spectra were obtained between 4000 and 600 cm^{-1} and at 2 cm^{-1} resolution.

5.2.3.2 Scanning microscopy

Dry macromolecules isolated were fixed for 2–3 h with 3% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2. The samples were then dehydrated in ethanol, sputtered with gold

under vacuum in a Fine Coat Ion Sputter JFC-1100 (Japan), and examined under a scanning electron microscope (JSM-6510LV).

5.2.4 HPLC analysis

The glucose and fructose residue, as well as the glycerol and ethanol production, were quantified by Waters 1525 HPLC binary pump with a 300×7.8 mm stainless steel column packed with Aminex HPX_87H 300×7.8 mm was used. Waters 2414 Refractive Index Detector, set at 600 nm wavelength for the determination of glucose glycerol and ethanol while for the acetic acid quantification a UV detector,which was run at 65°C with 5 mM H_2SO_4 as the mobile phase, with a flow rate of 0.5 mL/min. An additional internal standard solution of mannose, glucose, fructose, glycerol and ethanol were prepared in milQ water creating a calibration curve for each analyte to be quantified using linear.

Composition sugars was performed as reported above, with an external calibration curve prepared using an aqueous solution of 100 mM H2SO4 to same the conditions as describe above after the mannoprotein hydrolysis; concentrations of each compound ranged from 250 mg/L to 1 g/L.

5.2.5 Tartaric stability

The wine tartaric stability was measured by the mini contact test and was carried out by a Tartar Check (Ing.C.Bullio, San Prospero, Italy). The variation of electric conductivity (Δx), expressed in µs/cm, indicated the level of stability. Briefly, 20 mL of wine were brought to 0 °C, then a dose of 300 mg micronized potassium hydrogen tartrate was added (corresponding to a final concentration of 15 g/L) and the conductivity decrease due to tartaric acid precipitation was followed during 10 minutes. A very unstable wine (Prosecco

wine, alcohol content 11.3%, 267µS drop) was used to test the effect of yeast macromolecules, added to the wine at a final concentration of 200 mg/L before the mini contact test.

5.2.6 Protein stability

The protein stability was evaluated measuring the turbidity of wine after a treatment at 80 °C for 6 h followed by 4°C for 12 h (Pocock e Rankine, 1973). The turbidity was measured by a Nephelometer (HI83749, Hanna Instruments) and the difference between the value before and after the treatment was used for calculations. A very unstable wine (Traminer wine, alcohol content 12.4%, 225 NTU after heating) was used to test the effect of yeast macromolecules, added to the wine at a final concentration of 200 mg/L before the heating.

5.2.7 Statistical analysis

Scatter plot analysis, principal component analysis (PCA) and one-way analysis of variance (ANOVA) at 95 % accuracy level were made with XLSTAT software, vers.7.5.2 (Addinsoft, Paris, France).

5.3 Results and discussion

5.3.1 Fermentation performances and mannoproteins release

Fermentation performances of 7 strains of *S. bacillaris*, both in single-strain and in sequential fermentation together with *S. cerevisiae*, were evaluated in MS300 synthetic must. The industrial wine strain *S. cerevisiae* EC1118 was used as control. The total

macromolecules obtained from the culture filtrates (i.e. mainly mannoproteins) were recovered, freeze dried and weighed.

The growth kinetics of the fermentation were represented by fermentation vigor, in terms of CO_2 production after 48 h of incubation, CO_2 production after 312 h and at the end of fermentation (624), together with sugars consumption and secondary metabolite production, were considered to assess fermentation performances (Table 2 and 3).

All the fermentations trails were stopped after 624h, when the fermentation of *S. cerevisiae* EC1118 was completed.

As regards the single fermentation, Strains FRI729, FRI751, FRI754, FRI779 and FRI7199 showed high fermentation vigor (CO_2 production after 48 h) unlike the case of FRI728 and FRI719 evidenced a significant lower CO_2 production. On the contrary, after 321 and 624 hours not high differences were detected. As expected, glucose residue (from 80.63 to 91.50) and fructose residue (from 20.19 to 32.93) can be related to a limited ethanol production (from 4.15 to 5.25% v/v). Glycerol production by *S. bacillaris* was very high (from 5.58 to 7.16 g/L).

Sequential fermentations were performed adding *S. bacillaris* strain at first, followed, after 48 h, by *S. cerevisiae* EC1118.

Table 2. CO_2 production during fermentation, residual glucose and fructose concentrations and glycerol and ethanol concentrations of the main fermentation products at the end of the fermentation of *S. bacillaris* strains in MS300. Data are expressed as the average of three replicates ± standard deviations. Different letters indicate significant differences between values (Fisher's test, p=0.05).

Strain	CO2/100mL			Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (%v/v)
	48 h	312h	624h				
FRI719	0.03±0.00c	1.58±0.25b	3.63±0.58a	87.24±3.50ab	21.33±9.07bc	5.58±0.02c	4.69±0.20b
FRI728	0.00±0.00c	1.06±0.02c	2.97±0.10b	84.92±3.10bc	32.93±1.19a	4.77±0.30d	4.15±0.24c
FRI729	0.22±0.02b	1.57±0.12b	3.12±0.23ab	90.39±0.35°	29.07±1.16ab	6.03±0.15b	4.45±0.13cb
FRI751	0.26±0.04ab	1.81±0.29ab	3.28±0.50ab	80.63±4.82c	22.65±3.68bc	5.72±0.20bc	4.52±0.10bc
FRI754	0.23±0.01ab	1.63±0.06b	3.10±0.07ab	91.50±0.01°	31.51±0.56a	5.88±0.30bc	4.48±0.12bc
FRI779	0.23±0.01ab	1.85±0.06ab	3.60±0.11a	89.75±1.81°	22.01±2.06bc	7.05±0.12a	5.25±0.18a
FRI7100	0.27±0.04a	2.00±0.27a	3.74±0.50a	82.87±0.84bc	20.19±8.46c	7.16±0.18a	5.21±0.60a

As reported in table 2, *S. bacillaris* can be divided into two groups: based on the fermentation vigor (FRI719, FRI728, FRI751, FRI779), and (FRI729, FRI779, FRI7100) that produced up 0.18 to 0.31 of CO_2/100mL. Finally high fermentation vigor (EC1118) with CO_2 production values of 1.19. after the addition of EC1118 a high fermentation rate than that of *S. bacillaris* single-strain fermentation occurred. There is another points worth noting about the performance of strains.

All strains that showed low fermentation vigor at 312 and 624 were significantly similar to EC1118 CO_2 production. On the contrary, other *S. bacillaris* strains produce significantly less CO_2 than EC1118. Only was FRI719, FRI729, FRI754, FRI779, and FRI7100 were tested a limited sugar residue was (from 9.38 to 5.12 g1/L). Glycerol concentration in sequential was from 7.13 to 9.69 g/L whereas EC1118 single strain fermentation produce ongly 6.23 g/l of glycerol. Ethanol concentration ranged between 13.53 and 11.59% (v/v).

Significant differences were found between EC1118 fermentation and sequential fermentations for all strains tested except for FRI719 and FRI728. This effect can confirm the relation between fermentative vigor and metabolic fermentation production as describe previously.

Table 3. CO_2 production during fermentation, residual glucose and fructose concentrations and concentrations of the main fermentation products at the end of the sequential fermentation of *S. bacillaris* strains with EC1118 in MS300. Data are expressed as the average of three replicates ± standard deviations. Different letters indicate significant differences between values (Fisher's test, p=0.05).

Strain	CO2/100mL			Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Ethanol (%v/v)
	48 h	312h	624h				
EC1118	1.19±0.09a	8.10±0.78bcd	9.47±0.12a	-	-	6.23±0.32d	14.03±0.06a
FRI719	0.00±0.00d	8.85±0.12ab	9.45±0.07a	1.39±0.33c	3.73±1.76a	7.66±0.11bcd	13.56±0.23a
FRI728	00±0.00d	7.37±0.41d	9.16±0.08b	-	-	7.13±0.33cd	13.52±0.36a
FRI729	0.18±0.01c	8.20±0.46bc	8.73±0.09d	1.80±0.80cb	3.66±2.27a	7.41±0.07cd	12.12±0.51bc
FRI751	0.00±0.00d	9.09±0.26a	9.39±0.02a	-	-	9.44±0.67ab	12.50±0.05b
FRI754	0.31±0.00b	8.56±0.04abc	8.91±0.21c	3.32±2.32ba	2.87±1.69a	8.46±1.19abc	12.54±0.66b
FRI779	0.00±0.00c	9.11±0.19a	9.40±0.08a	4.44±1.16a	3.64±1.41a	8.59±0.65abc	12.21±0.24cb
FRI7100	0.22±0.039c	7.82±0.99cd	8.76±0.07cd	4.85±1.62a	4.53±2.24a	9.69±2.86a	11.59±0.25c

The fermentation was conducted from the MS300 synthetic without protein or polysaccharide, so at the end of fermentation every polysaccharide present at the end of the fermentation comes exclusively produced and left by the yeast (Figure 1). *S. cerevisiae* strain produced significantly more protein (119 mg/L) than *S. bacillaris* single inoculum (50 mg/L) confirmed by statistic analyses ($p<0.001$).

This effect can be explained by fermentation length in fact EC1118 after 26 days (Fig. 1). These results are different from those obtained by (Domizio, Liu, Bisson, & Barile, 2014) who described that *S. bacillaris* #22 showed a similar level of total polysaccharides (aourond 80 mg/l) compared to *S. cerevisiae* EC1118 (around 200 mg/L). However, the conditions of

fermentations were different such as temperature, agitation at 150 rpm, type of synthetic must may have influenced the different found among *S. cerevisiae* EC1118.

At the sequential fermentations (151-270 mg/L) showed higher polysaccharides compared to *S. cerevisiae* single fermentation (119 mg/L). These results are in agreement with Comitini et al., (2011) where the *S. bacillaris* in sequential fermentation with *S. cerevisae* produced more polysaccharides compared to *S. cerevisiae* single in natural grape juice.

The higher mannoprotein production in sequential inoculum cannot be ascribed just to the presence of two microorganisms in the same medium, as in some cases the total mannoprotein content is significantly higher than the sum of the mannoprotein released by the two microorganisms when individually cultured. This behavior indicates that an interaction exists between the two microorganisms during fermentation, leading to modification in the mannoprotein release into the culture medium.

The different mannoproteins obtained from the filtered media, were suspended in water at 10 mg/ml and then used for further characterization.

5.3.2 Mannoprotein identification

FRI751, FRI751 sequential fermentation and single EC1118 were used to confirm that the isolate samples were mannoproteins from yeast cell wall.

In Figure 1 similar spectra are observed for the three mannoproteins obtained, with no significant differences being evidenced visually. In general, two important bands can be observed in the infrared spectra of all secondary amides in the solid state. The first, denoted amide I band, is attributed to C=O stretching vibrations, and appears around 1640 cm^{1}. The other, known as amide II band, corresponds to a combination of N–H bending with C–H

stretching vibrations, and appears on the region 1570– 1515 cm^{1}. In proteins and peptides, these bands are structurally important because they give information on the conformation and structural stability (Gulão et al., 2016).

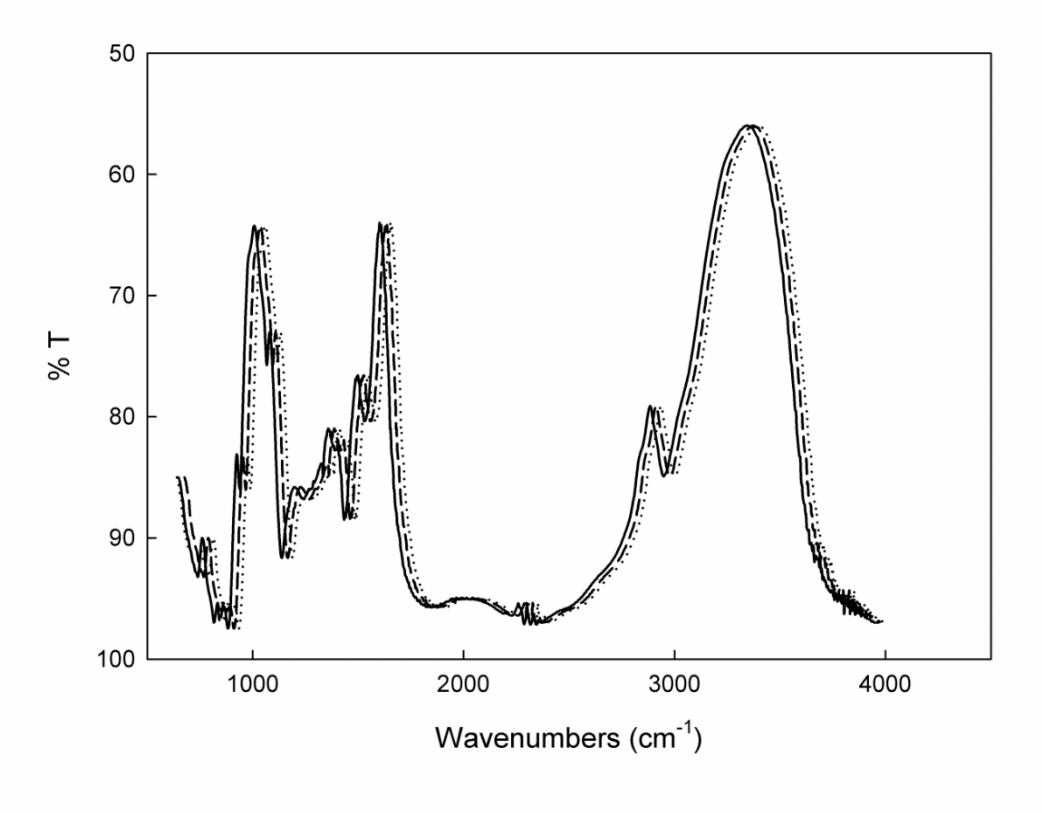


Figure 1. Infrared spectra obtained in the region of 4,000 to 600 cm of mannoproteins obtained from: (__) Starmarella bacillaris; (- - -) Scharomyces and (…) mixture of mannoproteins.

The polysaccharide region is observed in the spectral range 925-1190 cm^{-1}. The profile in this region probably represents the absorption of sugars that are present in the yeast cell wall (Galichet et al., 2001). The cell wall is a rigid structure that maintains the integrity of the cell and interacts with the medium it is in. It is located on the outside of the cell membrane and is composed mainly of glucans and mannan and small amounts of chitins and lipids. Glucans and mannan combine to form glycoproteins, mannoproteins, and glycanoproteins (Chaffin, 2008). A pronounced band around the spectrum region of 1.000 cm^{-1} is observed.

Adt et al. (2006) attributed this band to vibrations of the C-O-C bonds of the polysaccharide ring. According to the literature, the presence of α- (1 → 4) glucans can be identified in the fingerprint zone through the wave numbers 1150 and 998 cm^{-1} and the β- (1 → 4) glucans by the existence of two peaks in the wave number 1050 and 1041 cm^{-1} (Oliveira, et al., 2009). From the figure it is possible to visualize, according to the loadings, the existence of two peaks at 1091 and 938 cm^{-1}, close to the wave numbers of α- (1 → 4) glucans. According to Mantsch and Chapman, 1996 and Maquelin et al., 2002;, the band pronounced in the region of the spectrum between 3.000 and 4.000 cm^{-1} corresponds to the stretching of the O-H group of the hydroxyl group.

Figure 2 shows typical high-resolution images of the mannoproteins, recorded from single *S. bacillaris,* sequential fermentation and single EC1118 at the end fermentation in MS300. Mannoproteins produced by FRI751 and EC1118 showed that surface morphology remained apparently homogeneous during polysaccharide synthesis, whereas significant ultrastructural changes of the cell wall could be inferred in the cellular *responses* to oxidative and *osmotic stress*, both in single strain and sequential fermentation.

The difference among mannoproteins can be related to different genes that regulate stress response pathway such as HOG pathway, as describe by Fuchs & Mylonakis, (2009), this pathway to regulate osmolality is not conserved in non-*Saccharomyces* species.

