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Candida zemplinina can reduce acetic acid produced by *Saccharomyces cerevisiae* in sweet wine fermentations

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Running title: Acetic acid reduction in sweet wine fermentations by *C. zemplinina*

Abstract

In this study we have investigated the possibility of using *Candida zemplinina*, as a partner of *Saccharomyces cerevisiae*, in mixed fermentations of must with a high sugar content, in order to reduce its acetic acid production. Thirty five *C. zemplinina* strains, which were isolated from different geographic regions, were molecularly characterized and their fermentation performances were determined. Five genetically different strains were selected for mixed fermentations with *S. cerevisiae*. Two types of inoculation were carried out: co-inoculation and sequential inoculation. A balance between the two species was generally observed for the first 6 days, after which *C. zemplinina* started to decrease. Relevant differences were observed concerning the consumption of sugars, the ethanol and glycerol content and acetic acid production, according to which strain was used and which type of inoculation was performed. Sequential inoculation led to reduction of about a half of the acetic acid content, compared to the pure *S. cerevisiae* fermentation, but the ethanol and glycerol amounts were also low. A co-inoculation with selected couples of *S. cerevisiae* and *C. zemplinina* resulted in a decrease of about 0.3 g/L of acetic acid, maintaining high ethanol and glycerol levels. This study demonstrates that mixed *S. cerevisiae* and *C. zemplinina* fermentation could be applied in sweet wine fermentation to reduce the production of acetic acid, connected to the *S. cerevisiae* osmotic stress response.

Keywords: Sweet wine fermentation; *Candida zemplinina;* molecular characterization; phenotypic characterization; acetic acid.

Introduction

Candida zemplinina is a psycrotolerant and osmotolerant yeast, properties that can be advantageously exploited in sweet wine production, which is characterized by a high sugar concentration and low fermentation temperatures (26). Sipiczki recognized it as a distinct new species and named it *C. zemplinina* in 2003 (25). However, already back in 2002, in the Napa valley, California, Mills et al. isolated a strain of *Candida* sp., named *Candida* EJ1, from *Botrytis cinerea*-infected grapes, with interesting features, such as the capability of depleting fructose from a Chardonnay juice without affecting the glucose concentration (19).

The yeast ecology studies carried out over the last 5 years have highlighted the frequent presence of this species in wine fermentations (3, 16, 17, 20, 28, 29, 31, 33). Moreover, it has been demonstrated that strains of *C. stellata*, deposited in several culture collections and isolated from grapes, belong to the *C. zemplinina* species (10). Altogether, all this evidence points out the need for a better understanding of the role of this yeast during wine transformation.

Sweet wines, such as "Passito wines", "Icewines", Sauternes and others, are produced from must obtained from dried grapes, and are characterized by a very high sugar concentration. The *Saccharomyces cerevisiae* strains used for these fermentations should be able to promptly respond to osmotic stress, and be able to increase their load immediately after inoculation. It has been shown that the response of *S. cerevisiae* to osmotic stress can result in increased acetic acid contents, due to the upregulation of genes encoding for aldehyde dehydrogenases. In fact, yeast grown in 40% (w/v) sugar juice produced 1.35 g/L acetic acid, compared to 0.3 g/L at the lower sugar concentration (22% w/v) (12). The acetic taste in these wines is in part masked by the high residual sugars after fermentation. However, winemakers would like to reduce its content, which generally penalizes the final sensory quality of wines, becoming a limit to its commercialization, also in view of international legal limits for the acetic acid (11,

14).

Several studies have demonstrated that non-*Saccharomyces* yeasts are able to survive during alcoholic fermentations (9, 19). Such evidence opens the way towards new applications of non-*Saccharomyces* in wine fermentation, which would make the organoleptic profiles of the wines more complex due to enzymatic activities that such yeasts possess (27).

Mixed *Saccharomyces* and non-*Saccharomyces* fermentations have been tested since the 90's (32), but it is only in recent years that the interest of researchers has been growing, as witnessed by the recent review papers published on this subject (5, 13). *Hanseniaspora uvarum, Torulaspora delbrueckii, Lachancea* (*Kluyveromyces*) *thermotolerans* and *C. stellata* have been the main species investigated so far in mixed fermentations with the aim of adding complexity to the wine (4, 6, 9, 15, 32). The mixed fermentation strategy has also been used in high sugar musts, in order to reduce the acetic acid content of the final wine. For this purpose, strains of *T. delbruekii*, which have been described as low acetic acid producers (23), have been combined with *S. cerevisiae* and a 53% reduction in volatile acidity has been obtained (2).

In this context, one possibility that could be exploited is to combine *S. cerevisiae* with *C. zemplinina* during fermentation. Since the latter yeast is osmotolerant and fructophylic, and generally produces low amounts of acetic acid together with relevant quantities of glycerol from sugar fermentation (18, 29), it can be suggested that it would be able to consume sugars at the very beginning of fermentation, in this way alleviating the *S. cerevisiae* osmotic stress, thereby reducing production of acetic acid.

In this work, we have performed mixed fermentations, combining *C. zemplinina* obtained from grapes and wines of different origin with three strains of *S. cerevisiae* (one commercial and two wine isolates), in order to evaluate the potential effect on the reduction of acetic acid.

Materials and methods

Yeast strains

Thirty-five *C. zemplinina* isolates, mainly from Italy, but also including one isolate from Greece and one from the United States, were used in this study (Table 1). Three strains of *S. cerevisiae* were also selected for mixed fermentations. Two of these, coded FB40 and ELCF3WC, came from the spontaneous fermentation of sweet wines in the North of Italy, Picolit and Erbaluce, respectively. The third *S. cerevisiae*, Lalvin EC1118 (Lallemand, Montreal, Canada), a commercial active dried yeast (ADY), was selected because it is widely used by local wineries for the production of sweet wines.

All the isolates were identified through molecular methods and they are deposited in the culture collections of the Universities of Turin, Teramo and Verona (Italy). They were routinely cultivated in YPD broth (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, all from Oxoid, Milan, Italy) for 36-48 h at 30°C.

Molecular characterization of the *Candida zemplinina* strains

DNA was extracted from the *C. zemplinina* strains using a mechanical treatment in a bead-beader machine (FastPrep®-24, MP Biomedical, Solon, OH, USA) as describe elsewhere (7). The DNA was subsequently quantified using a NanoDrop instrument (Celbio, Milan, Italy) and standardized at 100 ng/μl. Molecular characterization of the isolates was carried out using RAPD and SAU-PCR methods, as described by Cocolin et al. (8). All the *C. zemplinina* strains were subjected to both methods at least twice.

