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The consequences of *Lactobacillus vini* and *Dekkera bruxellensis* as contaminants of the sugarcane-based ethanol fermentation

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Abstract This work describes the effects of the presence of the yeast *Dekkera bruxellensis* and the bacterium *Lactobacillus vini* on the industrial production of ethanol from sugarcane fermentation. Both contaminants were quantified in industrial samples, and their presence was correlated to a decrease in ethanol concentration and accumulation of sugar. Then, laboratory mixed-cell fermentations were carried out to evaluate the effects of these presumed contaminants on the viability of *Saccharomyces cerevisiae* and the overall ethanol yield. The results showed that high residual sugar seemed the most significant factor arising from the presence of *D. bruxellensis* in the industrial process when compared to pure *S. cerevisiae* cultures. Moreover, when *L. vini* was added to *S. cerevisiae* cultures it did not appear to affect the yeast cells by any kind of antagonistic effect under stable fermentations. In addition, when *L. vini* was added to *D. bruxellensis* cultures, it showed signs of being able to stimulate the fermentative activity of the yeast cells in a way that led to an increase in the ethanol yield.

Keywords Lactic acid bacteria · Microbial interaction · Contaminant yeast

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Introduction

The industrial fermentation process for ethanol production in Brazil is characterized by the reuse of yeast biomass during the harvesting season, which favors constant replacement of the yeast starter and the settlement of yeast and bacterial contaminants in a non-sterile substrate [1, 2, 8, 14]. It is generally believed that the installation of a particular bacterial species, or even a particular bacterial strain, is responsible for the instability of the yeast population and for problems in yield and productivity, because it reduces yeast growth and viability, and affects yeast fermentation capacity [6, 9, 13, 15, 16]. These findings are in marked contrast with those of the established *Dekkera bruxellensis/Lactobacillus vini* fermentation consortium [10]. We have shown that both yeasts and the bacterium are present in sugarcane-based ethanol fermentations in Brazil [7, 8]. *Lactobacillus vini* is a homofermentative lactic acid bacterium that is found in fermented grape must and can ferment glucose, fructose and cellobiose as well as produce lactate from pentoses [12]. So far, its role and/or effect on ethanol fermentations have not been determined.

In this study, we analyzed a distillery that made extensive use of *D. bruxellensis* in its production processes and re-evaluated distilleries that are constantly contaminated by *D. bruxellensis* for the presence of *L. vini*. Moreover, laboratory mixed-cell recycling fermentations were conducted to evaluate the effect of both yeast and bacterial

contaminants on the fermentation capacity and viability of *S. cerevisiae* cells. The results are presented in terms of the significant effect of the presence of *D. bruxellensis* and *L. vini* on both the industrial process and the fermentative activity of *S. cerevisiae*.

Materials and methods

Industrial sampling and microbial identification

Industrial samples were collected and used for the isolation, molecular identification by DNA sequencing and quantification of the yeast [5, 7, 11] and Lactobacilli [8]. Industrial data were provided by the distilleries and represent samples taken from continuous fermentation processes with cell recycle. The differential growth rates of the *D. bruxellensis* population were calculated as previously reported [7]:

Selected sets of the industrial population dynamics data depicted in Fig. 1 were used to estimate the growth rate gap between the two subpopulations. The two cell types were considered to be growing at constant but different growth rates in a continuous fermentation system with cell recycle by centrifugation. In this system, the dynamics of each subpopulation can be written as follows:

$$\frac{\mathrm{d}(VX_i)}{\mathrm{dt}} = \mu_i(VX_i) - QX_{Vi} \quad (1)$$

where μ_i is the specific growth rate of the subpopulation; i, X_i and X_{Vi}, respectively, are the cell counts in the fermentation system and in the centrifugation efflux stream; V is the system volume; and Q is the volumetric flow rate leaving the system from the centrifuge. For each set of dynamic data, V and Q can be considered to be constant during the observation period. Considering, in addition, that for each subpopulation the cell count in the efflux stream is proportional to the cell count in the system (i.e. the centrifuge operates with a constant concentration factor $\alpha i = X_{Vi}/X_i$, Eq. 1 can be rewritten as follows:

$$\frac{\mathrm{d}X_i}{\mathrm{dt}} = (\mu_i - \alpha_i q)X_i \quad (2)$$

where $q = Q/V$. For each subpopulation, the integration of Eq. 2 over time gives

$$X_i = X_{i0}e^{(\mu_i - \alpha_i q)t} \quad (3)$$

The fraction of contaminant cells in the population (F_C) is then given by the following equation, where the subscripts S and C refer to the subpopulations of *S. cerevisiae* and the contaminant species:

$$F_C = \frac{X_C}{X_S + X_C} = \frac{X_{C0}e^{(\mu_C - \alpha_C q)t}}{X_{S0}e^{(\mu_S - \alpha_S q)t} + X_{C0}e^{(\mu_C - \alpha_C q)t}} \quad (4)$$