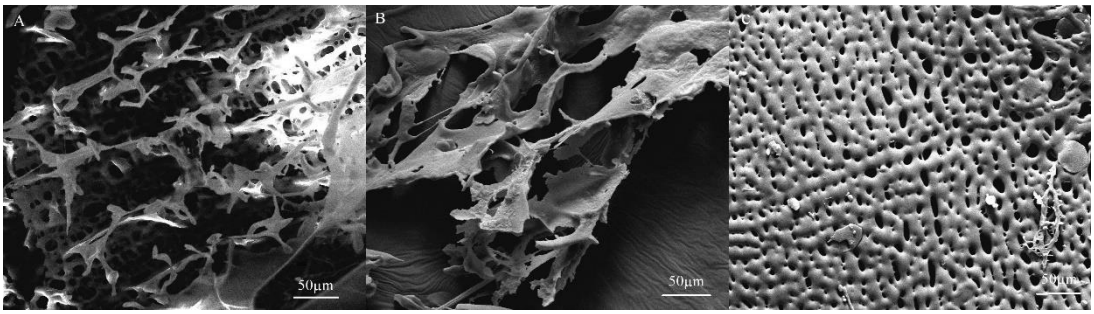


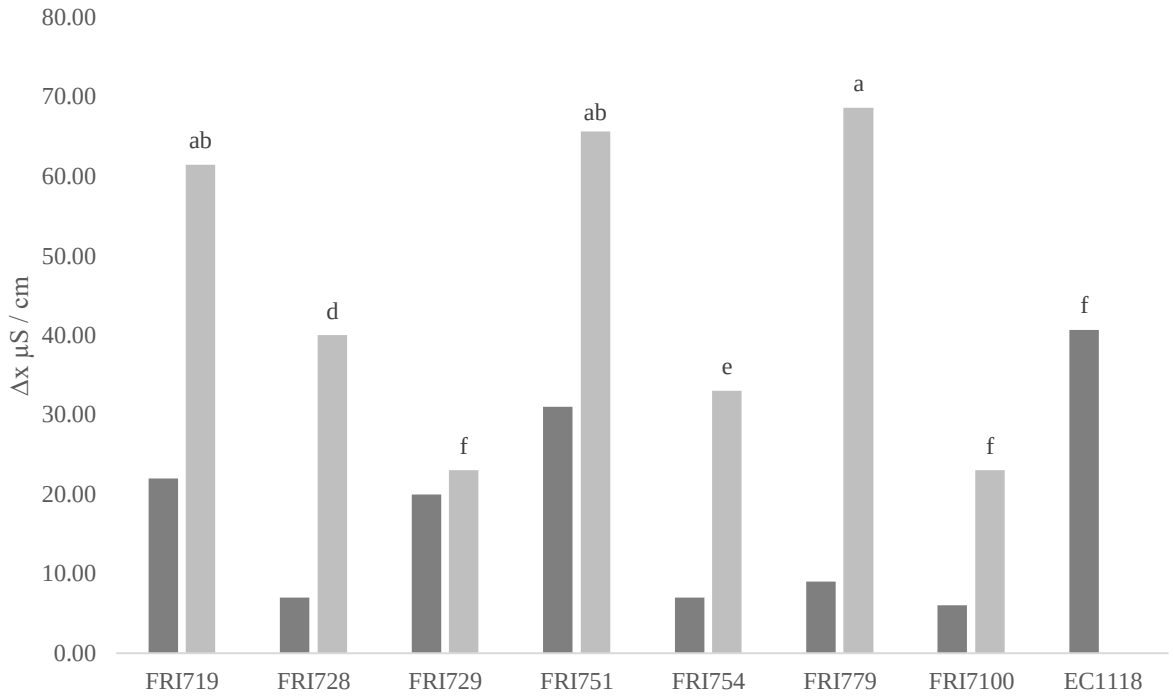
Figure 2. Scanning microscopy of pool mannoprotein at the end fermentation (A) EC118; (B)FRI751; (C) sequential fermentation first FRI751 after 48 hours EC1118.

5.3.3 Tartaric stabilization

First of all, the mannoproteins were tested for the ability to decrease the tartaric instability, by addition to a very unstable wine at a final concentration of 200 mg/L.

The tendency of tartaric acid to precipitate was determined by measuring the conductivity drop after addition of potassium hydrogen tartrate (crystallization nuclei) at 0 °C. The values are showed in Figure 3A. All the *S. bacillaris* strains single produced mannoproteins with low inhibition capacity (from 6.00 to 31.00 µS) when compared with EC1118 alone (41 µS) ANOVA $p<0.001$. The mannoproteins recovered after the sequential inoculum fermentation showed a significant higher activity, indicating a better capacity to inhibit the salt crystallization. It is possible because mannoproteins have polymeric structure acts as a ‘‘protective colloid’’, which prevents crystallization of potassium hydrogen tartrate (Ribéreau-Gayon et al., 2006).

A



B

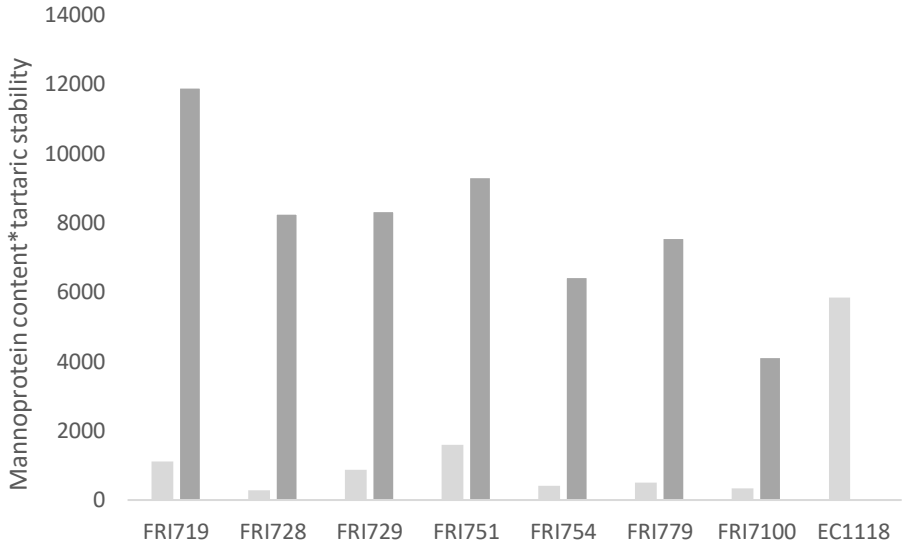


Figure 3 (A) Tartaric stabilization in the two conditions: single-strain (■) and sequential inoculum (■) fermentation. (B) Relation between mannoprotein production and to increase the tartaric stability: single-strain (■) and sequential inoculum (■) fermentation.

Considering the simultaneous increase of mannoprotein content and their ability to reduce tartaric instability, the effect of the sequential inoculum became even more evident. The multiplication of the two factors (Figure 3B) show clearly that some *S. bacillaris* strain in combination with *S.cerevisiae* is able to increase the tartaric stability in comparison with the *S. cerevisiae* alone, reaching to double the total stabilization effect in the case of FRI719. Mannoproteins produced by any *S. bacillaris* strans showed less Δx than carboxylmethylcellulose, arabic gum and mannoproteins applicated in red and white wine by (Guise et al., 2014).

5.3.4 Protein stabilization

The mannoproteins were also tested for their ability to affect the protein instability, by addition to an unstable wine at a final concentration of 200 mg/L. After addition, the wines were heated at 80°C (Pocock & Rankine, 1973) in order to induce the aggregation and precipitation of grape unstable proteins.

The induced turbidity was measured by nefelometry and the difference between the initial turbidity and that obtained after heating was taken in account. The wine used for the experiment was very unstable, giving a value of 225 NTU. In all cases the addition of mannoproteins caused a reduction of turbidity, confirming the ability of these molecules to decrease the protein precipitation (Figure 4) (Waters et al., 1993, 1994).

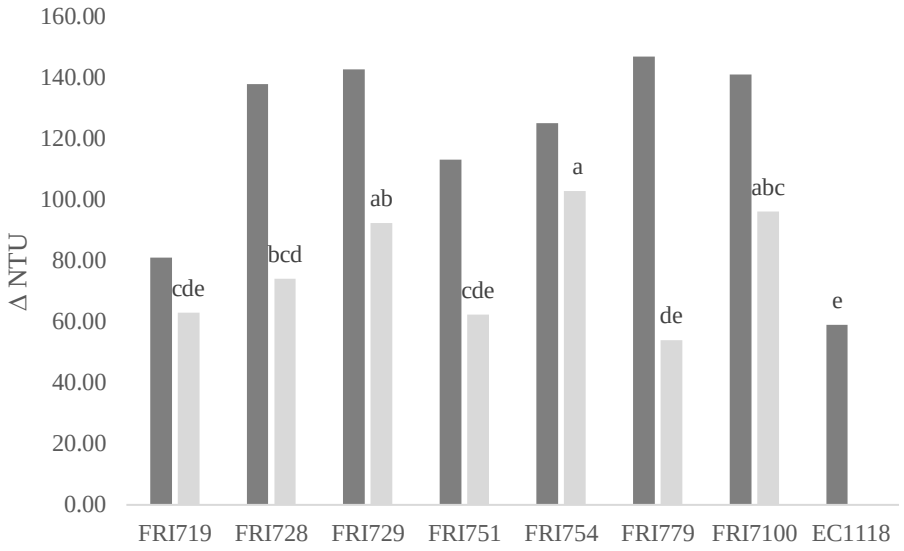


Figure 4 Protein stabilization in the two conditions: single-strain (■) and sequential inoculum (■) fermentation.

Considering the mannoproteins released by the *S. bacillaris* strains when cultured alone, the protective activity was always significantly higher than the mannoproteins produced by the EC1118 strain ($p<0.001$). Even though the *S. bacillaris* strains are not able

to complete a grape juice fermentation, this particular ability of their mannoproteins could be of great interest for the wine industry, as the selection of yeast strains producing mannoproteins with high stabilizing effect could lead to the manufacturing of new oenological products. When the same *S. bacillaris* strains were cultured in sequential inoculum with EC1118, the protein stabilization effect of the obtained mannoproteins was reduced, but in general, the stabilizing effect is comparable to that of the commercial strain alone, and in some case even higher.

It is interesting to note that the ability to decrease the tartaric instability in general do not coexist with the ability to improve the protein stability. A PCA analyses (Figure 5). Function (F1) accounted for 84.97% of the total variance, second function (F2) explained 11.51% of the total variance and was correlated ($\alpha < 0.01$). The analysis confirmed the high level of difference between the strains with different genetic profiles when they are tested as single or sequencital starter. *S. bacillaris* confirmed to be the genus with the better protein stabilizations aptitudes correleted with terms of quantity production. On the contrary sequential fermentation showed the best quantity of totally manoprotein production at the end fermentation.

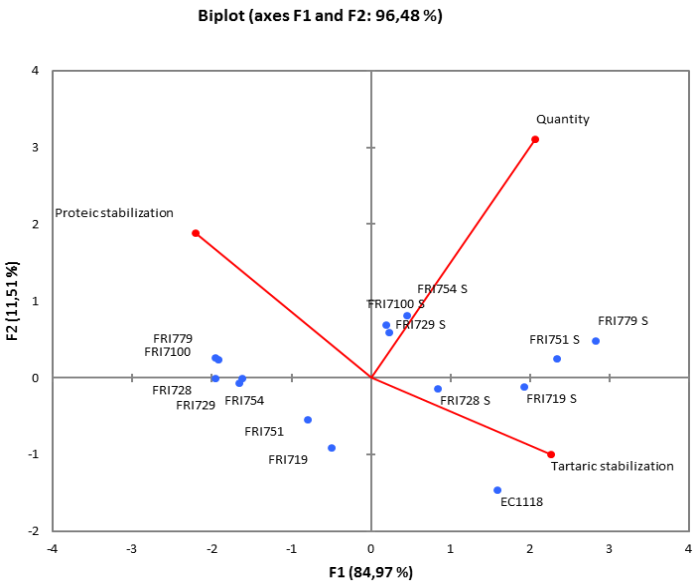


Figure 5 Principal component analysis (PCA) biplot showing fermentation performances (mannoprotein production, tartaric and protein stabilization) of single *S. bacillaris* strains and in sequential fermentation with EC1118 in MS300, single fermentation of EC1118 was used as control. Only variables that showed significant correlations are reported.

This behavior confirms that the tartaric and protein stabilization are based on different mechanisms and, consequently, differently affected by the mannoprotein structure. When an aged wine has not undergone a separation process from the less, it can be assumed that 31.8 kDa N-glycosylated, MP32 is released by the combined action of b-glucanases on the cell wall and vacuolar and excreted protease. B-glucanases and acid protease into the environment during cell autolysis, thus exerting a heat stabilizing effect on the proteins in white wine (Moine-ledoux & Dubourdieu, 1999).

5.4 Conclusion

The use of *S. bacillaris* associated or not with *S. cerevisiae* can increase glycerol concentration and reduce the amount of ethanol in wine. In addition, it can produce different mannoproteins with functional characteristics in relation to wine stability. Mannoproteins from *S. bacillaris* single presented greater potential in the increase of protein stabilization,

while in sequential fermentation, *S. bacillars* strains increased in the tartaric stabilization. These mannoproteins can aid in the reduction of bentonite during the stabilization processes of the wines.

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CHAPTER 6

Evaluation of glutathione production in yeast lees for food and beverage

6.1. Introduction

Several studies have evaluated the production of metabolites by *Saccharomyces* and non-*Saccharomyces* species (Ciani et al., 2016). Non-conventional yeast may be present in oenological environment including ground, climate and variety of starting vine, influencing the quality of wine. Among the non-*Saccharomyces*, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum*, and *S. bacillaris* can confer specific characteristics to wines, altering aroma and flavour (Jolly et al., 2014).

Sequential or mixed fermentation using *S. bacillaris* (formerly *Candida zemplinina)* with *S. cerevisiae* has been widely investigated in the last years (Bovo et al., 2016; Ciani et al., 2016; Contreras et al., 2014; García et al., 2016; Tristezza et al., 2016).

The presence of *S. bacillaris* as inoculum has shown a complementary consumption of glucose and fructose; an increase of glycerol and succinic acid contents, biocontrol ability without increase acetoin contents in wines (Englezos et al., 2015; Lemos Junior et al., 2016; Rantsiou et al., 2012). However, the interaction between the yeasts during osmotic stress at the beginning of the fermentation process involves complex reactions. Antioxidant compounds such as glutathione can be produced by yeast during this process, which can help to understand the expression of genes among yeasts under these conditions (Li et al., 2004).

Glutathione or glutathione GSH (L-g-glutamyl-L-cysteinyl-glycine) is a hydrosoluble tripeptide synthesized through consecutive enzymatic reactions in the presence of ATP and it as an important non-protein molecule of the tiol group (Kiriyama et al., 2013).

GSH possess metabolic functions in cells such as protection against oxidative stress, amino acid transport across the cell membrane, elimination of heavy metals and of toxic endogenous metabolites, participating in the nitrogen metabolism and sulfur compounds(Lucas et al., 2010). Currently, GSH can be used as pharmaceutical compound, food additive and cosmetic industries as well as in sport nutrition (Jinap and Hajeb, 2010).

The use of inactive dry yeast (IDY) as source of GSH can be used in several application of food industry and winemaking, whose interest is currently increasing due to its wide range of claimed applications. During all steps of the wine fermentation, GSH can also to preserve the characteristic of aroma compounds, including volatile thiols, esters and terpenes (Jinap and Hajeb, 2010).

Usually at the end of the wine production process, yeast lees residues can be separated by various methods. In addition, this residue often is not used and, if managed incorrectly, it can serve as nutrient for undesirable microorganisms present in the wineries (Loureiro and Malfeito-Ferreira, 2003),because during the winemaking process produces large volumes of waste streams, including solid organic waste, wastewater, greenhouse gases, and packaging waste (Lucas et al., 2010). Then, in order to assess the content of GSH in yeast less at the end of wine fermentation, this chapter aims to evaluate the use of different *S. bacillaris* strains as potential source of GSH from yeast lees in food and beverage applications.

6.2. Materials and methods

6.2.1 *S. bacillaris strains*

Fourteen *Starmerella bacillaris* strains (FRI719, FRI728, FRI729, FRI751, FRI779,FRI7100, PAS 13, PAS 55, PAS 66, PAS 92, PAS 103, PAS151 and PAS173) were isolated from dried grape of Raboso piave variety as described by Lemos Junior et al. 2016.

EC1118 was used as control in sequential fermentation. The starter cultures were prepared from YPD agar plate (yeast extract 10 g/L, peptone 10 g/L, dextrose 20 g/L) to inoculate 5 mL of YPD broth in 15 mL tubes. A stationary phase culture with approximately 7.10^7 - 3.10^8 cells/mL was determined by OD measurements and confirmed by plate counts (CFU/ml), which was obtained after 24 hours of incubation at 30°C.

6.2.2 Fermentations trials

During the fermentation at 20 °C, the inoculum size was the same for all *S. bacillaris* and *S. cerevisiae* (EC1118) strain (1.5 $x10^6$ cells/ml). In the sequential fermentation, *S. bacillaris* was added in the synthetic must MS300 and the EC1118 was added latter after 48h. To undestend the impact of GSH production by *S. bacillaris* strains, single fermentation was carried out with only two strains sequence yet: FRI751 and PAS13 in other to figure out difference among genome level of the strains stuided. The MS300 was prepared with 100 g/L of glucose, 100 g/L of fructose and 6 g/L of malic acid with pH 3.3 according Bely et al. (1990). Then, when the weight loss was lower than 0.05g per day, the fermentations were considered concluded. All the experiments were performed in bottle (100 mL of must MS300) and in triplicate.

6.2.3 Thiols

Yeast lees from synthetic wine were collected at the end of fermentation, which were resuspended in 5 mL of buffer solution. This procedure was performed twice, adding the buffer solution before each centrifugation (15 mL tubes). The samples were weighted and resuspended in 0.1M HCl solution and glass beads were added to break the cells. After the cellular lysis, the total thiols (cysteinyl-glycine, γGluCys, GSH) were ascertained by

derivatization with the fluorescent dye SBD-F as described in Masi et al., 2002 and by reverse-phase HPLC separation equipped with a refractive index detector fluorescent (excitation wavelength: 386 nm; emission wavelength: 516 nm). The chromatographic conditions were realized with the LC C18 100Å column (150 mm × 4.6 mm I.D., 5µm particle size; Luna, Phenomenex, USA), which was run at a flow rate of 1mL/min and at room temperature. The mobile phase was $NH4^+$ formiate 50 mM pH 2.9 containing 3% methanol.

Calibration curves of cysteinyl-glycine, γGluCys, GSH were made before of sample injection on HPLC. Fisher's test was used as comparison test when samples were significantly different after ANOVA ($p < 0.05$).