Candida zemplinina fermentations

Laboratory fermentations were carried out in a total volume of 100 mL of an Erbaluce dried grape must, containing 210 g/L glucose, 193 g/L fructose, 3.5 g/L glycerol and 1.8 % vol. of ethanol. The pH and titratable acidity, expressed as g/L of tartaric acid, were 3.23 and 8.12, respectively. Before inoculation, the must was pasteurized at 90°C for 90 min and the absence of live yeast populations was assessed by plating 100 μl on YPD agar, incubated at 30°C for 48 h. Despite the long time necessary for the pasteurization, no caramelization of the sugars occurred. The *C. zemplinina* isolates were pre-adapted in the same sweet must for 48 h and then inoculated to reach a final concentration of 10^5-10^6 cells/mL, which was determined through a microscopic count. Fermentations were carried out at 25°C for 14 days. Samples were collected in triplicate at 0, 1, 2, 6, 8, 12 and 14 days of fermentation and microbiological counts and HPLC analysis were carried out. Colony forming units (cfu)/mL were determined by plating the serially diluted samples on the differential WLN medium (21) (Oxoid) and by incubating them for 3 to 5 days at 30°C. *S. cerevisiae* forms convex, creamy-white colonies in this medium, while *C. zemplinina* forms flat, light to intense green-ones, this difference enabling their concurrent enumeration on the same medium (31). The glucose and fructose consumption as well as the glycerol, ethanol and acetic acid production were quantified by means of HPLC (Thermo Electron Corporation, Waltham, MA, USA) equipped with a UV detector (UV100), set to 210 nm, and a refractive index detector (RI-150). The analyses were performed isocratically at 0.8 mL/min and 65 °C with a 300x7.8 mm i.d. cation exchange column (Aminex HPX-87H) and a Cation H^+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA), using 0.0026N H_2SO_4 as the mobile phase (14, 24).

The three *S. cerevisiae* were also tested for their fermentation performances in the dried grape must, as described for the *C. zemplinina* strains. All the fermentation trials were carried out twice.

Mixed fermentations

Three *S. cerevisiae* strains and 5 strains of *C. zemplinina* were selected and used for mixed fermentations in a total volume of 100 mL of the same must described above. Two different approaches were used: inoculation of both yeasts at the same time (co-inoculation), and addition of *S. cerevisiae* 48 h after *C. zemplinina* inoculation (sequential inoculation). All the strains were pre-adapted in the same sweet must and always added to a final concentration of 10^5-10^6 cells/mL. The fermentations lasted 14 and 16 days for the co-inoculation and sequential inoculation, respectively. Both the plate counts and HPLC analysis of these experiments were performed as described above. Samplings were carried out in triplicate at 0, 1, 6, 8, 12 and 14 days of fermentation. In the case of sequential inoculation, samples were also collected 48 and 24 h prior to inoculation of *S. cerevisiae* (-2 and -1 days of fermentation). All the fermentation trials were carried out twice.

Statistical analysis

Gels containing the RAPD and SAU-PCR profiles of the *C. zemplinina* isolates were normalized, using the 1 kb molecular ladder (Sigma, Milan, Italy) loaded in each gel, and subjected to cluster analysis with the BioNumerics software (Applied Maths, Kortrijk, Belgium). The Pearson product moment correlation coefficient was used to calculate the similarities in profile patterns, and dendrograms were obtained by means of the Unweighted Pair Group Method using the Arithmetic Average (UPGMA) clustering algorithm.

Statistical analyses of the chemical composition of the wines were performed using the SPSS statistical software package (version 17.0; SPSS Inc., Chicago, IL, USA). The Tukey-b test for $p<0.05$ was used in order to establish any statistical differences by one-way analysis of variance (ANOVA). A multi-factorial ANOVA test was used to explore the effect of the three

tested factors (strain of *S. cerevisiae*, strain of *C. zemplinina* and type of inoculum) and to verify the existence of any interaction between them.

Results

Molecular characterization of the *C. zemplinina* strains

The dendrogram, which combines the results of the RAPD and SAU-PCR molecular characterization of the *C. zemplinina* strains included in this study, is presented in Figure 1. As can be observed, 26 out of 35 strains clustered in two groups (1 and 2), while the remaining 9 formed three minor clusters, each including 2 to 4 strains, and one single-strain cluster. If the composition of the clusters is analyzed, it can be observed that no correlation could be found with the source of isolation; strains from different Italian regions, from Greece and USA in fact clustered together. Only cluster 4 was composed of 4 strains from the same geographic region in the Northeast of Italy and the majority of strains from winery G, in the Abruzzo region (Central Italy), were grouped in cluster 2.

Dynamics and analytical results of the fermentations inoculated with pure cultures of *C. zemplinina* and *S. cerevisiae*

The growth kinetics of the *C. zemplinina* strains, when inoculated in pure culture in must obtained from dried grapes, are summarized in Figure 2, where the counts of all the tested strains are taken into consideration and the results are shown as minimum, maximum and median growth. As can be seen, *C. zemplinina* was able to grow reaching a count of about 10^7 colony forming units (cfu)/mL in the first two days of fermentation, and then started to decrease from day 6 onwards. At the end of the monitored period, the strains still presented good vitality with counts spanning from 10^5 to 10^6 cfu/mL.

The results obtained from the HPLC analyses of the wines, after 14 days of fermentation, using either strains of *C. zemplinina* or *S. cerevisiae* in pure culture, are shown in Table 2. The *C. zemplinina* strains were characterized by a higher consumption of fructose than glucose, and some strains showed a total fructophylic character, having left the content of glucose originally found in the must before inoculation untouched (Table 2). The *C. zemplinina* strains generally produced relevant quantities of glycerol and low amounts of acetic acid and ethanol, although it should be underlined that some isolates were able to produce up to 8.0%vol. of ethanol. A completely different picture emerged from the chemical analysis of the wines obtained from the *S. cerevisiae* fermentation. Strains FB40 and ELCF3WC performed rather well, consuming more than half of the sugars present in the must and producing more than 13.5 %vol. of ethanol (Table 2). However, both strains showed a very high production of acetic acid, 1.54 and 1.29 g/L for FB40 and ELCF3WC, respectively. Different behavior was observed for the EC1118 commercial strain, which showed a limited consumption of sugars (about 130 g/L), which was correlated to a very low ethanol and acetic acid content. All the *S. cerevisiae* strains were able to produce a relevant amount of glycerol (Table 2).