Assuming that the concentration factors αS and αC of both cell types are equal (i.e., no preferential separation during centrifugation), after some rearrangement Eq. 4 can be simplified to

$$\ln\left(\frac{1}{F_C} - 1\right) = \ln\frac{X_{S0}}{X_{C0}} + (\mu_S - \mu_C)t \quad (5)$$

The difference between the growth rates of the two subpopulations can then be obtained from the slope of a straight line by plotting $\ln(1/F_C - 1)$ against time.

Strains and growth conditions

The industrial yeast strains *D. bruxellensis* GDB 248 [5, 11] and *S. cerevisiae* JP1 [11, 14] were maintained in YPD medium (10 g/l yeast extract, 20 g/l glucose, 20 g/l bacteriological peptone, 20 g/l agar) at 30 °C. The industrial bacterial strain *L. vini* TR7.5.7 [8] was maintained at 37 °C in MRS medium (10 g/l peptone, 8 g/l meat extract, 4 g/l yeast extract, 20 g/l glucose, 5 g/l sodium acetate trihydrate, 1 g/l Tween 80, 2 g/l K_2HPO_4, 0.2 % triammonium citrate, 0.2 g/l $MgSO_4{\cdot}7H_2O$, 0.05 g/l $MnSO_4{\cdot}4H_2O$, 10 g/l agar, pH adjusted to 6.2 at 25 °C). The Wallerstein Laboratories Nutrient Agar medium (WLN; 50 g/l glucose, 40 g/l yeast extract, 50 g/l casein, 0.55 g/l KH_2PO_4, 0.43 g/l KCl, 0.13 g/l $CaCl_2{\cdot}6H_2O$, 0.13 g/l $MgSO_4{\cdot}7H_2O$, 0.0025 g/l $FeCl_3{\cdot}6H_2O$, 0.0025 g/l $MnSO_4{\cdot}4H_2O$, 0.022 g/l Bromocresol green, 20 g/l Agar, pH adjusted to 5.5 at 25 °C) was used for yeast isolation and differentiation. Sugar cane juice as feeding substrate was supplied by the distillery no. 1 (see Fig. 2a), and prepared and diluted to 120 g sucrose/l as reported [5, 11]. All media components were from Himedia Laboratories (Mumbai, India). Actidione (cycloheximide) was used for yeast selection, since *D. bruxellensis* is resistant at 1 g/l in the medium [7].

Recycling fermentative assays

Recycled batch fermentations were carried out as described earlier [11], with minor modifications. Cell pre-inoculum was prepared in YPD (for yeasts) or MRS (for *L. vini*) to generate enough biomass for the fermentations. The cells were recovered by centrifugation and suspended in diluted sugarcane juice, and the cell density was determined by microscopic count in a Neubauer chamber. The cell viability was evaluated after dyeing with methylene blue before the microscopy analysis. Fermentations in 50-ml conical tubes were started by mixing the cells of yeast and bacteria to ca. 10^8 cell/ml each, and diluted sugarcane juice was added to 50 ml final volume. The following combinations were prepared: mix 1 (*S. cerevisiae/D. bruxellensis*), mix 2 (*S. cerevisiae/L. vini*), mix 3 (*D. bruxellensis/*