The thiols were quantified using a calibration curve; the concentration of thiols injected was in the range of γ-glutamyl-cysteine, 0.01–0.5µmol/L cysteinyl –glycine and 0.5 –25µmol/L glutathione.

6.2.4 Statistical Analysis

The statistical data analysis was performed with XLSTAT software, vers.7.5.2 (Addinsoft, Paris, France) the one-way analysis of variance (ANOVA) at 95% accuracy level. Fisher's test was used as comparison test when samples were significantly different after ANOVA ($p < 0.05$).

6.3. Results and discussion

6.3.1 GSH production by S. bacillaris and EC1118 in sequential fermentation

Yeasts can synthetized Glutathione (GSH) through three pathways in different organisms such as a bifunctional γ-glutamylcysteine synthetase/glutathione synthetase (GshF); an alternative condensation of γ-glutamyl phosphate synthesized by γ-glutamyl

kinase (Pro1 or ProB) with cysteine to form γ-glutamylcysteine which was further conjugated to glycine by glutathione synthetase (Tang et al., 2015).

However, conventional GSH biosynthetic pathway from two consecutive enzymatic reactions catalyzed by γ-glutamylcysteine synthetase (Gsh1 or GshA) and glutathione synthetase (Gsh2 or GshB) was evaluated.The sequential performance fermentation of the 14 strains of *S. bacillaris* was carried as described by (Lemos Junior et al., 2016). Precursors γ-glutamylcysteine, cysteinyl-glycine and total GSH were analysed. These results are shown in Table 1. As can the concentration of cysteinyl-glycine was different among the samples obtained from sequential fermentation and the control (from 0.79 to 2.53 nmol g^{-1} FW). The γGluCys content was from 0.10 to 1.22 nmol g^{-1} FW. Regarding GSH compound, its production was strongly dependent of strain. The results showed a high variability among yeast lees (0.92 to 6.39). PAS13 and PAS92 showed more GSH production than EC1118. Due to high variation in GSH production between the samples in the sequential fermentations, two *S. bacillaris* were chosen by the following criteria: high (PAS13) and low (FRI751) GSH production, using as reference the EC1118 GSH production. Single fermentations of these strains were run in the same condition used for the synthetic must.

Table 1: Thiol content at the end (624h) single and sequential fermentation.

Sequencial Fermentation				Single Fermentation			
Strain	**CysGly**	**gGluCys**	**GSH**	**Strain**	**CysGly**	**gGluCys**	**GSH**
EC1118	1.87±0.41^{b}	1.96±0.03^{ab}	5.19±0.43bcd	PAS13	2.19±0.03^{a}	2.38±1.01^{a}	8.64±0.24^{a}
FRI719	0.91±0.28^{f}	0.39±0.05^{d}	2.21±0.78f	FRI751	1.25±0.35^{b}	1.54±4.03^{b}	7.36±0.49^{b}
FRI728	0.79±0.08^{f}	0.10±0.03^{e}	2.34±0.32f				
FRI729	0.97±0.08^{f}	0.37±0.03^{d}	2.46±1.26f				
FRI751	0.93±0.04^{f}	0.28±0.04^{d}	1.73±0.36f				
FRI754	1.61±0.35^{bc}	0.70±0.04^{c}	3.80±0.094e				
FRI779	1.47±0.25^{bcde}	4.36±0.05^{d}	4.59±1.184cde				
FRI7100	1.62±0.66^{bc}	0.80±0.10^{c}	5.71±0.71ab				
PAS13	1.00±0.09^{ef}	0.72±0.12^{c}	6.21±0.15a				
PAS55	2.53±0.083^{a}	1.13±0.19^{ab}	4.34±0.31de				
PAS66	1.17±0.12^{cdef}	0.78±0.12^{c}	5.49±0.00abc				
PAS92	1.47±0.22^{bcd}	1.22±0.187^{a}	6.39±0.25a				
PAS103	1.83±0.24^{b}	1.10±0.08^{ab}	5.47±0.54abc				
PAS151	1.79±0.29^{b}	1.09±0.36^{ab}	4.00±0.30e				
PAS173	1.11±0.24^{def}	1.05±0.14^{b}	4.06±0.37e				

Levels of glutathione (GSH), cysteinyl-glycine (Cys-Gly) and cysteine (Cys) (nmol mL^{-1}), from total cell extract derivatized and quantified by reverse-phase HPLC; (n = 3), (means ± SD)

The results showed high variability which was confirmed by statistical analysis (table 1). *S. bacillaris* single after 26 days of the fermentation produced more GSH precursors than EC1118. CysGly showed values of 2.19 and 1.25, gGluCys of 2.38 and 1.54, and GSH of 8.64 and 7.36 for PAS13 and FRI751, respectively. Effectively, PAS13 had the higher capacity of producing GSH precursors and GSH than FRI751.

When compared the mean of the GSH production from yeast less at the end fermentation showed less GSH production 4.72 nmol/mL then 24 and 79 nmol/mL produced by *S. cerevisiae* and *Candida utilis* respectively (Liang et al., 2008; Zhang et al., 2007). These yeasts are the most commonly used microorganisms on an industrial scale (Li et al., 2004).

However, these authors used synthetic must different in less quantity. GSH production from yeast less at the end fermentation are in agreement with previous findings

where the residue product from residue of winereis can improve environmental sustainability (Da Ros et al., 2015; Lannone et al., 2016).

This is in agreement with previously published works in which in a screening of commercial enological IDY preparations, only those claimed to be GSHenriched IDY preparations released reduced GSH into synthetic wines Rodríguez-Bencomo et al., 2014. Thus, a very high glutathione content was demonstrated in *S. bacillaris* cells after fermentation. Therefore, yeast lees obtained from *S. bacillaris* fermentations can be proposed as glutathione source.

6.4 References

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CHAPTER 7

Draft genome sequence of the yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) FRI751 isolated from fermenting must of dried raboso grapes

7.1. Genome announcements - FRI751

The ascomycetous yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) is frequently found in spontaneous must fermentation usually at relatively high population level of 10^4–10^6 cells/ml (Masneuf-Pomarede et al., 2015), in grape marcs (Bovo, Giacomini, & Corich, 2011) and is also normally present on botrytized grapes.

This species was isolated for the first time in Napa Valley (California, USA) in 2002 (Mills, Johannsen, Cocolin, 2002) and one year later, Sipiczki, (2003) assigned this *Candida* sp. to a novel species under the name *C. zemplinina*, due to the significant differences observed in the ribosomal RNA sequence from that of the related species *Candida stellata* (Duarte et. al., 2012). For a long time, *C. zemplinina* has been confounded with its close species *C. stellata* which shares similar ecological niches, particularly grape and wine environments. Finally it was established as *Starmerella bacillaris* (Duarte et. al., 2012).

S. bacillaris is able to ferment glucose, sucrose and raffinose, but not galactose, maltose or lactose (Duarte et. al., 2012). Unable to grow in vitamin-free medium, it develops well in the presence of high glucose concentration, up to 50 % (w/v) (6). It is highly fructophilic and high glycerol producer (Englezos et. al., 2015).

It is a psichrotolerant and osmotolerant species (Duarte et. al., 2012) and among the non-*Saccharomyces* yeasts of enological interest, *S. bacillaris* is considered one of the most promising species to satisfy modern market and consumers' preferences.

Lemos Junior, W. J. F., Treu, L., Duarte, V. da S., Campanaro, S., Nadai, C., Giacomini, A., & Corich, V. (2017). Draft Genome Sequence of the Yeast *Starmerella bacillaris* (syn., *Candida zemplinina*) FRI751 Isolated from Fermenting Must of Dried Raboso Grapes. *Genome Announcements*, *5*(17), e00224–17. http://doi.org/10.1128/genomeA.00224-17

Lemos Junior, W. J. F., Treu, L., Duarte, V. da S., Campanaro, M. Carlot, S., Nadai, C., Giacomini, A., & Corich, V. (2017). Whole genome sequence of *Starmerella bacillaris* PAS13, a non-conventional yeast with antifungal activity. *Genome Announcements*.

In particular, it produces less ethanol from must fermentation with respect to *Saccharomyces cerevisiae*, low levels of biogenic amines and average volatile acidity (Lemos Junior et al., 2016). It is also being tested in association with *Saccharomyces cerevisiae* in mixed o sequential fermentations to reduce alcohol content and to increase organoleptic properties of wines Englezos et. al., 2015 and its possible use in the vineyard as antifungal agents against *Botrytis* is under study Lemos Junior et al., 2016.

In this work, the first genome sequence for a *S. bacillaris* strain is released. Strain FRI751 was isolated from fermentation of dried grapes of Raboso wine, a vine variety cultivated mainly in the North East of Italy for the production of passito wines.

S. bacillaris FRI751 genomic DNA was prepared by zymolyase digestion followed by standard phenol–chloroform extraction as described in Vaughan-Martini & Martini (1996). Genome sequence was generated using an Illumina NextSeq 500 platform (1-kb mate-pair libraries) at the Ramaciotti Centre, Sydney, Australia. The sequencing generated 45-folds coverage with 2,871,108 paired-end (2x150 bp) and 102,368 unpaired reads (after quality filtering) that were used for the *de novo* assembly by SPAdes 3.10 software (Bankevich et al., 2012) (with option -k 21,33,55,77,99,127). The genome size of *S. bacillaris* FRI751 was 9.3 Mbp, divided into 106 contigs longer than 100 bp (N_{50} length of 208744 bp), and the GC content was 39.4%. Protein-coding gene (CDS) prediction was performed using GeneMark-ES (Ter-Hovhannisyan et al., 2008) and resulted into 4028 CDSs and a total of 4315 exons. Gene annotation was obtained combining two strategies: 1) BlastKOALA (Kanehisa et al., 2016) as used to searches against a nonredundant set of KEGG genes and selecting *Saccharomycetaceae* as taxonomy group; 2) RPS BLAST was used to compare protein sequences with Eukaryotic Orthologous Groups of proteins (KOG) (Tatusov et al., 2003).

Lemos Junior, W. J. F., Treu, L., Duarte, V. da S., Campanaro, S., Nadai, C., Giacomini, A., & Corich, V. (2017). Draft Genome Sequence of the Yeast *Starmerella bacillaris* (syn., *Candida zemplinina*) FRI751 Isolated from Fermenting Must of Dried Raboso Grapes. *Genome Announcements*, *5*(17), e00224–17. http://doi.org/10.1128/genomeA.00224-17

Lemos Junior, W. J. F., Treu, L., Duarte, V. da S., Campanaro, M. Carlot, S., Nadai, C., Giacomini, A., & Corich, V. (2017). Whole genome sequence of *Starmerella bacillaris* PAS13, a non-conventional yeast with antifungal activity. *Genome Announcements*.

The data reported here represents a useful resource to increase the knowledge of *S. bacillaris* metabolism and of its potential technological characteristics applied to enology.

Whole genome sequence of *Starmerella bacillaris* PAS13, a non-conventional yeast with antifungal activity

7.2 Genome announcements - PAS 13

Biocontrol agents have been developed as potential alternatives to agrochemicals in integrated crop management Bai et al., 2008, with the attempt to reduce pesticide use. They can be a relevant part in an effective strategy to improve sustainable agriculture systems. In this scenario, several non-conventional yeasts like *Candida intermedia*, *Sporidiobolus pararoseus*, *Saccharomyces cerevisiae*, and *S. bacillaris* (formerly *Candida zemplinina*) were studied for their capability to produce volatile organic compounds that are involved in biocontrol activity against *B. cinerea*. Moreover, *S. bacillaris* activity against gray mold has also been demonstrated in vivo on grape berries (Belda et al., 2017, Bovo et al., 2016). Furthermore, wine co-fermentations (sequential or mixed inoculums) using *S. bacillaris* and *S. cerevisiae* have been widely investigated in the last years. These fermentations determined a complementary consumption of glucose and fructose, with the consequent increase in glycerol and succinic acid contents, associated with low ethanol and acetoin production. This is an interesting feature for the indirect improvement of wine quality and the reduction of wine ethanol content. (Bovo et al., 2016; Englezos et al., 2015; Rantsiou et al., 2012).

Lemos Junior, W. J. F., Treu, L., Duarte, V. da S., Campanaro, S., Nadai, C., Giacomini, A., & Corich, V. (2017). Draft Genome Sequence of the Yeast *Starmerella bacillaris* (syn., *Candida zemplinina*) FRI751 Isolated from Fermenting Must of Dried Raboso Grapes. *Genome Announcements*, *5*(17), e00224–17. http://doi.org/10.1128/genomeA.00224-17

Lemos Junior, W. J. F., Treu, L., Duarte, V. da S., Campanaro, M. Carlot, S., Nadai, C., Giacomini, A., & Corich, V. (2017). Whole genome sequence of *Starmerella bacillaris* PAS13, a non-conventional yeast with antifungal activity. *Genome Announcements*.

Here we present for the first time the draft genome sequence of *S. bacillaris* PAS13, isolated from destemmed dried grape of Raboso Piave variety, grown on Bagnoli DOC (Guaranteed Origin Name) area (North-East of Italy).

For genomic DNA extraction, zymolyase digestion followed by standard phenol–chloroform purification as described in Vaughan-Martini & Martini (1996) was used. Illumina 1-kb mate-pair libraries were prepared at the Ramaciotti Centre for Genomics (Sydney, Australia) and run on a Illumina NextSeq 500 platform with. The results of sequencing produced 147-folds genome coverage using high quality 9651388 paired-end (2x150 bp) and unpaired reads. The *de novo* assembly was performed using SPAdes software (version 3.10, with option -k 21,33,55,77,99,127) (Bankevich et al., 2012) and generated the draft genome of *S. bacillaris* PAS13 of 9.4 Mbp with GC content of 39.45%. The high quality of the assembly was proven by the presence of only 67 contigs composing the genome (N_{50} length of 318510 bp). GeneMark-ES software was used for protein-coding sequences (CDS) prediction [10] and the results indicated the presence of 4321 CDSs and a 4322 exons. Two different approaches were used for gene annotation, BlastKOALA (Kanehisa et al., 2016) and RPS BLAST. The first used Saccharomycetaceae as the taxonomy group to generate a nonredundant set of KEGG genes. The second was used to compare protein sequences with Eukaryotic Orthologous Groups of proteins (KOG) (Tatusov et al., 2003). *S. bacillaris* PAS13 genome reported in this manuscript will help understanding the metabolism of this yeast and its potential role as a biocontrol agent in vineyards.

Lemos Junior, W. J. F., Treu, L., Duarte, V. da S., Campanaro, S., Nadai, C., Giacomini, A., & Corich, V. (2017). Draft Genome Sequence of the Yeast *Starmerella bacillaris* (syn., *Candida zemplinina*) FRI751 Isolated from Fermenting Must of Dried Raboso Grapes. *Genome Announcements*, *5*(17), e00224–17. http://doi.org/10.1128/genomeA.00224-17

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7.3 Accession number.

The Whole Genome Shotgun project of *S. bacillaris* FRI751 and *S. bacillaris* PAS13 has been deposited in DDBJ/ENA/GenBank under the accession no. MWSF00000000, MWPI00000000. The version described in this capitulo is the first version, MWSF01000000 and MWPI01000000.

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Lemos Junior, W. J. F., Treu, L., Duarte, V. da S., Campanaro, S., Nadai, C., Giacomini, A., & Corich, V. (2017). Draft Genome Sequence of the Yeast *Starmerella bacillaris* (syn., *Candida zemplinina*) FRI751 Isolated from Fermenting Must of Dried Raboso Grapes. *Genome Announcements*, *5*(17), e00224–17. http://doi.org/10.1128/genomeA.00224-17

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CHAPTER 8

Whole genomic comparison of two newly sequenced *Starmerella bacillaris* strains and its technological functional categorization comparison among *Saccharomyces* and non-*Saccharomyces* species

8.1 Introduction

Alcoholic beverages are worldwide consumed, but its overconsumption is related to health and financial issues (Goold et al., 2017). Among these types of drinks, wine is a product coming from a complex fermentation process that, if consumed in a moderate way, can bring health benefits (WHO, 2014). In this context, a great number of wine consumers that are concerned about theirs long-life and wellness have been raised, creating a new food and beverage demand to be supplied by the canteens (Goold et al., 2017).

Natural or inoculated yeasts display a crucial role during the fermentation and are responsible to promote wine sensory quality and terroir (Ciani et al., 2016). Regard the spontaneous fermentation, the first days are characterized by a rapid switch of *Saccharomyces* and non-*Saccharomyces* yeasts usually found in a grape resident microbiota such as *Torulaspora*, *Kluyveromyces*, *ZygoSaccharomyces*, *Pichia*, *Hanseniaspora*, *Metschnikowia* and *Starmerella* (Jolly et al. 2014; Manzanares 2016; Comitini et al. 2017).

Starmerella bacillaris (synonym *Candida zemplinina*) is known early colonizing wine yeast with interesting technological properties related to flavor development. With the purpose to improve the winemaking process, *S. bacillaris* can mainly contributes to increase glycerol and acetic acid contents and decreases pH and ethanol levels maintaining a

moderate volatile acid production even if it is used as a co-inoculum in a sequential fermentation with *S. cerevisiae* (Magyar and Tóth 2011; Masneuf-pomarede et al. 2015; Englezos et al. 2015).

In spite of the high knowledge around *S. bacillaris* capability to enhance glycerol content of the wine, little is known about the genomic level related to this pathway or others when a global comparison is performed between *Saccharomyces* and non-*Saccharomyces* yeasts or even into the Starmerella genus. Englezos et al. (2015) identified a high genetic homogeneity among 63 *S. bacillaris* strains isolated in different regions using SAU-PCR and Rep-PCR, while (Masneuf-Pomarede et al. (2015) found a defined geographical pattern among 163 strains of *S. bacillaris* when 10 microsatellite markers were used for the molecular characterization.