Five strain of *C. zemplinina* were selected from the 35 that were tested, on the basis of the fermentation performances and their genetic diversity. The details regarding sugar consumption, yield and glycerol, acetic acid and ethanol production are reported in Table 2. These strains were chosen because of the low acetic acid, low ethanol and relevant glycerol content of the final wines, and the preferred consumption of fructose with respect to glucose. The *C. zemplinina* EJ1strain did not comply with the above mentioned criteria; however, it was selected due to its origin (from a different continent) and limited genetic similarity with the other *C. zemplinina* isolates, as shown in Figure 1.

S. cerevisiae **and** ***C. zemplinina*** **mixed fermentations**

The microbial dynamics of the mixed fermentations are shown in Figure 3. Two different strategies were tested, a co-inoculation of the two species and a sequential inoculation, with a delay of 48 h in the addition of *S. cerevisiae* with respect to *C. zemplinina*. The data are presented separately for the three *S. cerevisiae* strains, and the results of the counts (cfu/ml) are expressed as means ± standard deviation. The *C. zemplinina* trends are the results of the five fermentations conducted in duplicate with the selected strains. The counts of *S. cerevisiae* FB40 when mixed separately with the 5 *C. zemplinina* strains are reported in panel A. In this case in either the co-inoculated or the sequential inoculation, the *C. zemplinina* strains were not able to compete with *S. cerevisiae*, although a balance of the two species can be observed in the first 6 days. After this point, the *C. zemplinina* counts started to decrease and reached 10^3 – 10^4 cfu/mL after 14 days, compared to a population of *S. cerevisiae* that remained stable at 10^6 – 10^7 cfu/mL throughout the whole period. Very similar behavior was observed for *S. cerevisiae* ELCF3WC (Fig. 3B). Again in this case, the *C. zemplinina* strains could not compete and started to decrease in numbers after 6 days. In order to exclude possible killer toxin activity of *S. cerevisiae* FB40 and ELCF3WC towards the *C. zemplinina* strains, tests were carried out as described by Pérez et al. (22), and resulted in no inhibition in any of the cases (data not shown). A completely different picture emerged for the EC1118 commercial strain, which was only able to dominate the fermentation in the case of the co-inoculation (Fig. 3C). It is interesting to note that in the case of the sequential inoculation, the *C. zemplinina* strains were able to dominate EC1118 until day 12, and this was most probably due to the scarce capability of growth that this *S. cerevisiae* showed when inoculated after *C. zemplinina.*

The chemical composition of the wines obtained from the fermentations carried out by co-inoculation and sequential inoculation are presented in Tables 3 and 4, respectively. In the co-inoculated fermentations, the three different *S. cerevisiae* strain/*C. zemplinina* combinations, resulted in a significantly different consumption of sugars. As can be observed, the EC1118

strain combination always performed poorly, leaving high quantities of sugars at day 14, regardless of what *C. zemplinina* was used. On the contrary, the ELCF3WC combination always showed good fermentation properties, and this resulted in high ethanol content and low residual sugars. The Glucose/Fructose (G/F) ratio was similar for strains FB40 and ELCF3WC, while it was significantly higher for all the wines produced with EC1118. The acetic acid content was influenced to a great extent by the *S. cerevisiae* strain that was used. It is interesting to observe that *S. cerevisiae* EC1118, which offered the worst performance, also resulted to be the producer of the highest amount of acetic acid, unless it was inoculated with *C. zemplinina* T1Y3. This observation could be considered in conflict with its behavior in pure culture, however, in such a condition, the strain consumed very little sugar and produced limited amounts of ethanol and acetic acid. On the contrary, wines fermented with the ELCF3WC strain always contained higher amounts of alcohol (>12.6 %vol.) and glycerol (>15 g/L), while strain FB40 always produced low quantities of glycerol. However it did not always produce low quantities of alcohol.

In the case of the sequential inoculation (Table 4), *S. cerevisiae* EC1118 was again the worst, producing wines with high residual sugars and with a G/F ratio of between 1.85 and 2.13. When this inoculation approach was used, it is important to note that the acetic acid content was not connected to the *S. cerevisiae* strain, but was influenced by the *S. cerevisiae*/*C. zemplinina* strain combination used in the fermentation process. *Candida zemplinina* EJ1 was associated to wines with a low ethanol and generally high acetic acid content, and similar results were also obtained for the BC60 strain. The combination *C. zemplinina* PEDRO10 and *S. cerevisiae* ELCF3WC produced wines with less acetic acid (< 0.40 g/L) without affecting the ethanol (11.4 %vol.) or glycerol (12 g/L) contents.

If the chemical parameters determined for the wines obtained with the two different inoculation approaches are analyzed together, significant differences emerge in their

compositions, especially for the G/F ratio, glycerol and acetic acid (Table 5). The influence of the strains (both *S. cerevisiae* and *C. zemplinina*), their interaction, as well as the type of inoculation used, always resulted in significant differences ($p<0.001$) (data not shown). The wines obtained with sequential inoculation resulted to have higher residual sugars, with an increased G/F ratio, thereby potentially influencing their final organoleptic properties. In these cases, the fermentation products, such as ethanol and glycerol, decreased. However, the quantity of acetic acid produced was between 0.60 – 0.75 g/L, and was half that of the fermentations conducted inoculating only the *S. cerevisiae* strains or both species at the same time.

Discussion

In the last couple of years, a number of papers that have focused on the potential application of *C. zemplinina* in wine fermentations have been published (1, 18, 29, 30), mainly due to its ethanol and low temperature tolerance, osmotic resistance and fructophylic character.

In this study, we have specifically investigated the possibility of using *C. zemplinina* in sweet wine fermentations, sequentially or co-inoculated with *S. cerevisiae*. All the fermentations were carried out in natural must obtained from dried grapes, in order to mimic the real conditions encountered during the production of sweet wines and avoid the use of laboratory media that can give a totally different picture in terms of yeast fermentative behavior.