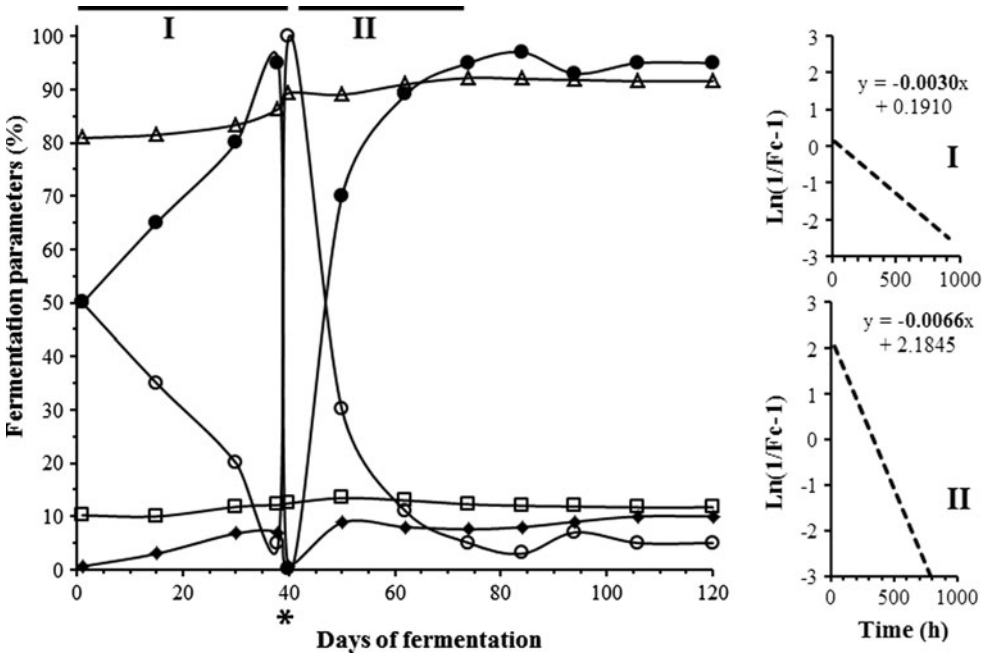

Fig. 1 Industrial data for continuous fermentation in the harvest season 2007–2008 in a distillery that operates with a sugar concentration at 120 g/l. The percentage of *Dekkera bruxellensis* (*closed circles*) and *Saccharomyces cerevisiae* (*open circles*) cells in the yeast population, as well as the percentage of biomass concentration (*open squares*), residual sugar (*closed diamonds*) and the fermentation efficiency (*open triangles*) were plotted. The *asterisk* indicates the complete change of the biomass on the 39th day of the operation. Inserts I and II refers to the calculation of the differential growth rate of the *D. bruxellensis* population during two periods of the fermentation (see Ref. [7] for calculations)

L. vini) and mix 4 (*S. cerevisiae/D. bruxellensis/L. vini*). The cultures were incubated for 12 h at 33 °C without agitation. At the end of each batch, the cells were recovered by centrifugation (1,200 g for 5 min) and suspended in the same medium to 50 ml final volume for a new batch of fermentation. This procedure was repeated for five consecutive cycles. All the mixed recycle fermentations were conducted in biological triplicates, with technical replicates for each sample, and the results obtained were averaged (±SD).

At the end of each batch, samples were taken for cell concentration, viability and counting onto WLN + 1 g/l ampicillin plates (for total yeast count), WLN + 1 g/l ampicillin + 1 g/l actidione (for *D. bruxellensis* count) and MRS + 5 g/l actidione (for bacterial count). The remaining volume was centrifuged, and the fermented worts were used for metabolite analysis by HPLC (Waters Co., USA) using an Aminex HPX-87H column (BioRad, USA).

Results and discussion

Effect of *D. bruxellensis* on the industrial fermentation efficiency

We carried out an analysis of one sugarcane juice-based distillery in the season of 2007–2008, in which the presence of *D. bruxellensis* was reported by the technical staff as extremely high (Fig. 1). Cell count results demonstrated that this yeast achieved levels similar to those reported by Passoth et al. [10], which had not been seen before in our local distilleries. At the beginning of the study, the population of *S. cerevisiae* and *D. bruxellensis* was already in a 1:1 ratio, and the fermentation efficiency was 81 % (ethanol yield of 0.41 g/g for 120 g sucrose/l substrate). Very surprisingly, the fermentation efficiency slightly increased as the *D. bruxellensis* count increased (Fig. 1), despite the fall in the ethanol concentration in the wort to 50 g/l. The explanation for this lies in the increase in residual sugar (Fig. 1), which exactly reflects the complaint made by the industry operators. The bacterial population was in the range of 10^7–10^8 CFUs/ml. Unfortunately, as we did not get direct access to the samples, it was not possible to identify the bacterial species at that time. The *Dekkera bruxellensis* population grew at 0.003 h^{-1} (Fig. 2, insert I) in the first 38 days of fermentation, four times lower than previously calculated [5, 7]. The biomass was then replaced by a pure batch of *S. cerevisiae* at the time when *D. bruxellensis* reached 95 % of the population; this increased the ethanol concentration to 54 g/l and used up all the sugar (Fig. 2) (ethanol yield of 0.46 g/g). Afterwards, a second event involving a faster rate of *D. bruxellensis* overgrowth at 0.006 h^{-1} was observed over a period of 35 days (Fig. 1, insert II). This two-fold increase in cell growth indicated that some sort of adaptation had occurred, followed by a high count stabilization that brought ethanol production back to 51 g/l and increased the level of residual sugar, although high fermentation efficiency was maintained (Fig. 1) (ethanol yield of 0.45 ± 0.02 g/g). This overgrowing phenomenon was reported when the *D. bruxellensis* population took control of the fermentation process in Sweden [10]. Yeast biomass used in the process remained around 120 (± 0.91) g/l. As a result, despite the maintenance of a high ethanol yield, we