At the metabolic point of view, glycerol production is well-understood in the model organism *S. cerevisiae*, and occurs using as precursor the glycolytic intermediate dihydroxyacetone phosphate (DHAP). In *S. cerevisiae*, glycerol-3-phosphate dehydrogenase (GPD) and glycerol-3-phosphatase (GPP) are the mainly involved enzymes in this pathway and have two isoforms that arised from a gene duplication event, the osmotically induced (GPD1 and GPP2) and the constitutive (GPD2 and GPP1) (Oliveira et al., 2014).

Cytosolic glycerol content is positively correlated with stress conditions such as intracellular phosphate levels, NAD^+/NADH redox balance and osmotic stress (Shen et al., 2006). With regards the osmotic stress, glycerol accumulation is linked with the activation proteins kinases from the High-Osmolarity-Glycerol (HOG) response, one pathway that belongs to the MAPK module (Chen and Thorner, 2007). Furthermore, secondary metabolic

products like acetaldehyde and acetic acid are unable to be used as an electron acceptor for cytosolic NADH, which lead to the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate and, consequently, increased glycerol production(Shen et al., 2006).

In the present work, two newly sequenced *Starmerella bacillaris* strains, namely *S. bacillaris* FRI751 and PAS13, were firstly annotated (Lemos Junior et al., 2017) and compared to each other and among *Saccharomyces* and non-*Saccharomyces* species based on technological functional categories, being highlighted differences on the glycerol pathway.

8.2 Material and Methods

8.2.1 S. bacillaris FRI751 and PAS13 technological features

Starmerella bacillaris FRI751 and PAS13 used in this work were isolated from fermenting must obtained from dried grape of Raboso piave variety as described by (Lemos Junior et al., 2016). The starter cultures were prepared from YPD agar plate (yeast extract 10 g/L, peptone 10 g/L, dextrose 20 g/L) to inoculate 5 mL of YPD broth in 15 mL tubes. A stationary phase culture with approximately 7.10^7 - 3.10^8 cells/mL, determined by OD measurements and confirmed by means plate counts (CFU/ml) was obtained after 24 hours of incubation at 30°C.

During the fermentation, the same inoculum size for both *S. bacillaris* strain ($1x10^6$ cells/ml) was used. The synthetic must MS300 was prepared with 100 g/L of glucose, 100 g/L of fructose and 6 g/L of malic acid with pH 3.3 Bely et al. (1990). Then, when the weight loss was lower than 0.05 g per day, the fermentations were considered concluded.

All the experiments were performed in triplicate. At the end of the fermentation, each sample were filtered (0.22 µm, WWR filters) and 10 µl were analyzed by HPLC (Shimadzu,

Japan) equipped with a refractive index detector, set at 600 nm wavelength for the determination of glucose glycerol and ethanol was used, while for the acetic acid quantification a UV detector was used. Calibration curves of glucose, fructose, glycerol, acetic acid and ethanol were made before of sample injection on HPLC. The chromatographic conditions were realized with the ROA-Organic Acid H+ column (Phenomenex, USA), which was run at 65°C with 5 mM H_2SO_4 as the mobile phase, with a flow rate of 0.5 mL/min. Fisher's test was used as comparison test when samples were significantly different after ANOVA ($p < 0.05$).

8.2.2 Pairwise comparison between S. bacillaris strains

Single nucleotide polymorphism (SNPs), insertions and deletions (INDELs) and multiple nucleotide polymorphisms (MNPs) in different genomic locations that could affect *S. bacillaris* FRI751 and PAS13 phenotypes were evaluated.

Reads with high-quality (above 50 bp) were used and aligned using the read-aligner Bowtie2 software (Langmead and Salzberg, 2012), being the output file used by Mpileup tool to extract the variants from the generated aligned reads (SAMtools package). The Binary Call Format (BCF) generated previously was forwarded and analyzed by BCFtools and the reads visualized by Tview (SAMtools package).

SnpEff program (Cingolani et al., 2012) was run using *S. bacillaris* PAS13 as reference genome. Only genes with putative impact variants considered high or moderate were selected and clustered according their functional functions using the pathway reconstruction of KEGG.

Progressive MAUVE (Darling et al., 2004) was used to manually evaluate genomes collinearity and the presence of translocations between *S. bacillaris* FRI751 and PAS13.

8.2.3 Orthologous analyze and ITS-1 based phylogenetic tree

Orthologous analyze tool was used to annotate orthologous from *S. bacillaris* FRI751 and PAS13, *S. bomcicola* NBRC10243 and JCM9596 and *H. uvarum* DSM2768 using the proteins sequences of *S.cerevisiae* S228c as templates with the aim to better understand the presence and absence of proteins in pathways with technological interest.

A global ITS1-based phylogenetic tree was constructed with 174 *Saccharomyces* and non-*Saccharomyces* yeasts ITS sequences, downloaded from the National Center for Biotechnology Information (NCBI database) (Coordinators, 2017), plus *S. bacillaris* FRI751 and PAS13. In order to perform the final tree, a subset of yeasts was selected. The trees were drawn using Dendroscope (Huson et al., 2007) and aligned.

8.2.4 Prediction of regulation sites and protein structures

Online tools were used to predict proteins structure homologies (Biasini et al., 2014), promoters regions (Reese, 2001) and binding sites for transcriptional factors (TF) (Teixeira et al., 2014).

8.3 Results and discussion

8.3.1 S. bacillaris FRI751 and PAS13 technological features

S. bacillaris FRI751 and PAS13 were isolated from fermenting musts obtained of dried grape of Raboso piave variety (Veneto region, Italy) during wine production.

Through CO_2 measurement it was possible to monitor fermentation kinetics of the 2 strains of *S. bacillaris* in synthetic must MS300 at 20°C, being stopped after 624 hours, period necessary to the industrial wine strain *S. cerevisiae* EC1118, used here as reference strain, requires to finish the fermentation. *S. bacillaris* FRI751 and PAS13 showed low CO_2 production (CO_2/100mL) when compared to the *S. cerevisiae* EC1118 (Table 1), but not significant differences ($p < 0.05$) after 48 and 624 h of incubation were noticed between *S. bacillaris* strains, which indicates a very similar growth behavior.

Regarding the capability to use glucose and fructose as carbon sources and ethanol content by *S. bacillaris* FRI751 and PAS13, significant differences ($p < 0.05$) were displayed only for glucose (78.22 ± 2.41 g/L to 85.46 ± 2.10 g/L), while fructose residue (24.49 ± 1.84 g/L to 23.87 ± 1.31 g/L) and ethanol level (4.52 ± 0.024 % v/v to 4.94 ± 0.01 % v/v) weren't shown any significant statically difference. Conversely, *S. cerevisiae* EC1118 showed the capability to totally consume both carbohydrates and produce high amounts of ethanol (13.16 ± 0.02 % v/v).

Concerning glycerol and acetic acid concentrations, *S. bacillaris* PAS13 is a better producer when compared to *S. bacillaris* FRI751 (5.72 ± 0.20 g/L to 7.81 ± 0.36 g/L) and *S. cerevisiae* EC1118 (5.77±0.14 to 7.81 ± 0.36), while *S. cerevisiae* EC1118 showed the ability to produce more acetic acid than *S. bacillaris* strains (0.51 ± 0.02).

Although several parameters like pH, must, carbon source and temperature are take in consideration when the metabolic system of yeasts during the alcoholic fermentation is studied, this work focus on *S. bacillaris* capability to increase glycerol and reduce ethanol contents, being highlighted the yeast *S. bacillaris* PAS13, able to produce, approximately,

36% more glycerol than *S. bacillaris* FRI751 and *S. cerevisiae* EC1118 consuming 9% less glucose than *S. bacillaris* FRI751 maintaining an excellent ethanol level near of 5 % (v/v), once that values around 14 % (v/v) have been reported on the literature (Englezos et al. 2015).

Table 1. CO_2 production during fermentation, residual glucose and fructose concentrations and acetic acid, glycerol, ethanol concentrations of the main fermentation products at the end of the fermentation of *S. bacillaris* and sequential fermentation of *S. bacillaris* strains with EC1118 in MS300. Data are expressed as the average of three replicates ± standard deviations. Within the column, mean values followed by the same letter are not significantly different according to Fisher's test ($p \leq 0.05$).

Strains	CO_2/100mL		Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Ethanol (% v/v)
	48 h	624h					
FRI751	0.26 ± 0.40^{B}	3.28 ± 0.50^{B}	78.22 ± 2.41^{B}	24.49 ± 1.84^{A}	5.72 ± 0.20^{A}	0.38 ± 0.05^{B}	4.52 ± 0.10^{C}
PAS13	0.28 ± 0.04^{B}	3.54 ± 0.33^{B}	85.46 ± 2.10^{A}	23.87 ± 1.31^{A}	7.81 ± 0.36^{B}	0.40 ± 0.26^{B}	4.94 ± 0.24^{B}
EC1118	1.10 ± 0.14^{A}	9.57 ± 0.20^{A}	-	-	5.77 ± 0.14^{A}	0.51 ± 0.02^{A}	13.16 ± 0.02^{A}

8.3.2 Bioinformatics analysis

8.3.2.1 Genome-wide and phylogenetic tree

S. bacillaris FRI751 and PAS13 whole-genomes assembly and annotation features are described according to Lemos Junior et al. (2017).

High throughput sequencing technology has afforded the sequencing of a great number of yeast genomes. Currently, tools from the comparative genomic help us in the comprehension of why some phenotypic differences rise among similar fungal species (Dujon, 2010; Mohanta and Bae, 2015).

S. bacillaris genome, as others non-conventional yeasts with interesting technological features, has its genetic structure like the number of genes encoding enzymes, synteny and pathways to be elucidated (Albertin et al., 2016).

S. bacillaris FRI751 and PAS13 genomes were not aligned in a unique consensus sequence, an evidence of divergence with regarding the genomic level (Mohanta and Bae, 2015). In fact, 5 five translocations were identified and are discussed on the item 3.1. Genomes size of 9.3 and 9.4 Mbp were generated, respectively, for *S. bacillaris* FRI751 and PAS13 and are very close to the 8.97 Mb *Hansenula polymorpha* genome, an Ascomycota with the smallest genome from 172 fungal species studied by Mohanta and Bae (2015) and so far from the average genome size (36.91 Mb) of Ascomycota phylum. GC content of approximately 39.4% for both *S. bacillaris* strains is less than that found for *S. bombicola*, (Table 2), a close related yeast that belong to the same clade. Interestingly, *S. bacillaris* FRI751 and PAS13 showed average exons per gene, respectively, of 1.07 and 1.00, values in accordance with others members of the Ascomycota group (Mohanta and Bae, 2015). In a review about the diversity of fungal genome, Mohanta and Bae (2015) report that reduced genomes sizes, as found for *S. bacillaris*, are associated with a quick evolution in terms of phenotypic diversification provoked by adaptive exigencies or due natural selection.

Table 2: *Saccharomyces* and non-*Saccharomyces* whole-genomes features

Species	Genome size (Mb)	Number of protein-coding gene	Averege GC content (%)
Hanseniaspora optunie	8.83	4,167	34.7
Hanseniaspora osmophila	11.45	4,657	36.7
Hanseniaspora uvarum	8.81	3,552	32
Starmerella bombicola	9.46	4,599	47.85
Saccharomyces cerevisiae	12.132	5,409	38.39

A total of 174 reference strains including *Starmerella* spp. strains (2 strains), 56 Candida species and 116 non-Candida species were used to perform ITS-1-based phylogenetic major and minor trees, which supported the relationship between *Starmerella bacillaris* FRI751 and PAS13 with others *Starmerella* spp. strains (Fig. 1).

Interestingly, enological (*Hanseniaspora uvarum* and *Hanseniaspora guilliermondii*) and non-enological yeasts (*Zygoascus hellenicus* and *Metschnikowia fructicola*) were isolated from the same environment that *S. bacillaris* FRI751 and PAS13, but ITS-1-based phylogenetic tree shows the clade which *S. bombicola* and *S. bacillaris* strains are grouped with a great distance to the group of yeasts isolated from enological environments.

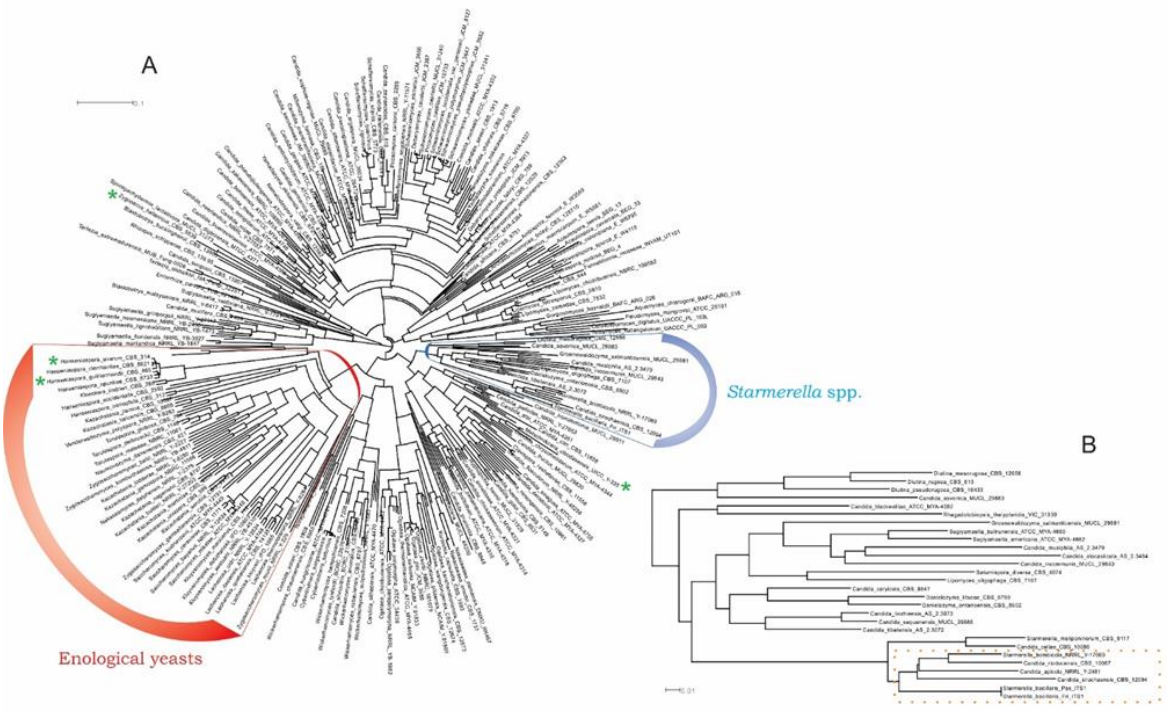


Figure 1. ITS1 sequence-based phylogenetic trees; (A) major phylogenetic tree of 174 reference yeast strains (56 candida species, 116 non candida species and *S. bacillaris* FRI751 and PAS13. Green asterisks (*) mean that enological and non-enological yeasts were isolated from the same environment that *S.*

bacillaris FRI751 and PAS13; (B) minor phylogenetic tree showing Starmerella clade. *S. bacillaris* FRI751 and PAS13 are highlight.

8.3.2.2 Orthologous analyze

Orthologous analysis is in accordance to the evolutionary distance between *Saccharomyces* and non-*Saccharomyces* species (Table 3) with *S. bacillaris* strains very close to *S. bombicola* ones, here evidenced by the total number of proteins identified for each strain using *S. cerevisiae* S288C proteins as templates. Interestingly, mainly three *S. bacillaris* pathways displayed a great amount of proteins that were not classified as orthologous: MAPK signalling (29), carbon metabolism (24) and amino acid biosynthesis (13).

Table 3: Orthologous analyses considering *Saccharomyces* and non-*Saccharomyces*.