A set of 35 isolates of *C. zemplinina* was first molecularly characterized and the results obtained underlined a relative genetic homogeneity within the strains tested. There were no differences, in terms of clustering, on the basis of the geographic distribution, and most of the strains formed two large clusters. When these strains were tested in fermentation trials of must obtained from dried grapes, their fructophylic character was confirmed, as previously described (25, 26). Moreover, their capability to produce relevant quantities of glycerol and

low amounts of acetic acid was also confirmed, in agreement with other studies (18, 29). Interestingly, the behavior of *S. cerevisiae* was different between the commercial strain and the wild isolates from sweet wine fermentations. As shown in Table 2, the EC1118 commercial strain performed worse than the two wine isolated strains, since a high residual sugar, associated with lower ethanol production, was detected at the end of the monitoring period. A notable production of acetic acid was concomitantly observed, most likely due to the osmotic stress provoked by the high concentration of sugars, responsible for the upregulation of the genes encoding for aldehyde dehydrogenases (12). It should be underlined that differences in the vitality counts were observed for the three *S. cerevisiae* strains when inoculated as pure culture in the must utilized in this study. While the wild strains were able to promptly increase in cell counts, reaching 10^8 cfu/mL at day 1, EC1118 never reached a load of 10^7 cfu/mL and started to decrease after 6 days of fermentation (data not shown). This evidence allows us to speculate that the wild strains may adapt well to an environment, similar to the one from where they were isolated (musts with high sugar concentration), and underlines the need for better performing strains than the commercial one used in this study. Five strains of *C. zemplinina* were selected, on the basis of their genetic characteristics and fermentation performances, for the mixed fermentation experiments. As shown in Figure 1, the BC60 and PEDRO10 strains were located in cluster 1 (although in two different subclusters), while the T1Y3 and L37 strains grouped in cluster 2 (but again in two different subclusters). The last strain selected, namely EJ1, was genetically far from the first 4 described. Considering the chemical parameters of the wines obtained with these strains (Table 2), it can be observed that they all resulted to be homogeneous, apart from *C. zemplinina* EJ1, which again showed relevant differences with respect of the other strains selected.

Remarkably, all the selected *C. zemplinina* showed comparable growth kinetics, regardless of which *S. cerevisiae* strain was used. This aspect is highlighted by the contained standard deviations reported in Figure 3. In other words, the 5 selected *C. zemplinina* showed similar growth curves when coupled with *S. cerevisiae*, in both the case of the co-inoculation and the sequential inoculation. The main differences were observed in the trends of *S. cerevisiae* EC1118, which was not able to dominate the fermentation in the case of sequential inoculation. It is interesting to underline that the counts reached by this strain in mixed fermentations were higher than in pure culture, underlining that *C. zemplinina* strains could facilitate the growth of *S. cerevisiae* EC1118.

While no variations were observed, in terms of growth, in the mixed fermentation experiments for each strain of *S. cerevisiae*, relevant differences were detected for the chemical composition of the wines. Again in this case, *S. cerevisiae* EC1118 showed poor fermentation power, while *S. cerevisiae* ELCF3WC was able to produce more ethanol and glycerol and less acetic acid. However, as described above, *C. zemplinina* influenced the production of acetic acid by *S. cerevisiae*. More specifically, a co-inoculation of *C. zemplinina* T1Y3 or L37 with *S. cerevisiae* ELCF3WC resulted in wines with a high ethanol content (>14.5 %vol.) and acetic acid of about 1 g/L (21% acetic acid reduction, compared to the pure ELCF3WC fermentation).

This study has demonstrated that the fermentation of musts, characterized by a high sugar content, with *S. cerevisiae* and *C. zemplinina* mixtures, may contribute to control the acetic acid production by *S. cerevisiae*. The data presented in this study support the use of this non-*Saccharomyces* species, but at the same time the specific *S. cerevisiae*/*C. zemplinina* combination is important. As shown, the possibility to reduce the acetic acid content is closely connected to the strain combination and the type of inoculation performed. Moreover, other chemical parameters, such as higher alcohols and acetaldehyde should be monitored, since it

has been pointed out that *C. zemplinina* can contribute to a great extent to their increase or decrease, respectively (1, 18). Since all the data presented here were obtained from pasteurized must, more investigations are necessary to assess the capability of *C. zemplinina* to compete with the natural microbiota of grape musts and to confirm that the mechanism responsible for the acetic acid reduction is due to *S. cerevisiae* osmotic stress relief.

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Table 1. Source of isolation of the *C. zemplinina* strains used in this study

Geographical region (Country)	**Winery**	**Source**	**Strain**
Abruzzo (Italy)	G	Grape juice	L37
		Cooked must	L191, L35, L491, L23. L34, L364, L344, L36, L365, L477
Friuli Venezia Giulia (Italy)	B	Picolit grapes	BC16, BC20
		Picolit grape juice	BC55, BC60
		Picolit fermentation (3 days)	BC115, BC116
		Picolit fermentation (14 days)	BC224, BC226
	F	Picolit grapes	FC50, FC54
	R	Ramandolo grapes	R1, R5
Trentino Alto Adige (Italy)	To	Nosiola dried grapes	TOHA07
	T	Nosiola dried grapes	TOBLINO02
	Pd	Nosiola dried grapes	PEDRO10
	Pi	Nosiola grapes	PISO02, SANTA01
Veneto (Italy)	M	Amarone grape juice	C2CY2, C1AY2, T1Y3
		Amarone fermentation (7 days)	C2AY9, C2BY10
California (USA)	nd[a]	Botritized grapes	EJ1
Attika (Greece)	nd	Grape Juice	D1

[a]nd, not defined

Table 2. HPLC analyes of the wines obtained from pure cultures of *C. zemplinina* and *S. cerevisiae.* Results are shown as minimum, maximum and average amounts for the 35 strains of *C. zemplinina.* Moreover, the analytical results of the three *S. cerevisiae* and the five *C. zemplinina* selected in this study are presented as the average of two fermentation trials. The glucose and fructose content of the sweet must used in the fermentations was 210 g/L and 193 g/L, respectively.

	Residual reducing sugars (g/L)	Consumption		Production			Ethanol / Consumed sugars yield (g/g)
		Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Ethanol (% vol.)	
All *C. zemplinina* strains							
Average	297.49	23.76	82.33	5.99	0.31	5.07	0.373
Minimum	229.75	ndc[a]	29.22	1.84	0.19	1.91	0.154
Maximum	360.60	67.82	107.34	10.10	0.79	8.44	0.504
S. cerevisiae strains							
FB40	179.47	158.32	65.21	10.89	1.54	13.70	0.484
EC1118	269.97	89.56	43.47	10.33	0.95	7.83	0.464
ELCF3WC	181.15	157.37	64.48	12.34	1.29	13.54	0.482
Selected *C. zemplinina* strains							
PEDRO10	328.31	ndc	74.69	6.16	0.33	4.71	0.498
L37	300.45	16.07	86.48	5.60	0.21	4.67	0.459
BC60	305.16	22.03	75.81	3.82	0.20	2.01	0.162
EJ1	234.13	67.82	101.05	7.73	0.39	6.88	0.321
T1Y3	271.87	33.77	97.36	6.01	0.19	5.48	0.330

[a]ndc, not detectable.