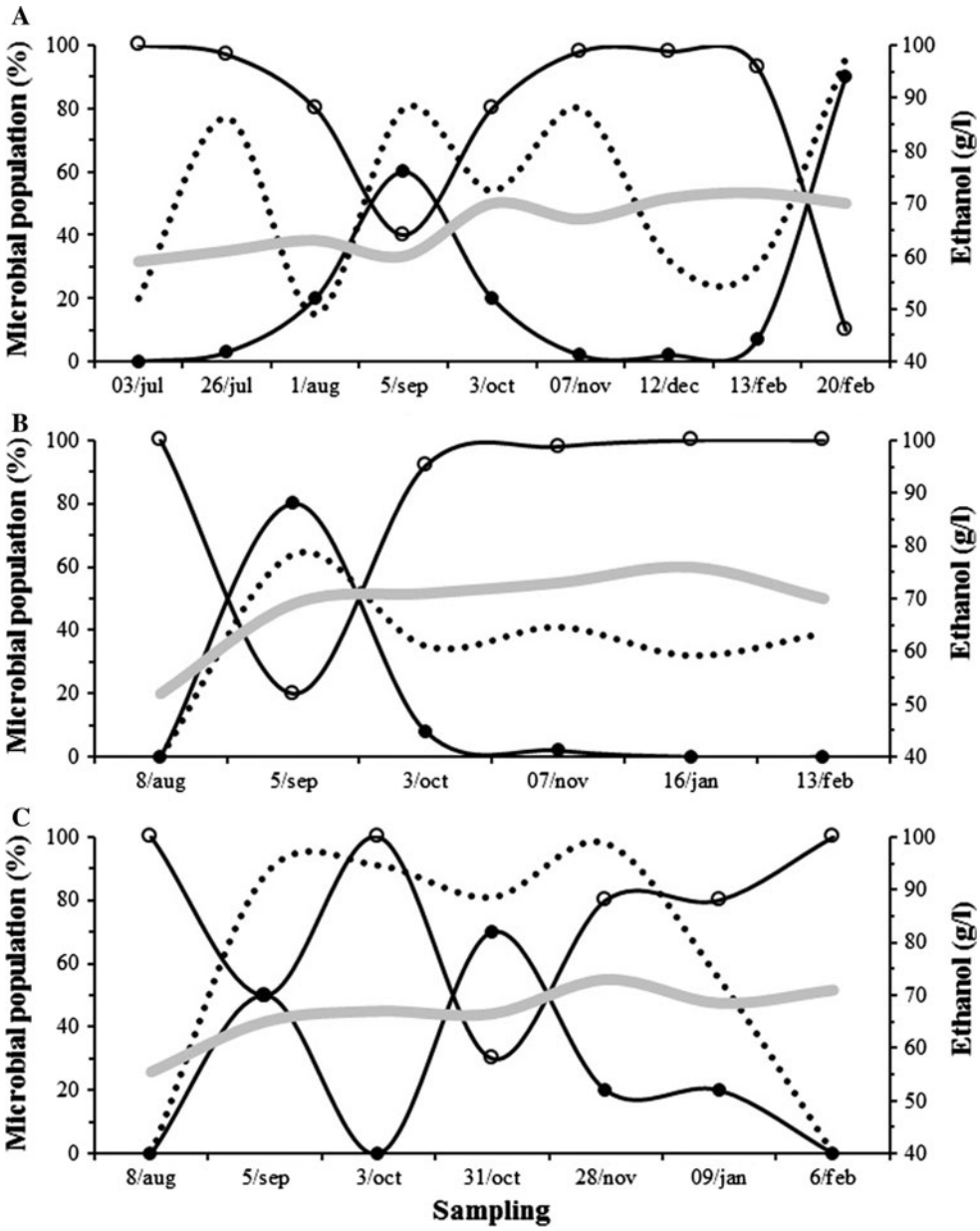

Fig. 2 Dynamics of the yeast population was plotted as the percentage of *Saccharomyces cerevisiae* (*open circle*), *Dekkera bruxellensis* (*closed circle*) and *Lactobacillus vini* (*dotted line*) in three distilleries (**a**, **b** and **c**) that operated with a sugar concentration of 140 g/l during the harvest season 2008–2009. The ethanol concentration of the industrial wort from the collected samples was plotted (*gray line*)