Strain	Strain	Number **orthologous**
S. cerevisiae S228c	*S. bacillaris FRI751*	2198
S. cerevisiae S228c	*S. bacillaris PAS13*	2200
S. cerevisiae S228c	*H. uvarum DSM2768*	1691
S. cerevisiae S228c	*S. bombicola JCM9596*	2387
S. cerevisiae S228c	*S. bombicola NBRC10243*	2389
S. bacillaris FRI751	*S. bacillaris PAS13*	3898
S. bacillaris FRI751	*H. uvarum DSM2768*	1194
S. bacillaris FRI751	*S. bombicola JCM9596*	3252
S. bacillaris FRI751	*S. bombicola NBRC10243*	3261
S. bacillaris PAS13	*H. uvarum DSM2768*	1200
S. bacillaris PAS13	*S. bombicola JCM9596*	3262
S. bacillaris PAS13	*S. bombicola NBRC10243*	3272
H. uvarum DSM2768	*S. bombicola JCM9596*	1285
H. uvarum DSM2768	*S. bombicola NBRC10243*	1284
S. bombicola JCM9596	*S. bombicola NBRC10243*	4412

MAPK is an important regulation system related to stress response of living cells mainly regarding pathways as pheromone, cell wall stress, starvation and high osmolarity (Chen

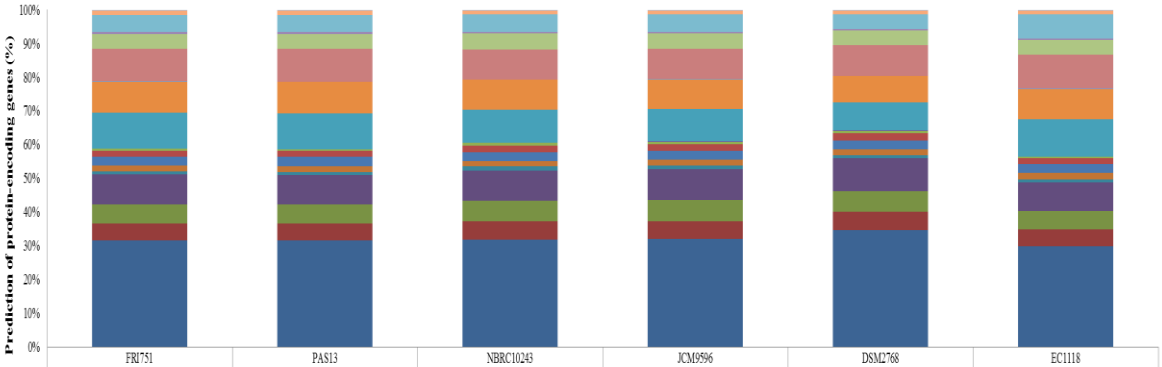
and Thorner, 2007; Yang et al., 2013). Especially considering the High-Osmolarity-Glycerol (HOG) response, Hog1 can be activated by two upstream routes of the HOG pathway (branches Sln1 or the Sho1) (Chen and Thorner, 2007). In this study, Sho1p were annotated as orthologous only for *H. uvarum* and *S. bombicola*, while Sln1 were not annotated even for non-*Saccharomyces* strains. This analyze brings a new insight into HOG pathway for *S. bacillaris* understanding, once Sln1 and Sho1 are essential proteins for *S. cerevisiae* survival (Cherry et al., 2012)

Regarding amino acids metabolism, the enzyme branched-chain amino acid aminotransferase, which is related to the valine, leucin and isoleucine biosynthesis, has not shown an orthologous with *S. cerevisiae* S288C. Ramified amino acids are responsible to produce higher alcohols (Ehrlich pathway) in different concentrations by genus *Saccharomyces* during the fermentation. This difference could be used to justify the production of some specific high ethanol compounds. 2-phenylethanol, for example, can be used as an antagonist agent against in *Botrytis cinerea* (Lemos Junior et al., 2016; Tofalo et al., 2016).

8.3.2.3 Functional categorization among Saccharomyces and non-Saccharomyces yeasts

Gene functional classification of *S. bacillaris* FRI751, *S. bacillaris* PAS13, *S. bombicola* NBRC10243, *S. bombicola* JCM9596, *H. uvarum* DSM2768 and *S. cerevisiae* EC1118 was done using, respectively, 2149, 2159, 2382, 2383, 1440 and 3124 protein-enconding genes annotated using KEGG (Supplementary material Table S1). *S. bacillaris* FRI751 and *S. bacillaris* PAS13 displayed high abundance in proteins regarding,

metabolism, transcription, folding, sorting and degradation and signal transduction pathways (Figure 2).



	FRI751	PAS13	NBRC10243	JCM9596	DSM2768	EC1118
Cellular community - prokaryotes	0,42%	0,42%	0,38%	0,38%	0,35%	0,29%
Cellular community - eukaryotes	2,00%	1,95%	1,76%	1,76%	2,01%	1,50%
Cell growth and death	7,68%	7,69%	8,02%	8,02%	7,08%	8,83%
Cell motility	0,84%	0,83%	0,76%	0,76%	0,90%	0,61%
Transport and catabolism	6,51%	6,58%	7,14%	6,97%	7,08%	5,54%
Signal transduction	14,66%	14,96%	13,85%	14,06%	15,28%	12,58%
Membrane transport	0,19%	0,19%	0,21%	0,21%	0,07%	0,16%
Folding, sorting and degradation	14,01%	14,03%	13,60%	13,60%	12,99%	11,33%
Transcription	16,15%	16,12%	15,03%	14,90%	13,82%	13,89%
Xenobiotics biodegradation and metabolism	0,14%	0,14%	0,25%	0,25%	0,21%	0,13%
Biosynthesis of other secondary metabolites	0,79%	0,79%	1,05%	1,05%	1,11%	0,64%
Metabolism of terpenoids and polyketides	2,61%	2,59%	3,11%	3,15%	3,54%	2,08%
Metabolism of cofactors and vitamins	4,19%	4,12%	4,03%	4,07%	4,38%	3,20%
Glycan biosynthesis and metabolism	2,51%	2,64%	2,56%	2,56%	2,99%	2,53%
Metabolism of other amino acids	1,44%	1,39%	1,76%	1,80%	1,53%	1,09%
Nucleotide metabolism	13,40%	13,34%	13,94%	14,14%	16,18%	10,66%
Energy metabolism	8,61%	8,66%	9,45%	9,57%	10,21%	6,91%
Carbohydrate metabolism	7,54%	7,50%	8,19%	8,18%	8,89%	6,21%
Metabolism	48,07%	47,99%	49,24%	49,81%	57,71%	37,68%

Figure 2 Total number of protein-encoding genes normalized based on KEGG annotation were grouped according to *Saccharomyces* and non-*Saccharomyces* yeasts.

S. bacillaris FRI751 and PAS13 highlighted high abundance in proteins related to stress response in comparison to *Saccharomyces* and non-*Saccharomyces* yeasts used in this study. According to Gonçalves et al. (2016), signal transduction develop a crucial role on the mechanisms involved in a coordinated cell response to environmental changes, that occurs by a variety of processes, but is highly dependent of transcriptional factors. In this way, taking into consideration the high amount of these protein-encoding genes found in each general categories for *S. bacillaris* FRI751 and PAS13 compared to the others

Saccharomyces and non-*Saccharomyces* yeasts, is reasonable suppose that *S. bacillaris*, an environmental fungal, can react and elaborate a response to an outside stress more readily due this high genic content. In fact, along the whole genome duplication (WGD) and the domestication process in *S. cerevisiae*, a huge number of genes were lost, showing a fungal genome dynamic under a determined condition (Mohanta and Bae, 2015).

8.3.2.4 Genomic variants annotation and translocations among S. bacillaris FRI751 and PAS13

Genomic variant annotation were performed between the two *S. bacillaris* strains under investigation in order to identify and calculate the effects under coding sequences that potentially can alter proteins functions. A high number of SNPs (33,771 high-quality variants) in 1,146 genes were identified and 543 known protein-coding genes that displayed high or moderate variations were grouped according to theirs functional properties (Figure 3).

Using microarrays to verify the frequency and distribution of variants in 8 unrelated *S. cerevisiae strains*, Schacherer et al. (2007) demonstrated a positively correlation with variants and the distance among the strains, with 37,424 SNPs identified between *S. cerevisiae* S288c and SK1, the most distant strains in that study. In this study, the divergence between *S. bacillaris* strains is 0.36 % of the genome and is accordance to Schacherer et al. (2007).

Still according to Schacherer et al. (2007), the interpretation of biological assays, as discussed on the item 3.1, can be facilitated by the understanding the number and the position of variants between strains that show different phenotypes, which could be extrapolated for this study.

Pathway	Protein-encoding genes	(%)
Signal transduction	67	12.34
Translation	66	12.15
Transport and catabolism	51	9.39
Replication and repair	46	8.47
Cell growth and death	45	8.29
Carbohydrate metabolism	41	7.55
Folding, sorting and degradation	41	7.55
Aminoacid metabolism	27	4.97
Metabolism of cofactors and vitamins	25	4.60
Lipid metabolism	24	4.42
Nucleotid metabolism	21	3.87
Transcription	20	3.68
Glycan biosynthesis and metabolism	19	3.50
Energy metabolism	17	3.13
Metabolism of other amino acids	7	1.29
Metabolism of terpenoids and polyketides	7	1.29
Cellular community - eukaryotes	7	1.29
Xenobiotics biodegradation and metabolism	5	0.92
Cell motility	3	0.55
Membrane transport	2	0.37
Biosynthesis of other secondary metabolites	1	0.18
Cellular community - prokaryotes	1	0.18

Figure 3. Functional annotation based in high and moderate variants among *S. bacillaris* PAS13 and *S. bacillaris* FRI751 were ranked. Pathway reconstruction from KEGG showed that Environmental Information Processing and Genetic Information Processing are the main genic-clusters that potentially are related to polymorphism and, consequently, different phenotypes between this strains.

Notwithstanding variants distribution were found for throughout the genome, high or moderate variants were mainly related to signal transduction and translation genes. Based in these variations, differences on the degree's response regarding each pathway could be observed between *S. bacillaris* PAS13 and FRI751 as discussed on the item 3.2.3.

Considering chromosomal translocations analyze in *S. bacillaris* strains, five regions were identified by visual investigation of the genome alignment performed using MAUVE software. 12 genes are involved in *S. bacillaris* PAS13, while 11 genes were found in *S. bacillaris* FRI751, both located close to the translocations (Figure 4).

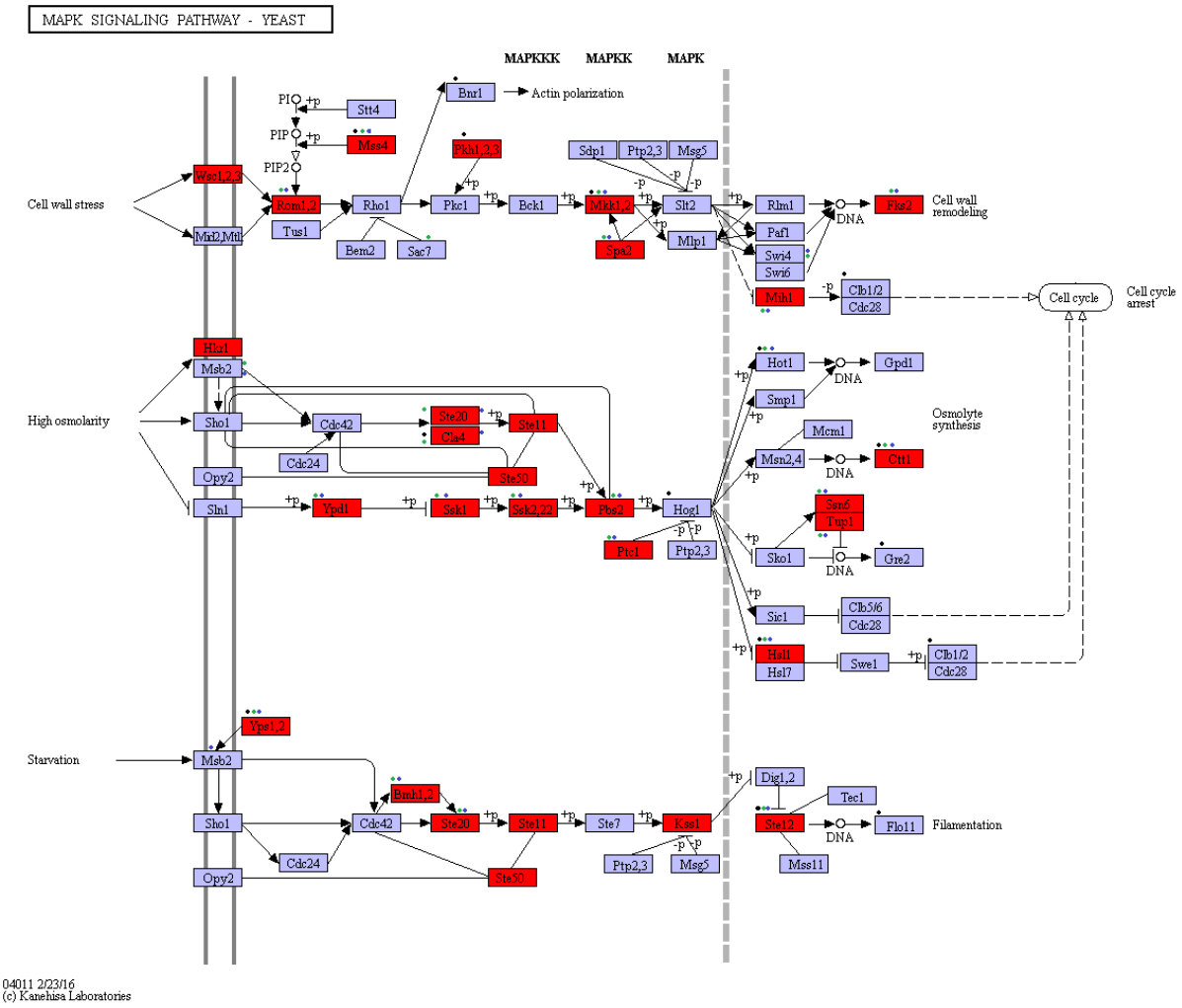


Figure. 4 MAPK module and its three stress response pathways. Characters represent, respectively, orthologous absence in *S. bacillaris* PAS13/FRI751**,** *S. bacillaris* PAS13**,** *H. uvarum DSM2768, S. Bombicola JCM9596, S. Bombicola NBRC10243*

Ten genes haven't been annotated, but coding sequences for endoplasmic reticulum protein-retaining receptor (KDELR), biosynthesis of aromatic amino acids (ARO1), farnesyl diphosphate synthetase (FDPS), dUTP pyrophosphatase, cytochrome c oxidase subunit 2 and mitochondrial ribosomal protein of the large subunit are present on double-strand DNA breakpoints (Supplementary material Table S2).

Hotspots sites are essential for chromosomal rearrangements like duplication, insertion-deletion and translocations, being the former favorable to happen when fragile sites are replicated and with strong impact in yeast evolution (Mohanta and Bae 2015; Lemoine et al. 2005). Five translocations were identified (Figure 5) in genes mainly involved in mitochondrial and nucleotides metabolisms. Treu et al. (2014) performed a comparison among fermentation performance and genetic variations of four *Saccharomyces cerevisiae* strains isolated from vineyard environment, been found genes adjacent to the translocations that were involved mainly in stress response that determine an adaptation to enological environments.

For *S. bacillaris* the functional role considering the translocation that involves the gene coding for cytochrome oxidase subunit 2 seems to be related to a better ability regard to *S. bacillaris* PAS13 to perform respiration in the first 24 hours of growth (unpublished data).

Few works in the last years bring the genetic diversity of *S. bacillaris* strains, but none at genome level, probably due the lack of *S. bacillaris* genomes available on the databases. Using two different strategies (SAU-PCR and Rep-PCR) for the molecular characterization of 63 *S. bacillaris* strains, Englezos et al. (2015) identified a high genetic homogeneity among these species even when isolated in different regions, however the phenotypic characterization regard ethanol production showed high levels (14.0% v/v) for all isolates, which is an uncommon feature for this specie. Controversially, Masneuf-Pomarede et al. (2015) analyzed the genetic diversity of 163 strains of *S. bacillaris* by 10 microsatellite markers and identified a defined pattern among these isolates, in part, due to the geographical distribution, however no selective pressure was correlated with the place were these strains were isolated.

Figure 5. Translocations identified between *S. bacillaris*.(A,B,C,D,E) show double-strand DNA breakpoints in genes with unknow function (A), cytochrome oxidase subunit 2 (B), Mitochondrial ribosomal protein of the large subunit/sUTP pyrophosphatase (C), coaE/FDPs/VPS54/AR01 (D) and KDELR (E). Bold asterisk means the selected genome regions were translocations have been identified.

8.3.2.5 S. bacillaris FRI751 and Pas 13 glycerol pathway comparison with Saccharomyces and non-Saccharomyces yeasts

Considering the most important characteristic of *S. bacillaris* in technological terms, the glycerol production, specific analyses using *S. bacillaris* FRI751 and PAS13, *S. bombicola* JCM9596, *S. bombicola* NBRC10243, *H. uvarum* DSM2768 and *Saccharomyces cerevisiae* EC1118 were used.

Gpd1 and Gpd2 are a NAD-dependent glycerol-3-phosphate dehydrogenase key enzymes of glycerol synthesis, being the first one the product of a genic duplication present in *S. cerevisiae*. Only one copy of *gpd1* was found in *S. bombicola* JCM9596, *S. bombicola* NBRC10243 and *H. uvarum* DSM2768. In *S. cerevisiae*, Gpd1 and Gpd2 show different performance under aerobically and high osmolarity conditions, as the overexpression of the first one determines increased glycerol accumulation, undergoing different regulation pattern, being inactivated, respectively, by YpK1p/YpK2p and SNF1 (Lee et al., 2012; According to Påhlman et al., 2001). Regarding *gpd1* and *gpd2* in *S. bacillaris* FRI751/PAS13, only one copy of *gpd1*, the major form found in *S. cerevisiae*, was found in both strains, but curiously neither regulators *ypk1p* and *ypk2p* were identified, just *snf1p*, which negatively regulates *gpd2*.

Concerning Gpp1 and Gpp2, a glycerol-3-phosphate phosphatase enzyme, *gpp2* was not identified for *S. bacillaris* FRI751 and PAS13, but two copies of *gpp1*, an essential gene to growth in anoxic condition, were found as a tandem array in both strains with an intergenic region of 918 bp among these genes. These two copies showed different lengths (723 bp/720 bp) and identities (62%) when compared, but none differences were found between *S. bacillaris* strains. In the intergenic region, a promoter has been predicted and two SNPs

identified within, followed by a homopolimeric region in both strains with a deletion identifed in *S. bacillaris* PAS13. This gene duplication event is also found in *S. cerevisiae*, but *gpp1* and *gpp2* are found in different chromosomes (V and IX), however no similar organization was observed in *S. bombicola* and *H. uvarum* (Table 4). Even without undergo WGD, the presence of two copies of *gpp1* with different identities can suggest a process of functional diversification associated to a rapid adaptive evolution (Chin et al., 2012; Dujon, 2010).