Table 3. Chemical composition of wines obtained from the co-inoculated fermentations of the *S. cerevisiae* and *C. zemplinina* strains. The ethanol content of the initial must was 1.8% vol.

C. zemplinina	*S. cerevisiae*	Reducing Sugar (g/L)	Glucose/Fructose (-)	Ethanol (% vol.)	Ethanol / Consumed sugars yield (g/g)	Glycerol (g/L)	Acetic acid (g/L)
EJ1	FB40	$240\pm2^{b,\gamma}$	$0.69\pm0.01^{a,\alpha}$	$11.2\pm0.1^{a,\alpha}$	$0.454\pm0.009^{a,\alpha}$	$13.2\pm0.1^{a,\alpha}$	$1.21\pm0.01^{b,\beta}$
EJ1	EC1118	$255\pm5^{c,\beta}$	$0.87\pm0.07^{b,\alpha}$	$10.6\pm0.4^{a,\alpha}$	$0.467\pm0.028^{a,\alpha}$	$14.0\pm0.2^{b,\alpha}$	$1.12\pm0.07^{b,\beta}$
EJ1	ELCF3WC	$223\pm4^{a,\delta}$	$0.70\pm0.05^{a,\alpha}$	$12.6\pm0.3^{b,\alpha}$	$0.471\pm0.023^{a,\alpha}$	$15.2\pm0.1^{c,\alpha}$	$0.92\pm0.05^{a,\alpha}$
Sig[1]		***	**	***	ns	***	***
T1Y3	FB40	$228\pm3^{b,\beta}$	$0.66\pm0.04^{a,\alpha}$	$12.3\pm0.2^{b,\beta}$	$0.471\pm0.018^{a,\alpha}$	$14.2\pm0.1^{a,\delta}$	$1.50\pm0.04^{b,\gamma}$
T1Y3	EC1118	$235\pm4^{c,\alpha}$	$0.91\pm0.05^{b,\alpha}$	$11.6\pm0.3^{a,\beta}$	$0.462\pm0.025^{a,\alpha}$	$14.7\pm0.1^{b,\beta}$	$0.96\pm0.05^{a,\alpha}$
T1Y3	ELCF3WC	$183\pm3^{a,\alpha}$	$0.60\pm0.04^{a,\alpha}$	$14.9\pm0.2^{c,\gamma}$	$0.468\pm0.015^{a,\alpha}$	$15.2\pm0.1^{c,\alpha}$	$1.02\pm0.04^{a,\beta}$
Sig[1]		***	***	***	ns	***	***
BC60	FB40	$206\pm3^{a,\alpha}$	$0.61\pm0.05^{a,\alpha}$	$13.4\pm0.3^{b,\gamma}$	$0.465\pm0.019^{a,\alpha}$	$13.8\pm0.1^{a,\gamma}$	$1.12\pm0.05^{a,\alpha\beta}$
BC60	EC1118	$248\pm5^{b,\beta}$	$0.83\pm0.07^{b,\alpha}$	$11.1\pm0.4^{a,\alpha\beta}$	$0.462\pm0.037^{a,\alpha}$	$14.5\pm0.2^{b,\beta}$	$1.55\pm0.07^{b,\gamma}$
BC60	ELCF3WC	$197\pm4^{a,\beta}$	$0.65\pm0.06^{a,\alpha}$	$13.6\pm0.4^{b,\beta}$	$0.454\pm0.023^{a,\alpha}$	$15.1\pm0.2^{c,\alpha}$	$1.22\pm0.06^{a,\gamma}$
Sig[1]		***	*	***	ns	***	***
L37	FB40	$222\pm2^{b,\beta}$	$0.70\pm0.03^{a,\alpha}$	$12.3.\pm0.1^{b,\beta}$	$0.460\pm0.011^{a,\alpha}$	$13.5\pm0.1^{a,\beta}$	$1.04\pm0.03^{a,\alpha}$
L37	EC1118	$249\pm3^{c,\beta}$	$0.91\pm0.05^{b,\alpha}$	$10.8\pm0.3^{a,\alpha\beta}$	$0.463\pm0.025^{a,\alpha}$	$14.5\pm0.1^{b,\beta}$	$1.62\pm0.05^{b,\gamma}$
L37	ELCF3WC	$189\pm1^{a,\alpha}$	$0.63\pm0.02^{a,\alpha}$	$14.7\pm0.1^{c,\gamma}$	$0.474\pm0.007^{a,\alpha}$	$15.3\pm0.2^{c,}$	$1.02\pm0.02^{a,\beta}$
Sig[1]		***	***	***	ns	***	***
PEDRO10	FB40	$225\pm3^{b,\beta}$	$0.68\pm0.04^{a,\alpha}$	$12.0\pm0.2^{b,\beta}$	$0.453\pm0.017^{a,\alpha}$	$13.4\pm0.1^{a,\beta}$	$1.13\pm0.04^{a,\alpha\beta}$
PEDRO10	EC1118	$238\pm3^{c,\alpha}$	$0.82\pm0.04^{b,\alpha}$	$11.4\pm0.2^{a,\beta}$	$0.459\pm0.020^{a,\alpha}$	$14.6\pm0.1^{b,\beta}$	$1.22\pm0.04^{ab,\beta}$
PEDRO10	ELCF3WC	$209\pm2^{a,\gamma}$	$0.65\pm0.05^{a,\alpha}$	$13.8\pm0.1^{c,\beta}$	$0.488\pm0.011^{a,\alpha}$	$15.7\pm0.1^{c,\beta}$	$1.27\pm0.05^{b,\gamma}$
Sig[1]		***	**	***	ns	***	*
Sig[2]		***,**,***	ns,ns,ns	***,*,***	ns,ns,ns	***,**,***	***,***,***

All data are expressed as the average value ± standard deviation (n=2). Different Latin letters within the same column indicate significant differences (Sig[1]) among the different *S. cerevisiae* strains inoculated with the same strain of *C. zemplinina* (Tukey-b test; $p<0.05$). Different Greek letters within the same column indicate significant differences (Sig[2]) for the different *C. zemplinina* strains inoculated with the same strain of *S. cerevisiae* (Tukey-b test; $p<0.05$). *,**,*** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and no significant differences, respectively.