Table 4: GPP1 model prediction from non-*Saccharomyces* strains was generated using the SWISS MODEL server. GPP1 from EC1118 was used as template.

Features	**Strains**							
	S. cerevisiae	*S. bacillaris*		*S. bacillaris*		*S. bombicola*	*S. bombicola*	*H. uvarum*
	EC1118	Fri751		Pas13		JCM9596	NBRC10243	DSM 2768
Gene(s)	GPP1	GPP1_1	GPP1_2	GPP1_1	GPP1_2	GPP	GPP	GPP
Length (a.a)	250	241	240	241	240	240	240	254
Mol. Weight (KDa)	27.94	26.07	25.48	26.07	25.48	26.53	26.53	28.62
Isoleletric Point	5.24	5.34	5.03	5.34	5.03	5.71	5.71	5.14
Identity (%)		41.82	35.91	41.82	35.91	43.75	43.75	67.21
*GMQE		0.71	0.69	0.71	0.69	0.74	0.74	0.82
INDELs		6	6	6	6	6	6	0

*GMQE is the Swiss-Model global quality estimation and has a range from 0 to 1.

The comparison between *S. bacillaris* strains, taking into consideration the variants analyze for regulators involved in glycerol production (HOG pathway and *gpp1*/*gpd1* expression) according to *S. cerevisiae* EC1118 and S288C metabolic pathways, revealed that six molecules could be associated with the explanation for different glycerol levels found between these strains. 185 (2 with high effect, 12 with low effect, 5 with moderate effect and 166 with modifier effect), 146 (141 with modifier effect, 1 with moderate effect and 4 with low

effect), 146 (14 with low effect and 132 with modifier effect), 137 (29 with low effect, 27 with moderate effect, 81 with modifier effect), 44 (5 with low effect, 5 with moderate effect and 34 with modifier effect) and 14 (14 with modifier effect) variants, respectively, for SSK1, SNF1, SSK2, SIN3, STE11 and SPT3, were found for *S. bacillaris* FRI751 and potentially can affect these proteins functions.

SSK1, SSK2 and STE11 are response regulators involved in the HOG MAPK cascade, important component of the osmotic stress response. Unphosphorylated SSK1p is the target for ubiquitination and its degradation is associated with the downregulation of this osmosensing pathway (Sato et al., 2003).

SIN3 is a *gpp1* regulator and component of Rpd3S and Rpd3L histone deacetylase complexes acting upon in the transcriptional level, specifically in osmoresponsive promoters regions, that is recruited by HOG1p in situations of osmotic stress (Cherry et al., 2012; De Nadal et al., 2004).

The sucrose non-fermenting 1 (SNF1) is an AMP-activated serine/threonine-protein kinase α subunit involved in transcriptional activation and repression of cellular processes essentially related to glucose stress response (Sanz, 2003). *S. cerevisiae snf1*Δ mutants are more sensitive to heat stress, are unable to accumulate glycogen, are auxotrophs for inositol and have difficult to growth in the presence of 1.2 M NaCl and 0.3 M LiCl (Sanz, 2003) .

In *S. cerevisiae*, it was demonstrated that the improvement of ethanol production is related to the reduction of glycerol biosynthesis (Yu et al., 2010). In this context, SPT3, a subunit of the SAGA (Spt-Ada-Gcn5-acetyltransferase) transcriptional regulatory complex, is a relevant regulator that acts performing chromosomal modifications, being the first one

involved on *gpp2* expression (Cherry et al., 2012). Yu et al. (2012) demonstrate that the overexpression of both *SPT3* and *SPT15* was responsible to diminish glycerol production and, consequently, increase ethanol yield in engineered *S. cerevisiae* strains.

Finally, no differences were found in putative DNA binding-sites for transcription factors considering the intergenic region between the two *gpp1* copies and upstream the *gpp1_2* promoter region (Supplementary material table S3) in *S. bacillaris* strains.

8.4 Conclusions

Here, we reported the first work performing a genome comparison between two *Starmerella bacillaris* strains with different technological proprieties. *Saccharomyces* and non-*Saccharomyces* yeasts genomes were used as reference to functionally categorize *S. bacillaris* proteins, search for orthologous and better understand the glycerol production, an interesting compound produced in high amounts by this yeast. Analysis revealed that *S. bacillaris* strains have a special gene content related to stress response in comparison to *Saccharomyces* and non-*Saccharomyces* yeasts. Moreover, the absence of orthologous considering the canonical HOG pathway, *gpp1* tandem array and the high number of variants in regulators of *ggp1* and *gpd1* are new insights under the genomic level in order to understand the glycerol pathway and its yield in *S. bacillaris*.

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8.6 Supplementary Material

Supplementary material table 1:

	S. bacillaris Fri751	*S. bacillaris* Pas13	*S. bombicola* NBRC10243	*S. bombicola* JCM9596	*H. uvarum* DSM2768	*S. cerevisiae* EC1118	*S. bacillaris* Fri751	*S. bacillaris* Pas13	*S. bombicola* NBRC10243	*S.bombicola* JCM9596	*H. uvarum* DSM2768	*S. cerevisiae* EC1118
Total number of protein-encoding genes	**2149**	**2159**	**2382**	**2383**	**1440**	**3124**	**Percentage calculated on total number of protein-encoding**					
Metabolism	1033	1036	1173	1187	831	1177	48.07%	47.99%	49.24%	49.81%	57.71%	37.68%
01100 Metabolic pathways	438	441	506	515	344	517	20.38%	20.43%	21.24%	21.61%	23.89%	16.55%
01110 Biosynthesis of secondary metabolites	189	189	211	213	151	209	8.79%	8.75%	8.86%	8.94%	10.49%	6.69%
01120 Microbial metabolism in diverse environments	87	87	101	101	74	97	4.05%	4.03%	4.24%	4.24%	5.14%	3.10%
01130 Biosynthesis of antibiotics	141	141	154	157	115	156	6.56%	6.53%	6.47%	6.59%	7.99%	4.99%
01200 Carbon metabolism	59	59	69	69	50	67	2.75%	2.73%	2.90%	2.90%	3.47%	2.14%
01210 2-Oxocarboxylic acid metabolism	25	25	27	27	17	26	1.16%	1.16%	1.13%	1.13%	1.18%	0.83%
01212 Fatty acid metabolism	13	13	17	17	9	16	0.60%	0.60%	0.71%	0.71%	0.63%	0.51%
01230 Biosynthesis of amino acids	79	79	86	86	69	87	3.68%	3.66%	3.61%	3.61%	4.79%	2.78%
01220 Degradation of aromatic compounds	2	2	2	2	2	2	0.09%	0.09%	0.08%	0.08%	0.14%	0.06%
Carbohydrate metabolism	162	162	195	195	128	194	7.54%	7.50%	8.19%	8.18%	8.89%	6.21%
00010 Glycolysis / Gluconeogenesis	23	23	25	25	21	26	1.07%	1.07%	1.05%	1.05%	1.46%	0.83%
00020 Citrate cycle	20	20	21	21	14	20	0.93%	0.93%	0.88%	0.88%	0.97%	0.64%
00030 Pentose phosphate pathway	14	14	15	15	12	15	0.65%	0.65%	0.63%	0.63%	0.83%	0.48%
00040 Pentose and glucuronate interconversions	3	3	4	4	2	6	0.14%	0.14%	0.17%	0.17%	0.14%	0.19%
00051 Fructose and mannose metabolism	11	11	13	13	8	14	0.51%	0.51%	0.55%	0.55%	0.56%	0.45%
00052 Galactose metabolism	5	5	8	8	3	9	0.23%	0.23%	0.34%	0.34%	0.21%	0.29%
00053 Ascorbate and aldarate metabolism	1	1	1	1	1	1	0.05%	0.05%	0.04%	0.04%	0.07%	0.03%
00500 Starch and sucrose metabolism	10	10	14	14	12	17	0.47%	0.46%	0.59%	0.59%	0.83%	0.54%
00520 Amino sugar and nucleotide sugar metabolism	14	14	18	18	9	15	0.65%	0.65%	0.76%	0.76%	0.63%	0.48%

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00620 Pyruvate metabolism	20	20	22	22	13	21	0.93%	0.93%	0.92%	0.92%	0.90%	0.67%
00630 Glyoxylate and dicarboxylate metabolism	13	13	16	16	10	16	0.60%	0.60%	0.67%	0.67%	0.69%	0.51%
00640 Propanoate metabolism	8	8	10	10	4	9	0.37%	0.37%	0.42%	0.42%	0.28%	0.29%
00650 Butanoate metabolism	7	7	11	11	6	7	0.33%	0.32%	0.46%	0.46%	0.42%	0.22%
00660 C5-Branched dibasic acid metabolism	3	3	3	3	3	3	0.14%	0.14%	0.13%	0.13%	0.21%	0.10%
00562 Inositol phosphate metabolism	10	10	14	14	10	15	0.47%	0.46%	0.59%	0.59%	0.69%	0.48%
Energy metabolism	185	187	225	228	147	216	8.61%	8.66%	9.45%	9.57%	10.21%	6.91%
00190 Oxidative phosphorylation	39	41	56	56	33	51	1.81%	1.90%	2.35%	2.35%	2.29%	1.63%
00710 Carbon fixation in photosynthetic organisms	10	10	13	13	9	13	0.47%	0.46%	0.55%	0.55%	0.63%	0.42%
00720 Carbon fixation pathways in prokaryotes	8	8	8	8	5	7	0.37%	0.37%	0.34%	0.34%	0.35%	0.22%
00680 Methane metabolism	12	12	15	15	13	16	0.56%	0.56%	0.63%	0.63%	0.90%	0.51%
00910 Nitrogen metabolism	4	4	5	5	3	5	0.19%	0.19%	0.21%	0.21%	0.21%	0.16%
00920 Sulfur metabolism	10	10	11	11	9	10	0.47%	0.46%	0.46%	0.46%	0.63%	0.32%
00061 Fatty acid biosynthesis	6	6	6	6	3	7	0.28%	0.28%	0.25%	0.25%	0.21%	0.22%
00062 Fatty acid elongation	5	5	7	7	4	5	0.23%	0.23%	0.29%	0.29%	0.28%	0.16%
00071 Fatty acid degradation	5	5	10	10	5	8	0.23%	0.23%	0.42%	0.42%	0.35%	0.26%
00072 Synthesis and degradation of ketone bodies	2	2	3	3	2	2	0.09%	0.09%	0.13%	0.13%	0.14%	0.06%
00100 Steroid biosynthesis	16	16	14	15	11	15	0.74%	0.74%	0.59%	0.63%	0.76%	0.48%
00140 Steroid hormone biosynthesis	1	1	1	1	2	2	0.05%	0.05%	0.04%	0.04%	0.14%	0.06%
00561 Glycerolipid metabolism	14	14	16	17	12	17	0.65%	0.65%	0.67%	0.71%	0.83%	0.54%
00564 Glycerophospholipid metabolism	28	28	30	31	18	29	1.30%	1.30%	1.26%	1.30%	1.25%	0.93%
00565 Ether lipid metabolism	5	5	5	5	3	6	0.23%	0.23%	0.21%	0.21%	0.21%	0.19%
00600 Sphingolipid metabolism	10	10	10	10	7	8	0.47%	0.46%	0.42%	0.42%	0.49%	0.26%
00590 Arachidonic acid metabolism	3	3	4	4	2	3	0.14%	0.14%	0.17%	0.17%	0.14%	0.10%
00591 Linoleic acid metabolism	1	1	1	1	1	1	0.05%	0.05%	0.04%	0.04%	0.07%	0.03%

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00592 alpha-Linolenic acid metabolism	1	1	3	3	1	3	0.05%	0.05%	0.13%	0.13%	0.07%	0.10%
01040 Biosynthesis of unsaturated fatty acids	5	5	7	7	4	8	0.23%	0.23%	0.29%	0.29%	0.28%	0.26%
Nucleotide metabolism	288	288	332	337	233	333	13.40%	13.34%	13.94%	14.14%	16.18%	10.66%
00230 Purine metabolism	67	67	68	70	50	73	3.12%	3.10%	2.85%	2.94%	3.47%	2.34%
00240 Pyrimidine metabolism	55	55	54	56	42	59	2.56%	2.55%	2.27%	2.35%	2.92%	1.89%
00250 Alanine, aspartate and glutamate metabolism	19	19	22	22	17	22	0.88%	0.88%	0.92%	0.92%	1.18%	0.70%
00260 Glycine, serine and threonine metabolism	21	21	25	25	19	28	0.98%	0.97%	1.05%	1.05%	1.32%	0.90%
00270 Cysteine and methionine metabolism	22	22	29	30	19	31	1.02%	1.02%	1.22%	1.26%	1.32%	0.99%
00280 Valine, leucine and isoleucine degradation	7	7	12	12	4	8	0.33%	0.32%	0.50%	0.50%	0.28%	0.26%
00290 Valine, leucine and isoleucine biosynthesis	9	9	10	10	6	10	0.42%	0.42%	0.42%	0.42%	0.42%	0.32%
00300 Lysine biosynthesis	11	11	11	11	7	11	0.51%	0.51%	0.46%	0.46%	0.49%	0.35%
00310 Lysine degradation	9	9	13	13	9	12	0.42%	0.42%	0.55%	0.55%	0.63%	0.38%
00220 Arginine biosynthesis	12	12	16	16	11	15	0.56%	0.56%	0.67%	0.67%	0.76%	0.48%
00330 Arginine and proline metabolism	11	11	14	14	9	15	0.51%	0.51%	0.59%	0.59%	0.63%	0.48%
00340 Histidine metabolism	10	10	10	10	10	9	0.47%	0.46%	0.42%	0.42%	0.69%	0.29%
00350 Tyrosine metabolism	8	8	10	10	8	8	0.37%	0.37%	0.42%	0.42%	0.56%	0.26%
00360 Phenylalanine metabolism	5	5	9	9	5	6	0.23%	0.23%	0.38%	0.38%	0.35%	0.19%
00380 Tryptophan metabolism	10	10	14	14	6	11	0.47%	0.46%	0.59%	0.59%	0.42%	0.35%
00400 Phenylalanine, tyrosine and tryptophan biosynthesis	12	12	15	15	11	15	0.56%	0.56%	0.63%	0.63%	0.76%	0.48%
Metabolism of other amino acids	31	30	42	43	22	34	1.44%	1.39%	1.76%	1.80%	1.53%	1.09%
00410 beta-Alanine metabolism	7	7	10	10	3	7	0.33%	0.32%	0.42%	0.42%	0.21%	0.22%
00430 Taurine and hypotaurine metabolism	1	1	5	5	1	2	0.05%	0.05%	0.21%	0.21%	0.07%	0.06%
00440 Phosphonate and phosphinate metabolism	3	3	3	3	2	3	0.14%	0.14%	0.13%	0.13%	0.14%	0.10%
00450 Selenocompound metabolism	6	6	6	6	6	7	0.28%	0.28%	0.25%	0.25%	0.42%	0.22%
00460 Cyanoamino acid metabolism	1	1	3	3	3	2	0.05%	0.05%	0.13%	0.13%	0.21%	0.06%

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00480 Glutathione metabolism	13	12	15	16	7	13	0.60%	0.56%	0.63%	0.67%	0.49%	0.42%
Glycan biosynthesis and metabolism	54	57	61	61	43	79	2.51%	2.64%	2.56%	2.56%	2.99%	2.53%
00510 N-Glycan biosynthesis	18	19	20	21	16	27	0.84%	0.88%	0.84%	0.88%	1.11%	0.86%
00513 Various types of N-glycan biosynthesis	15	16	17	17	15	23	0.70%	0.74%	0.71%	0.71%	1.04%	0.74%
00515 Mannose type O-glycan biosynthesis	0	0	0	0	0	1	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%
00514 Other types of O-glycan biosynthesis	2	2	2	2	2	5	0.09%	0.09%	0.08%	0.08%	0.14%	0.16%
00531 Glycosaminoglycan degradation	0	0	1	1	0	0	0.00%	0.00%	0.04%	0.04%	0.00%	0.00%
00563 Glycosylphosphatidylinositol	18	19	20	19	9	22	0.84%	0.88%	0.84%	0.80%	0.63%	0.70%
00511 Other glycan degradation	1	1	1	1	1	1	0.05%	0.05%	0.04%	0.04%	0.07%	0.03%
Metabolism of cofactors and vitamins	90	89	96	97	63	100	4.19%	4.12%	4.03%	4.07%	4.38%	3.20%
00730 Thiamine metabolism	5	5	6	7	4	9	0.23%	0.23%	0.25%	0.29%	0.28%	0.29%
00740 Riboflavin metabolism	7	7	7	7	4	7	0.33%	0.32%	0.29%	0.29%	0.28%	0.22%
00750 Vitamin B6 metabolism	7	7	7	7	3	7	0.33%	0.32%	0.29%	0.29%	0.21%	0.22%
00760 Nicotinate and nicotinamide metabolism	12	12	13	13	7	14	0.56%	0.56%	0.55%	0.55%	0.49%	0.45%
00770 Pantothenate and CoA biosynthesis	12	12	12	12	9	13	0.56%	0.56%	0.50%	0.50%	0.63%	0.42%
00780 Biotin metabolism	2	2	3	3	1	5	0.09%	0.09%	0.13%	0.13%	0.07%	0.16%
00785 Lipoic acid metabolism	2	2	2	2	3	2	0.09%	0.09%	0.08%	0.08%	0.21%	0.06%
00790 Folate biosynthesis	7	6	9	9	3	7	0.33%	0.28%	0.38%	0.38%	0.21%	0.22%
00670 One carbon pool by folate	11	11	11	11	9	11	0.51%	0.51%	0.46%	0.46%	0.63%	0.35%
00830 Retinol metabolism	3	3	4	4	2	2	0.14%	0.14%	0.17%	0.17%	0.14%	0.06%
00860 Porphyrin and chlorophyll metabolism	15	15	15	15	14	16	0.70%	0.69%	0.63%	0.63%	0.97%	0.51%
00130 Ubiquinone and other terpenoid-quinone biosynthesis	7	7	7	7	4	7	0.33%	0.32%	0.29%	0.29%	0.28%	0.22%
Metabolism of terpenoids and polyketides	22	22	24	25	19	25	2.61%	2.59%	3.11%	3.15%	3.54%	2.08%
00900 Terpenoid backbone biosynthesis	16	16	16	17	12	17	0.74%	0.74%	0.67%	0.71%	0.83%	0.54%
00909 Sesquiterpenoid and triterpenoid biosynthesis	2	2	2	2	2	2	0.09%	0.09%	0.08%	0.08%	0.14%	0.06%