Table 4. Chemical composition of the wines obtained from sequential inoculated fermentations of the *S. cerevisiae* and *C. zemplinina* strains. The ethanol content of the initial must was 1.8% vol.

C. zemplinina	*S. cerevisiae*	Reducing Sugar (g/L)	Glucose/Fructose (-)	Ethanol (% vol.)	Ethanol / Consumed sugars yield (g/g)	Glycerol (g/L)	Acetic acid (g/L)
EJ1	FB40	$242\pm2^{b,\alpha}$	$2.06\pm0.03^{b,\gamma}$	$11.3\pm0.2^{b,\beta\gamma}$	$0.465\pm0.016^{a,\beta}$	$14.6\pm0.1^{c,\delta}$	$1.03\pm0.03^{b,\gamma}$
EJ1	EC1118	$288\pm3^{c,\gamma}$	$2.04\pm0.04^{b,\beta}$	$8.9\pm0.2^{a,\beta\gamma}$	$0.466\pm0.030^{a,\beta}$	$12.1\pm0.1^{a,\beta}$	$0.70\pm0.04^{a,\beta}$
EJ1	ELCF3WC	$228\pm3^{a,\beta\gamma}$	$1.56\pm0.04^{a,\alpha}$	$11.4\pm0.3^{b,\beta\gamma}$	$0.432\pm0.019^{a,\alpha}$	$13.5\pm0.1^{b,\beta}$	$0.75\pm0.04^{a,\gamma}$
Sig[1]		***	***	***	ns	***	***
T1Y3	FB40	$245\pm5^{b,\alpha}$	$1.54\pm0.07^{a,\alpha}$	$10.9\pm0.4^{b,\beta}$	$0.453\pm0.036^{b,\beta}$	$12.9\pm0.2^{b,\beta}$	$0.58\pm0.07^{a,\alpha}$
T1Y3	EC1118	$262\pm2^{c,\alpha}$	$2.13\pm0.03^{b,\beta}$	$8.4\pm0.1^{a,\alpha\beta}$	$0.373\pm0.013^{a,\alpha}$	$11.4\pm0.1^{a,\alpha}$	$0.50\pm0.03^{a,\alpha}$
T1Y3	ELCF3WC	$236\pm3^{a,\gamma}$	$1.56\pm0.04^{a,\alpha}$	$11.4\pm0.2^{b,\beta}$	$0.454\pm0.019^{b,\alpha}$	$13.9\pm0.1^{c,\beta\gamma}$	$0.56\pm0.04^{a,\beta}$
Sig[1]		***	***	***	**	***	ns
BC60	FB40	$247\pm4^{a,\alpha}$	$1.64\pm0.05^{a,\alpha}$	$9.1\pm0.3^{b,\alpha}$	$0.370\pm0.023^{a,\alpha}$	$12.1\pm0.1^{b,\alpha}$	$0.74\pm0.05^{b,\beta}$
BC60	EC1118	$271\pm5^{b,\alpha\beta}$	$1.85\pm0.07^{b,\alpha}$	$7.7\pm0.4^{a,\alpha}$	$0.355\pm0.035^{a,\alpha}$	$11.2\pm0.2^{a,\alpha}$	$0.61\pm0.07^{a,\alpha\beta}$
BC60	ELCF3WC	$250\pm4^{a,\delta}$	$1.51\pm0.05^{a,\alpha}$	$10.4\pm0.3^{c,\alpha}$	$0.444\pm0.026^{b,\alpha}$	$14.0\pm0.1^{c,\gamma}$	$1.01\pm0.05^{c,\delta}$
Sig[1]		***	***	***	**	***	*
L37	FB40	$247\pm3^{b,\alpha}$	$1.64\pm0.04^{a,\alpha}$	$9.6\pm0.2^{b,\alpha}$	$0.391\pm0.018^{a,\alpha}$	$12.2\pm0.1^{b,\alpha}$	$0.59\pm0.04^{ab,\alpha}$
L37	EC1118	$264\pm4^{c,\alpha}$	$2.04\pm0.06^{b,\beta}$	$8.5\pm0.3^{a,\alpha\beta}$	$0.378\pm0.029^{a,\alpha}$	$11.5\pm0.2^{a,\alpha}$	$0.50\pm0.06^{a,\alpha}$
L37	ELCF3WC	$214\pm5^{a,\alpha}$	$1.52\pm0.07^{a,\alpha}$	$11.9\pm0.4^{c,\beta}$	$0.422\pm0.029^{a,\alpha}$	$13.7\pm0.2^{c,\beta\gamma}$	$0.67\pm0.07^{b,\beta\gamma}$
Sig[1]		***	***	***	ns	***	***
PEDRO10	FB40	$242\pm3^{b,\alpha}$	$1.79\pm0.05^{a,\beta}$	$11.9\pm0.3^{b,\gamma}$	$0.475\pm0.023^{b,\beta}$	$14.1\pm0.1^{b,\gamma}$	$0.56\pm0.05^{b,\alpha}$
PEDRO10	EC1118	$278\pm4^{c,\beta}$	$2.09\pm0.06^{b,\beta}$	$9.4\pm0.4^{a,\gamma}$	$0.462\pm0.020^{b,\beta}$	$12.3\pm0.2^{a,\beta}$	$0.51\pm0.06^{b,\alpha}$
PEDRO10	ELCF3WC	$226\pm5^{a,\beta}$	$1.88\pm0.05^{a,\beta}$	$11.4\pm0.4^{b,\beta}$	$0.417\pm0.031^{a,\alpha}$	$12.0\pm0.2^{a,\alpha}$	$0.37\pm0.05^{a,\alpha}$
Sig[1]		***	**	***	*	***	*
Sig[2]		ns,***,***	***,**,***	***,**,**	***,**,ns	***,***,***	***,**,***

All data are expressed as the average value ± standard deviation (n=2). Different Latin letters within the same column indicate significant differences (Sig[1]) among the different *S. cerevisiae* strains inoculated with the same strain of *C. zemplinina* (Tukey-b test; $p<0.05$). Different Greek letters within the same column indicate significant differences (Sig[2]) for the different *C. zemplinina* strains inoculated with the same strain of *S. cerevisiae* (Tukey-b test; $p<0.05$). *,**,*** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and no significant differences, respectively

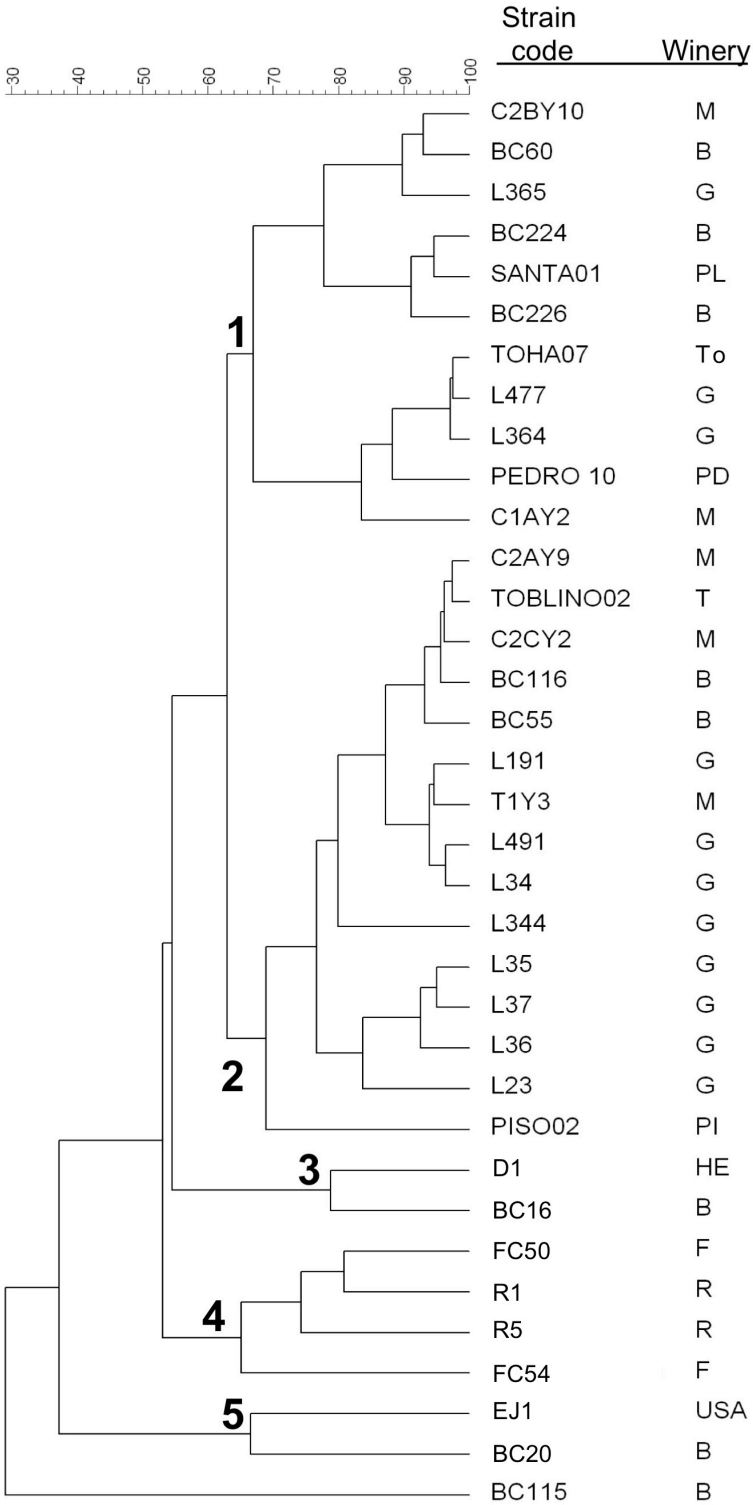
473 **Table 5**. Statistical differences of the chemical composition of the wines obtained from the
474 co-inoculated (data from Table 3) and sequential inoculated(data from Table 4)
475 fermentations of the *S. cerevisiae* and *C. zemplinina* strains.
476

C. zemplinina	***S. cerevisiae***	**Reducing Sugar (g/L)**	**Glucose/Fructose (-)**	**Ethanol (% vol.)**	**Ethanol / Consumed sugars yield (g/g)**	**Glycerol (g/L)**	**Acetic acid (g/L)**
EJ1	FB40	ns	***	ns	ns	***	**
EJ1	EC1118	**	***	**	ns	***	***
EJ1	ELCF3WC	ns	***	**	ns	**	*
T1Y3	FB40	*	***	**	ns	**	***
T1Y3	EC1118	***	***	***	**	***	***
T1Y3	ELCF3WC	***	***	***	ns	***	***
BC60	FB40	***	***	***	**	***	***
BC60	EC1118	**	***	***	*	***	***
BC60	ELCF3WC	***	***	***	ns	**	*
L37	FB40	***	***	***	**	***	***
L37	EC1118	**	***	**	*	***	***
L37	ELCF3WC	**	***	***	**	***	**
PEDRO10	FB40	**	***	ns	ns	**	***
PEDRO10	EC1118	***	***	**	ns	***	***
PEDRO10	ELCF3WC	**	***	**	*	***	***

477
478 *,**,***and ns indicate significance at $p < 0.05$, 0.01, 0.001 and no significant differences, respectively
479

480 **Figure legends**
481
482 **Figure 1.** Dendrogram of similarity constructed taking into consideration the RAPD and
483 SAU-PCR profiles of the *C. zemplinina* strains used in this study. Clusters are indicated with
484 Latin numerals.
485
486 **Figure 2.** Growth dynamics of *C. zemplinina* during fermentation of the must obtained from
487 dried grapes. The results of the 35 strains are reported as minimum, maximum and median
488 values.
489
490 **Figure 3.** Growth dynamics of the *S. cerevisiae* strains (Panel A, FB40; Panel B, ELCF3WC;
491 Panel C, EC1118) co-inoculated (solid symbols) or sequentially inoculated (empty symbols)
492 with *C. zemplinina*, as determined on the WLN medium. The mean (cfu/ml) ± standard
493 deviations of 5 fermentations (each with a different *C. zemplinina* strain) are shown for
494 each *S. cerevisiae*, while all the data were combined and are presented as the mean (cfu/ml)
495 ± standard deviations for the 5 selected strains of *C. zemplinina*. Abbreviations: Cz_C, *C.*
496 *zemplinina* in co-inoculation; Sc_C, *S. cerevisiae* in co-inoculation; Cz_S, *C. zemplinina* in
497 sequential inoculations; Sc_S, *S. cerevisiae* in sequential inoculations.
498