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00981 Insect hormone biosynthesis	1	1	1	1	1	1	0.05%	0.05%	0.04%	0.04%	0.07%	0.03%
00908 Zeatin biosynthesis	1	1	1	1	1	1	0.05%	0.05%	0.04%	0.04%	0.07%	0.03%
00903 Limonene and pinene degradation	1	1	1	1	1	1	0.05%	0.05%	0.04%	0.04%	0.07%	0.03%
00281 Geraniol degradation	0	0	1	1	0	0	0.00%	0.00%	0.04%	0.04%	0.00%	0.00%
01051 Biosynthesis of ansamycins	1	1	1	1	1	1	0.05%	0.05%	0.04%	0.04%	0.07%	0.03%
00940 Phenylpropanoid biosynthesis	0	0	1	1	1	2	0.00%	0.00%	0.04%	0.04%	0.07%	0.06%
Biosynthesis of other secondary metabolites	17	17	25	25	16	20	0.79%	0.79%	1.05%	1.05%	1.11%	0.64%
00950 Isoquinoline alkaloid biosynthesis	2	2	4	4	2	3	0.09%	0.09%	0.17%	0.17%	0.14%	0.10%
00960 Tropane, piperidine and pyridine alkaloid biosynthesis	3	3	5	5	3	4	0.14%	0.14%	0.21%	0.21%	0.21%	0.13%
00332 Carbapenem biosynthesis	2	2	2	2	2	2	0.09%	0.09%	0.08%	0.08%	0.14%	0.06%
00261 Monobactam biosynthesis	3	3	3	3	3	3	0.14%	0.14%	0.13%	0.13%	0.21%	0.10%
00232 Caffeine metabolism	0	0	2	2	1	0	0.00%	0.00%	0.08%	0.08%	0.07%	0.00%
00965 Betalain biosynthesis	0	0	1	1	0	0	0.00%	0.00%	0.04%	0.04%	0.00%	0.00%
00521 Streptomycin biosynthesis	3	3	4	4	2	4	0.14%	0.14%	0.17%	0.17%	0.14%	0.13%
00524 Neomycin, kanamycin and gentamicin biosynthesis	1	1	1	1	1	1	0.05%	0.05%	0.04%	0.04%	0.07%	0.03%
00401 Novobiocin biosynthesis	2	2	2	2	2	2	0.09%	0.09%	0.08%	0.08%	0.14%	0.06%
00254 Aflatoxin biosynthesis	1	1	1	1	0	1	0.05%	0.05%	0.04%	0.04%	0.00%	0.03%
Xenobiotics biodegradation and metabolism	22	22	29	29	19	27	0.14%	0.14%	0.25%	0.25%	0.21%	0.13%
00362 Benzoate degradation	1	1	3	3	1	1	0.05%	0.05%	0.13%	0.13%	0.07%	0.03%
00627 Aminobenzoate degradation	2	2	3	3	2	2	0.09%	0.09%	0.13%	0.13%	0.14%	0.06%
00364 Fluorobenzoate degradation	0	0	0	0	0	1	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%
00625 Chloroalkane and chloroalkene degradation	3	3	3	3	3	3	0.14%	0.14%	0.13%	0.13%	0.21%	0.10%
00361 Chlorocyclohexane and chlorobenzene degradation	0	0	0	0	0	1	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%
00623 Toluene degradation	0	0	0	0	0	1	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%
00643 Styrene degradation	0	0	0	0	0	1	0.00%	0.00%	0.00%	0.00%	0.00%	0.03%

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00930 Caprolactam degradation	0	0	2	2	0	0	0.00%	0.00%	0.08%	0.08%	0.00%	0.00%
00791 Atrazine degradation	1	1	1	1	1	2	0.05%	0.05%	0.04%	0.04%	0.07%	0.06%
00626 Naphthalene degradation	2	2	2	2	2	2	0.09%	0.09%	0.08%	0.08%	0.14%	0.06%
00980 Metabolism of xenobiotics by cytochrome P450	4	4	4	4	3	4	0.19%	0.19%	0.17%	0.17%	0.21%	0.13%
00982 Drug metabolism - cytochrome P450	4	4	4	4	3	4	0.19%	0.19%	0.17%	0.17%	0.21%	0.13%
00983 Drug metabolism - other enzymes	5	5	7	7	4	5	0.23%	0.23%	0.29%	0.29%	0.28%	0.16%
Genetic Information Processing												
Transcription	347	348	358	355	199	434	16.15%	16.12%	15.03%	14.90%	13.82%	13.89%
03020 RNA polymerase	24	24	22	22	19	29	1.12%	1.11%	0.92%	0.92%	1.32%	0.93%
03022 Basal transcription factors	26	25	27	27	13	30	1.21%	1.16%	1.13%	1.13%	0.90%	0.96%
03040 Spliceosome	34	34	41	42	15	60	1.58%	1.57%	1.72%	1.76%	1.04%	1.92%
03010 Ribosome	92	92	92	92	34	108	4.28%	4.26%	3.86%	3.86%	2.36%	3.46%
00970 Aminoacyl-tRNA biosynthesis	24	24	24	24	22	23	1.12%	1.11%	1.01%	1.01%	1.53%	0.74%
03013 RNA transport	57	58	59	57	30	75	2.65%	2.69%	2.48%	2.39%	2.08%	2.40%
03015 mRNA surveillance pathway	30	30	32	31	18	39	1.40%	1.39%	1.34%	1.30%	1.25%	1.25%
03008 Ribosome biogenesis in eukaryotes	60	61	61	60	48	70	2.79%	2.83%	2.56%	2.52%	3.33%	2.24%
Folding, sorting and degradation	301	303	324	324	187	354	14.01%	14.03%	13.60%	13.60%	12.99%	11.33%
03060 Protein export	15	15	16	16	7	18	0.70%	0.69%	0.67%	0.67%	0.49%	0.58%
04141 Protein processing in endoplasmic reticulum	48	49	53	54	33	60	2.23%	2.27%	2.23%	2.27%	2.29%	1.92%
04130 SNARE interactions in vesicular transport	9	11	14	14	7	16	0.42%	0.51%	0.59%	0.59%	0.49%	0.51%
04120 Ubiquitin mediated proteolysis	33	32	37	37	13	46	1.54%	1.48%	1.55%	1.55%	0.90%	1.47%
04122 Sulfur relay system	5	5	6	6	5	7	0.23%	0.23%	0.25%	0.25%	0.35%	0.22%
03050 Proteasome	30	32	33	33	23	33	1.40%	1.48%	1.39%	1.38%	1.60%	1.06%
03018 RNA degradation	37	35	39	37	27	48	1.72%	1.62%	1.64%	1.55%	1.88%	1.54%
03030 DNA replication	25	25	25	26	17	23	1.16%	1.16%	1.05%	1.09%	1.18%	0.74%

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03410 Base excision repair	15	15	15	15	7	13	0.70%	0.69%	0.63%	0.63%	0.49%	0.42%
03420 Nucleotide excision repair	31	31	31	31	16	30	1.44%	1.44%	1.30%	1.30%	1.11%	0.96%
03430 Mismatch repair	16	16	17	17	11	16	0.74%	0.74%	0.71%	0.71%	0.76%	0.51%
03440 Homologous recombination	14	14	15	15	8	17	0.65%	0.65%	0.63%	0.63%	0.56%	0.54%
03450 Non-homologous end-joining	5	5	5	5	1	10	0.23%	0.23%	0.21%	0.21%	0.07%	0.32%
03460 Fanconi anemia pathway	18	18	18	18	12	17	0.84%	0.83%	0.76%	0.76%	0.83%	0.54%
Environmental Information Processing												
Membrane transport	4	4	5	5	1	5	0.19%	0.19%	0.21%	0.21%	0.07%	0.16%
02010 ABC transporters	2	2	3	3	1	3	0.09%	0.09%	0.13%	0.13%	0.07%	0.10%
03070 Bacterial secretion system	2	2	2	2	0	2	0.09%	0.09%	0.08%	0.08%	0.00%	0.06%
Signal transduction	315	323	330	335	220	393	14.66%	14.96%	13.85%	14.06%	15.28%	12.58%
02020 Two-component system	12	12	12	12	7	11	0.56%	0.56%	0.50%	0.50%	0.49%	0.35%
04014 Ras signaling pathway	10	11	11	11	8	16	0.47%	0.51%	0.46%	0.46%	0.56%	0.51%
04015 Rap1 signaling pathway	8	9	9	9	5	11	0.37%	0.42%	0.38%	0.38%	0.35%	0.35%
04010 MAPK signaling pathway	11	11	11	11	10	14	0.51%	0.51%	0.46%	0.46%	0.69%	0.45%
04013 MAPK signaling pathway - fly	9	9	10	10	7	10	0.42%	0.42%	0.42%	0.42%	0.49%	0.32%
04016 MAPK signaling pathway - plant	3	4	3	4	3	4	0.14%	0.19%	0.13%	0.17%	0.21%	0.13%
04011 MAPK signaling pathway - yeast	37	38	39	41	32	69	1.72%	1.76%	1.64%	1.72%	2.22%	2.21%
04012 ErbB signaling pathway	6	6	6	6	5	7	0.28%	0.28%	0.25%	0.25%	0.35%	0.22%
04310 Wnt signaling pathway	12	12	13	13	7	12	0.56%	0.56%	0.55%	0.55%	0.49%	0.38%
04330 Notch signaling pathway	2	2	3	3	1	3	0.09%	0.09%	0.13%	0.13%	0.07%	0.10%
04340 Hedgehog signaling pathway	4	4	4	4	3	4	0.19%	0.19%	0.17%	0.17%	0.21%	0.13%
04341 Hedgehog signaling pathway - fly	6	6	6	6	4	6	0.28%	0.28%	0.25%	0.25%	0.28%	0.19%
04350 TGF-beta signaling pathway	7	7	7	7	4	7	0.33%	0.32%	0.29%	0.29%	0.28%	0.22%
04390 Hippo signaling pathway	8	8	8	8	4	8	0.37%	0.37%	0.34%	0.34%	0.28%	0.26%

04391 Hippo signaling pathway - fly	6	6	6	6	2	7	0.28%	0.28%	0.25%	0.25%	0.14%	0.22%
04392 Hippo signaling pathway -multiple species	3	3	3	3	1	4	0.14%	0.14%	0.13%	0.13%	0.07%	0.13%
04370 VEGF signaling pathway	8	8	8	8	5	8	0.37%	0.37%	0.34%	0.34%	0.35%	0.26%
04630 Jak-STAT signaling pathway	2	2	2	2	2	4	0.09%	0.09%	0.08%	0.08%	0.14%	0.13%
04064 NF-kappa B signaling pathway	3	3	4	4	2	4	0.14%	0.14%	0.17%	0.17%	0.14%	0.13%
04668 TNF signaling pathway	3	3	3	3	3	3	0.14%	0.14%	0.13%	0.13%	0.21%	0.10%
04066 HIF-1 signaling pathway	12	12	12	12	10	12	0.56%	0.56%	0.50%	0.50%	0.69%	0.38%
04068 FoxO signaling pathway	13	13	11	12	10	14	0.60%	0.60%	0.46%	0.50%	0.69%	0.45%
04020 Calcium signaling pathway	8	9	10	10	4	9	0.37%	0.42%	0.42%	0.42%	0.28%	0.29%
04070 Phosphatidylinositol signaling system	11	12	14	14	11	16	0.51%	0.56%	0.59%	0.59%	0.76%	0.51%
04072 Phospholipase D signaling pathway	10	10	10	10	7	12	0.47%	0.46%	0.42%	0.42%	0.49%	0.38%
04071 Sphingolipid signaling pathway	16	16	16	16	10	17	0.74%	0.74%	0.67%	0.67%	0.69%	0.54%
04024 cAMP signaling pathway	6	7	8	8	4	10	0.28%	0.32%	0.34%	0.34%	0.28%	0.32%
04022 cGMP-PKG signaling pathway	7	8	9	9	2	9	0.33%	0.37%	0.38%	0.38%	0.14%	0.29%
04151 PI3K-Akt signaling pathway	20	20	20	20	14	24	0.93%	0.93%	0.84%	0.84%	0.97%	0.77%
04152 AMPK signaling pathway	18	18	20	20	11	21	0.84%	0.83%	0.84%	0.84%	0.76%	0.67%
04150 mTOR signaling pathway	34	34	32	33	22	37	1.58%	1.57%	1.34%	1.38%	1.53%	1.18%
Cellular Processes												
Transport and catabolism	140	142	170	166	102	173	6.51%	6.58%	7.14%	6.97%	7.08%	5.54%
04144 Endocytosis	58	59	58	56	33	58	2.70%	2.73%	2.43%	2.35%	2.29%	1.86%
04145 Phagosome	25	25	28	27	21	31	1.16%	1.16%	1.18%	1.13%	1.46%	0.99%
04142 Lysosome	15	15	17	16	14	16	0.70%	0.69%	0.71%	0.67%	0.97%	0.51%
04146 Peroxisome	8	8	31	31	9	26	0.37%	0.37%	1.30%	1.30%	0.63%	0.83%
04140 Autophagy	11	11	11	11	7	15	0.51%	0.51%	0.46%	0.46%	0.49%	0.48%
04139 Mitophagy - yeast	23	24	25	25	18	27	1.07%	1.11%	1.05%	1.05%	1.25%	0.86%

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Cell motility	18	18	18	18	13	19	0.84%	0.83%	0.76%	0.76%	0.90%	0.61%
04810 Regulation of actin cytoskeleton	18	18	18	18	13	19	0.84%	0.83%	0.76%	0.76%	0.90%	0.61%
Cell growth and death	165	166	191	191	102	276	7.68%	7.69%	8.02%	8.02%	7.08%	8.83%
04110 Cell cycle	34	34	41	41	22	48	1.58%	1.57%	1.72%	1.72%	1.53%	1.54%
04111 Cell cycle - yeast	52	52	57	57	28	92	2.42%	2.41%	2.39%	2.39%	1.94%	2.94%
04112 Cell cycle - Caulobacter	0	0	0	0	1	1	0.00%	0.00%	0.00%	0.00%	0.07%	0.03%
04113 Meiosis - yeast	34	34	41	41	25	77	1.58%	1.57%	1.72%	1.72%	1.74%	2.46%
04114 Oocyte meiosis	20	21	26	25	8	30	0.93%	0.97%	1.09%	1.05%	0.56%	0.96%
04210 Apoptosis	9	9	9	10	6	10	0.42%	0.42%	0.38%	0.42%	0.42%	0.32%
04214 Apoptosis - fly	10	10	11	11	8	10	0.47%	0.46%	0.46%	0.46%	0.56%	0.32%
04215 Apoptosis - multiple species	2	2	1	1	1	2	0.09%	0.09%	0.04%	0.04%	0.07%	0.06%
04115 p53 signaling pathway	4	4	5	5	3	6	0.19%	0.19%	0.21%	0.21%	0.21%	0.19%
Cellular community - eukaryotes	43	42	42	42	29	47	2.00%	1.95%	1.76%	1.76%	2.01%	1.50%
04510 Focal adhesion	10	10	9	10	7	12	0.47%	0.46%	0.38%	0.42%	0.49%	0.38%
04520 Adherens junction	7	7	7	7	4	6	0.33%	0.32%	0.29%	0.29%	0.28%	0.19%
04530 Tight junction	15	14	15	14	8	16	0.70%	0.65%	0.63%	0.59%	0.56%	0.51%
04540 Gap junction	6	6	6	6	6	8	0.28%	0.28%	0.25%	0.25%	0.42%	0.26%
04550 Signaling pathways regulating pluripotency of stem cells	5	5	5	5	4	5	0.23%	0.23%	0.21%	0.21%	0.28%	0.16%
Cellular community - prokaryotes	9	9	9	9	5	9	0.42%	0.42%	0.38%	0.38%	0.35%	0.29%
02024 Quorum sensing	7	7	7	7	3	7	0.33%	0.32%	0.29%	0.29%	0.21%	0.22%
02025 Biofilm formation - Pseudomonas aeruginosa	2	2	2	2	2	2	0.09%	0.09%	0.08%	0.08%	0.14%	0.06%

Supplementary material table 2: Positions and genes in translocation regions

Translocations				
	Strains			
Translocation 1	***S. bacillaris* Pas13**		***S. bacillaris* Fri751**	
Genome position	8452014-8462522		9013137 - 9013802	
Gene(s) on the border	CDS: 24883-30140			
	3678_g (Unknown)			
Scaffold number		30		
Genome position	1208978-1211262		1241870-1244152	
Gene(s) on the border	CDS: 339006-342647		CDS: 135715-139356	
	496_g (Unknown function)		66_g (Unknown function)	
Scaffold number		2		1
Translocation 2	***S. bacillaris* Pas13**		***S. bacillaris* Fri751**	
Genome position	9133110-9133152		9239864-9242502	
Gene(s) on the border	CDS: 697-999		CDS: 19317-19619	
	3957_g (cytochrome c oxidase subunit 2)		3909_g (cytochrome c oxidase subunit 2)	
Scaffold number		44		62
Translocation 3	***S. bacillaris* Pas13**		***S. bacillaris* Fri751**	
Genome position	3980566-3980988			
Gene(s) on the border	CDS: 9-1044		CDS: 642-1532	
	1683_g (Mitochondrial ribosomal protein of the large subunit)		3990_g (Unknown function)	
	CDS: 2620-4194		CDS:1740-2081	
	1684_g (Unknown function)		3991_g (Unknown function)	
			CDS: 4008-4967	
			3992_g (Unknown function)	
Scaffold number		7		79
Genome position	*9314618-			
Gene(s) on the border	CDS487-1446		1956_g (HSPA_8)	
	4028_g (Unknown function)		1957_g (dut; dUTP pyrophosphatase)	
Scaffold number		56		13
Translocation 4	***S. bacillaris* Pas13**		***S. bacillaris* Fri751**	
Genome position				
Gene(s) on the border	2302_g (coaE)		2399_g	
	2301_g (Unknown function)		2398_g (FDPS)	
	2358_g (VPS54)		2403_g (AR01)	
Scaffold number		11		18
Genome position				
Gene(s) on the border	3836_g (DHX16)		Without break	
	3837_g (Unknown function)			

Scaffold number		35	57
Translocation 5	***S. bacillaris* Pas13**		***S. bacillaris* Fri751**
Genome position	3606076-3611718		731437
Gene(s) on the border and function	1523_g (Unknown function)		3403_g (KDELR)
Scaffold number		6	102/96

Supplementary material table 3: Gene Variations

PAS-Intergenic

Transcription Factor	Consensus	Position	Strand
Target Sequence: intergenic_region_Pas (size 919) Back to top			
Ace2p, Swi5p	ACCAGC	-701	F
Adr1p	TTGGRG	-114	F
Aft2p, Aft1p	TGCACCC	-575	R
Ash1p	YTGAT	-816	F
Ash1p	YTGAT	-258	F
Ash1p	YTGAT	-236	R
Ash1p	YTGAT	-246	R
Azf1p	AAGAAAAA	-368	F
Bas1p, Gcn4p	TGACTC	-616	F
Bas1p, Gcn4p	TGACTC	-523	R
Fkh1p, Fkh2p	RTAAAYAA	-712	F
Fkh1p, Fkh2p	RYMAAYA	-712	F
Fkh1p, Fkh2p	RYMAAYA	-458	F
Fkh1p, Fkh2p	RYMAAYA	-49	F
Fkh1p, Fkh2p	RYMAAYA	-729	R
Gat1p, Gln3p, Gzf3p	GATAAG	-359	F
Gcn4p	TGACTMT	-616	F
Gcn4p	TGACTMT	-79	F
Gcn4p	TGASTCA	-530	F
Gcn4p	TGASTCA	-523	R
Gcr1p	CTTCC	-663	F
Gcr1p	CWTCC	-663	F
Gcr1p	CWTCC	-105	R

Gis1p, YER130C	AGGGG	-584	F
Hac1p	CCAGC	-700	F
Hac1p	CCAGC	-261	R
Mot3p	CAGGYA	-686	R
Mot3p	AAGAGG	-356	F
Mot3p	ATGGAT	-391	F
Mot3p	ATGGAT	-102	F
Mot3p	TMGGAA	-871	F
Mot3p	AAGGWT	-166	R
Msn2p, Msn4p, Nrg1p, Rph1p	CCCCT	-579	R
Nrg1p	CCCTC	-855	R
Pho4p	CACGTK	-149	F
Rtg1p, Rtg3p	GTCAC	-75	R
Rtg1p, Rtg3p	GGTAC	-831	F
Rtg1p, Rtg3p	GGTAC	-641	F
Stb5p	CGGNS	-864	F
Stb5p	CGGNS	-834	F
Stb5p	CGGNS	-796	R
Ste12p	ATAAAACA	-708	F
Xbp1p	CTCGA	-462	F
Yap1p, Cad1p, Yap3p, Cin5p, Yap5p	TTACTAA	-795	F
Yap1p, Yap3p	TGACTCA	-523	R
Yrr1p	WCCGYKKWW	-30	F
Haa1p	SMGGSG	-835	F

FRI-Intergenic

Transcription Factor	Consensus	Position	Strand
Target Sequence: intergenic_region (size 918)			
Back to top			
Ace2p, Swi5p	ACCAGC	-701	F
Adr1p	TTGGRG	-113	F
Aft2p, Aft1p	TGCACCC	-575	R
Ash1p	YTGAT	-816	F
Ash1p	YTGAT	-257	F
Ash1p	YTGAT	-235	R
Ash1p	YTGAT	-245	R
Azf1p	AAGAAAAA	-367	F
Bas1p, Gcn4p	TGACTC	-616	F
Bas1p, Gcn4p	TGACTC	-523	R
Fkh1p, Fkh2p	RTAAAYAA	-712	F
Fkh1p, Fkh2p	RYMAAYA	-712	F
Fkh1p, Fkh2p	RYMAAYA	-458	F
Fkh1p, Fkh2p	RYMAAYA	-48	F
Fkh1p, Fkh2p	RYMAAYA	-729	R
Gat1p, Gln3p, Gzf3p	GATAAG	-358	F
Gcn4p	TGACTMT	-616	F
Gcn4p	TGACTMT	-78	F
Gcn4p	TGASTCA	-530	F
Gcn4p	TGASTCA	-523	R
Gcr1p	CTTCC	-663	F
Gcr1p	CWTCC	-663	F
Gcr1p	CWTCC	-104	R
Gis1p, YER130C	AGGGG	-584	F
Hac1p	CCAGC	-700	F
Hac1p	CCAGC	-260	R
Mot3p	CAGGYA	-686	R
Mot3p	AAGAGG	-355	F
Mot3p	ATGGAT	-390	F

Mot3p	ATGGAT	-101	F
Mot3p	TMGGAA	-871	F
Mot3p	AAGGWT	-165	R
Msn2p, Msn4p, Nrg1p, Rph1p	CCCCT	-579	R
Nrg1p	CCCTC	-855	R
Pho4p	CACGTK	-148	F
Rtg1p, Rtg3p	GTCAC	-74	R
Rtg1p, Rtg3p	GGTAC	-831	F
Rtg1p, Rtg3p	GGTAC	-641	F
Stb5p	CGGNS	-864	F
Stb5p	CGGNS	-834	F
Stb5p	CGGNS	-796	R
Ste12p	ATAAAACA	-708	F
Xbp1p	CTCGA	-462	F
Yap1p, Cad1p, Yap3p, Cin5p, Yap5p	TTACTAA	-795	F
Yap1p, Yap3p	TGACTCA	-523	R
Yrr1p	WCCGYKKWW	-29	F
Haa1p	SMGGSG	-835	F

Fri_GPP1_1

Transcription Factor	Consensus	Position	Strand
Target Sequence: Fri_GPP1_1 (size 919) Back to top			
Abf1p	TNNCGTNNNNNNTGAT	-537	R
Ash1p	YTGAT	-861	F
Ash1p	YTGAT	-852	F
Ash1p	YTGAT	-465	F
Ash1p	YTGAT	-335	R
Bas1p, Gcn4p	TGACTC	-229	R
Cup2p	HTHNNGCTGD	-299	R
Fkh1p, Fkh2p	RTAAAYAA	-50	F
Fkh1p, Fkh2p	RTAAAYAA	-909	R
Fkh1p, Fkh2p	RYMAAYA	-340	F
Fkh1p, Fkh2p	RYMAAYA	-115	F
Fkh1p, Fkh2p	RYMAAYA	-50	F
Fkh1p, Fkh2p	RYMAAYA	-363	R
Fkh1p, Fkh2p	RYMAAYA	-588	R
Fkh1p, Fkh2p	RYMAAYA	-819	R
Fkh1p, Fkh2p	RYMAAYA	-875	R
Fkh1p, Fkh2p	RYMAAYA	-909	R
Fkh1p, Fkh2p	RYAAACAWW	-588	R
Gcn4p	TGASTCA	-236	F
Gcn4p	TGASTCA	-229	R
Gcn4p	CACGTG	-762	F
Gcn4p	CACGTG	-756	R
Gis1p, YER130C	AGGGG	-897	F
Mcm1p	DCCYWWWNNRG	-252	R
Mcm1p	CCYWWWNNRG	-264	F
Mcm1p	CCYWWWNNRG	-253	R
Mcm1p	CCYWWWNNRG	-893	R
Mot3p	CAGGYA	-169	R
Mot3p	TAGGAT	-344	F

Msn2p, Msn4p, Nrg1p, Rph1p	CCCCT	-892	R
Pho4p	CACGTK	-762	F
Pho4p	CACGTK	-712	F
Pho4p	CACGTK	-544	F
Pho4p	CACGTK	-479	R
Pho4p	CACGTK	-756	R
Rtg1p, Rtg3p	GTCAC	-487	R
Rtg1p, Rtg3p	GTCAC	-711	R
Rtg1p, Rtg3p	GGTAC	-220	F
Rtg1p, Rtg3p	GGTAC	-642	R
Stb5p	CGGNS	-475	F
Stb5p	CGGNS	-76	F
Stb5p	CGGNS	-74	R
Stb5p	CGGNS	-473	R
Tec1p	RMATTCYY	-525	F
Tec1p	RMATTCYY	-43	F
Xbp1p	CTCGA	-305	F
Yap1p, Yap3p	TGACTCA	-229	R
Yap1p	TGACTAA	-491	F
Yap1p	TKACAAA	-732	F
Yap1p	TTACTCA	-913	F
Yap1p	TTACTCA	-313	F

Saccharomyces GPP2 (We choosed the intergenic region)

Transcription Factor	Consensus	Position	Strand
Target Sequence: NC_001137.3:279930-281710 (size 1781)			
Back to top			
Ace2p, Swi5p	ACCAGC	-177	F
Ace2p, Swi5p	ACCAGC	-969	R
Ace2p, Swi5p	ACCAGC	-1108	R
Adr1p	TTGGRG	-25	F
Adr1p	TTGGRG	-514	R
Adr1p	TTGGRG	-1192	R
Ash1p	YTGAT	-1693	F
Ash1p	YTGAT	-1522	F
Ash1p	YTGAT	-1423	F
Ash1p	YTGAT	-993	F
Ash1p	YTGAT	-423	F
Ash1p	YTGAT	-406	R
Ash1p	YTGAT	-840	R
Ash1p	YTGAT	-1203	R
Azf1p	AAAAGAAA	-928	F
Azf1p	AAAAGAAA	-842	F
Azf1p	AAAAGAAA	-817	F
Bas1p, Gcn4p	TGACTC	-122	F
Cbf1p	RTCACGTG	-779	F
Cbf1p	RTCACRTG	-779	F
Cup2p	HTHNNGCTGD	-154	F
Cup2p	HTHNNGCTGD	-9	R
Fkh1p, Fkh2p	RYMAAYA	-985	F
Fkh1p, Fkh2p	RYMAAYA	-411	F
Gcn4p	TGACTMT	-122	F
Gcn4p	RRTGACTC	-124	F
Gcn4p	CACGTG	-777	F
Gcn4p	CACGTG	-771	R
Gcr1p	CTTCC	-160	R

Gcr1p	CTTCC	-1371	R
Gcr1p	CTTCC	-1533	R
Gcr1p	CTTCC	-1639	R
Gcr1p	CWTCC	-716	F
Gcr1p	CWTCC	-93	R
Gcr1p	CWTCC	-160	R
Gcr1p	CWTCC	-413	R
Gcr1p	CWTCC	-1371	R
Gcr1p	CWTCC	-1472	R
Gcr1p	CWTCC	-1533	R
Gcr1p	CWTCC	-1639	R
Gis1p, YER130C	AGGGG	-759	F
Hac1p	CCAGC	-568	F
Hac1p	CCAGC	-247	F
Hac1p	CCAGC	-176	F
Hac1p	CCAGC	-19	F
Hac1p	CCAGC	-578	R
Hac1p	CCAGC	-970	R
Hac1p	CCAGC	-1109	R
Hac1p	CCAGC	-1568	R
Hap2p, Hap3p, Hap4p, Hap5p	TNATTGGT	-1608	R
Ime1p	TTTTCHHCG	-1294	F
Ime1p	TTTTCHHCG	-397	F
Mcm1p	CCYWWWNNRG	-755	R
Mcm1p	CCYWWWNNRG	-756	R
Met31p, Met32p	CTGTGGC	-1362	R
Met4p	TCACGTG	-778	F
Mot3p	WAGGTA	-616	R
Mot3p	WAGGTA	-1544	R
Mot3p	CAGGYA	-1572	R
Mot3p	AAGAGG	-343	R
Mot3p	AAGAGG	-378	R
Mot3p	ATGGAT	-1690	F

Mot3p	ATGGAT	-1300	F
Mot3p	ATGGAT	-420	F
Mot3p	TAGGAT	-905	R
Mot3p	TAGGAT	-1506	R
Mot3p	TMGGAA	-1646	F
Mot3p	TMGGAA	-437	F
Mot3p	TMGGAA	-732	R
Mot3p	TMGGAA	-785	R
Mot3p	TMGGAA	-1023	R
Mot3p	AAGGKA	-616	R
Mot3p	AAGGKA	-1544	R
Mot3p	AAGGWT	-1155	F
Mot3p	AAGGWT	-916	R
Msn2p, Msn4p, Nrg1p, Rph1p	CCCCT	-754	R
Ndt80p, Sum1p	GNCRCAAAW	-1390	R
Nrg1p	CCCTC	-1134	F
Nrg1p	CCCTC	-385	F
Nrg1p	CCCTC	-466	R
Pho4p	CACGTK	-777	F
Pho4p	CACGTK	-771	R
Pho4p	CACGTK	-1169	R
Pho4p	CACGTGGG	-777	F
Reb1p	CCGGGTAA	-209	R
Rgt1p	CGGANNA	-644	F
Rgt1p	CGGANNA	-733	R
Rgt1p	CGGANNA	-1585	R
Rlm1p	TAWWWWTAGM	-955	R
Rtg1p, Rtg3p	GTCAC	-1664	F
Rtg1p, Rtg3p	GTCAC	-779	F
Rtg1p, Rtg3p	GTCAC	-118	R
Rtg1p, Rtg3p	GGTAC	-1382	F
Rtg1p, Rtg3p	GGTAC	-1094	F
Rtg1p, Rtg3p	GGTAC	-1088	R
Rtg1p, Rtg3p	GGTAC	-1376	R
Stb5p	CGGNS	-1599	F
Stb5p	CGGNS	-1587	F
Stb5p	CGGNS	-1071	F
Stb5p	CGGNS	-289	F
Stb5p	CGGNS	-212	F
Stb5p	CGGNS	-250	R

Stb5p	CGGNS	-388	R
Stb5p	CGGNS	-674	R
Stb5p	CGGNS	-762	R
Stb5p	CGGNS	-1275	R
Stb5p	CGGNS	-1285	R
Ste12p	ATAAAACA	-1002	R
Tec1p	CATTCT	-1122	R
Tec1p	CATTCC	-1503	F
Tec1p	CATTCC	-1031	F
Tec1p	RMATTCYY	-428	R
Uga3p	SGCGGNWTTT	-1598	F
Upc2p	TCGTTYAG	-560	F
Xbp1p	CTCGA	-1409	F
Xbp1p	CTCGA	-230	R
Yap1p	TKACAAA	-78	F
Yrr1p	WCCGYKKWW	-681	R
Gsm1p	CGGNNNNNNNNCGG	-685	F
Gsm1p	CGGNNNNNNNNNCGG	-1599	F
Gsm1p	CGGNNNNNNNNNCGG	-1596	F
Gsm1p	CGGNNNNNNNNNCGG	-1585	R
Haa1p	SMGGSG	-1600	F
Haa1p	SMGGSG	-1588	F
Haa1p	SMGGSG	-1072	F
Haa1p	SMGGSG	-213	F
Haa1p	SMGGSG	-673	R

Saccharomyces GPP1

Transcription Factor	Consensus	Position	Strand
Target Sequence: NC_001141.2:254860-255115 (size 256) Back to top			
Ash1p	YTGAT	-134	F
Ash1p	YTGAT	-100	F
Ash1p	YTGAT	-32	R
Azf1p	AAGAAAAA	-80	R
Azf1p	AAGAAAAA	-200	R
Azf1p	AAGAAAAA	-223	R
Azf1p	AAAAGAAA	-198	R
Azf1p	AAAAGAAA	-216	R
Azf1p	AAAAGAAA	-221	R
Azf1p	AAAAGAAA	-229	R
Fkh1p, Fkh2p	RYMAAYA	-22	F
Mot3p	AAGAGG	-236	R