Figure 1


Strain code
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L365 G
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SANTA01 PL
BC226 B
TOHA07 To
L477 G
L364 G
PEDRO 10 PD
C1AY2 M
C2AY9 M
TOBLINO02 T
C2CY2 M
BC116 B
BC55 B
L191 G
T1Y3 M
L491 G
L34 G
L344 G
L35 G
L37 G
L36 G
L23 G
PISO02 PI
D1 HE
BC16 B
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BC20 B
BC115 B
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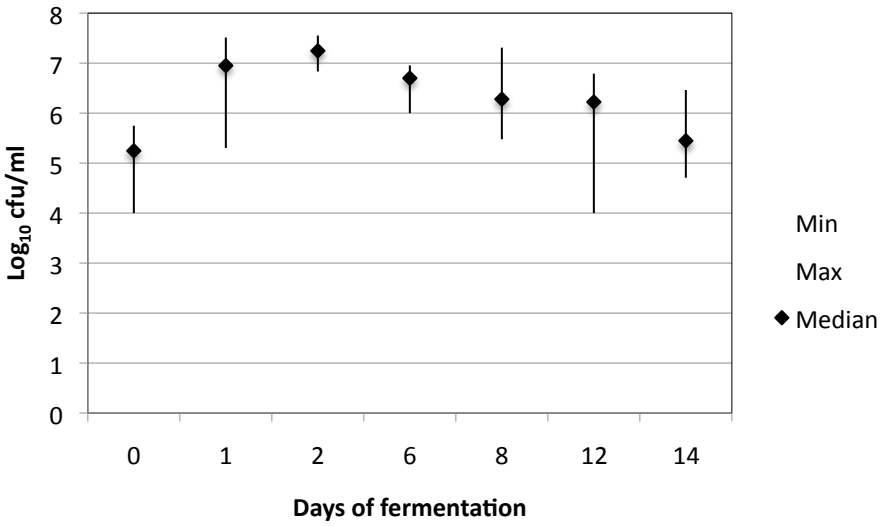
Figure 2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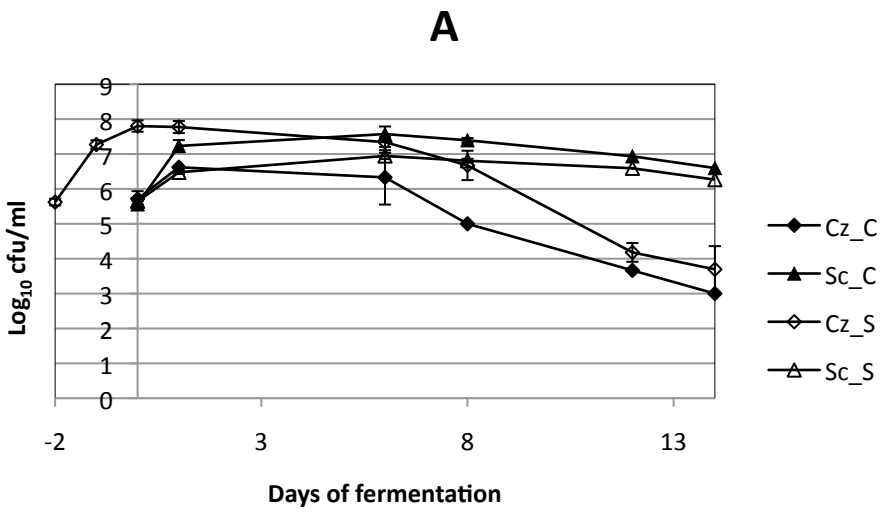
506 **Figure 3**
507



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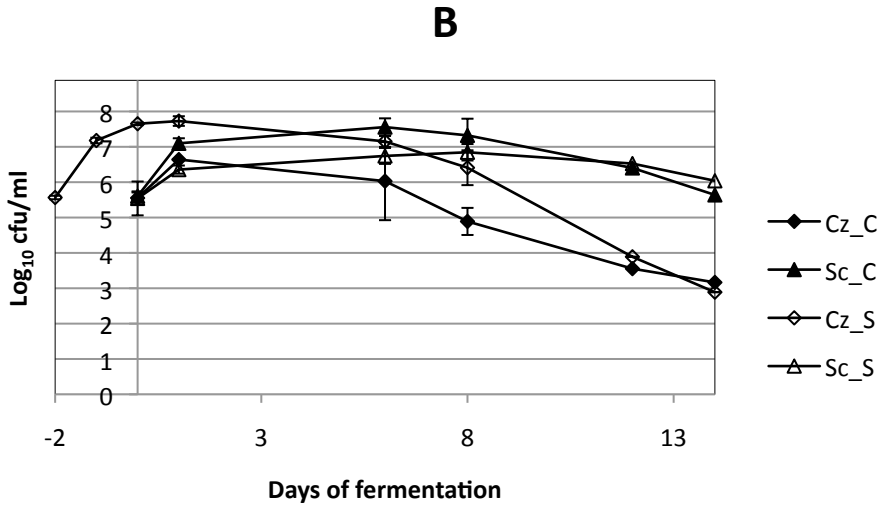

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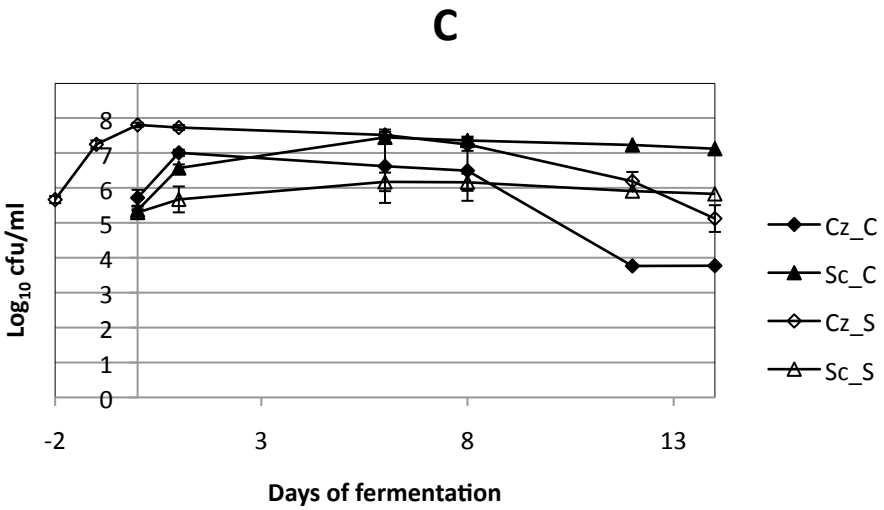

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