concluded that the loss of residual sugar and decrease in ethanol productivity in the bioethanol industry are the most significant effects of the presence of *D. bruxellensis* in industrial processes.

The dynamics of the *L. vini* population in industrial fermentations

Three distilleries were studied in the harvest season 2008–2009 when the bacterial population in the distilleries under study varied between 10^7 and 3×10^8 CFUs/ml, in the range of what had been previously observed [4], while the yeast population remained stable at around 10^8 CFUs/ml. This represented bacteria/yeast ratio in the range of 1:1–1:100. Lactobacilli predominated in the fermentation processes, as has been observed previously [4, 6, 13]. Overall, more than 50 % belonged to *L. fermentum* and *L. vini* species, as we recently reported [8]. These bacteria were apparently resistant to ethanol since the average alcohol concentration in the fermented wort was 65 g/l (Fig. 2) for substrates containing 140 g sugar/l. The *L. vini* population was predominant in the fermentation processes, but was not stable and varied between the different distilleries (Fig. 2). Despite some points of coincidence, there was no absolute correspondence between the fluctuations of *D. bruxellensis* and *L. vini* populations in all the distilleries that were studied (Fig. 2). It should be noted that the largest amount of *L. vini* was observed in distillery no. 3

(Fig. 2c), where sugarcane juice was processed by thermal treatment in the presence of flocculation agents to remove suspended solids and to reduce the spread of microorganisms coming from the fields. By eliminating the incoming flow, this operation may encourage the settlement of *L. vini* in the process. Recent results have shown that *L. vini* is not present at relevant counts in the feeding substrates (ongoing research study in preparation). The presence of *L. vini* in the industrial process in whatever amount has, by itself, had no significant effect on ethanol production (Fig. 2). Slight decreases in ethanol concentration were only observed when the *D. bruxellensis* population surpassed that of *S. cerevisiae* (Fig. 2). Thus, the presence of *L. vini* in high counts was not related to falls in ethanol production or a loss of fermentation efficiency in sugarcane fermentations.

Recycled mixed-cell laboratory batch fermentations

In vitro mixed fermentation experiments were conducted to mimic the conditions that prevail in the industry (continuous cell recycling, high cell density and high initial sugar content) without taking account of the effects of other industrial parameters. Recent work has shown that the viability of *S. cerevisiae* cells was affected by some, but not all, the strains of *D. bruxellensis* that had been isolated from the ethanol fermentation processes in Brazil [11]. For this study, we chose the GDB 248 strain that hardly has any influence on the viability of *S. cerevisiae,* solely to test the effects of *L. vini* on this parameter. The presence of *D. bruxellensis* and *L. vini*, either separately (mixes 1 and 2) or in combination (mix 4), did not affect the viability of *S. cerevisiae*, and the number of *D. bruxellensis* cells tended to increase during the recycles (mix 3) (Fig. 3A), as previously reported [11]. The number and viability of the *L. vini* cells remained stable at 10^7 cell/ml during the recycled batches (mixes 2, 3 and 4) (data not shown). Bayrock and Ingledew [3] used the term "self-regulation of the system" to describe this phenomenon of keeping the bacterial population under control without biocidal treatment. Thus, the presence of *L. vini* in 1:1 proportions did not affect the yeast viability. Similar results were reported in *S. cerevisiae/L. paracasei* where there was a 1:1 mixed population in continuous culture fermentations [3].

The final concentration of ethanol in the fermented wort reached 48 g/l, and the sugar was totally consumed when in the presence of *S. cerevisiae* in any combination (mixes 1, 2 and 4) (Fig. 3B), which resulted in ethanol yield in the range of 0.47 (±0.02) g/g observed for pure cultures of JP1 cells [11]. We recently demonstrated that in vitro ethanol yield fell with the increase of *D. bruxellensis* cells count in *S. cerevisiae* fermentation [11]. In the present case, the population of *D. bruxellensis* remained constant over the

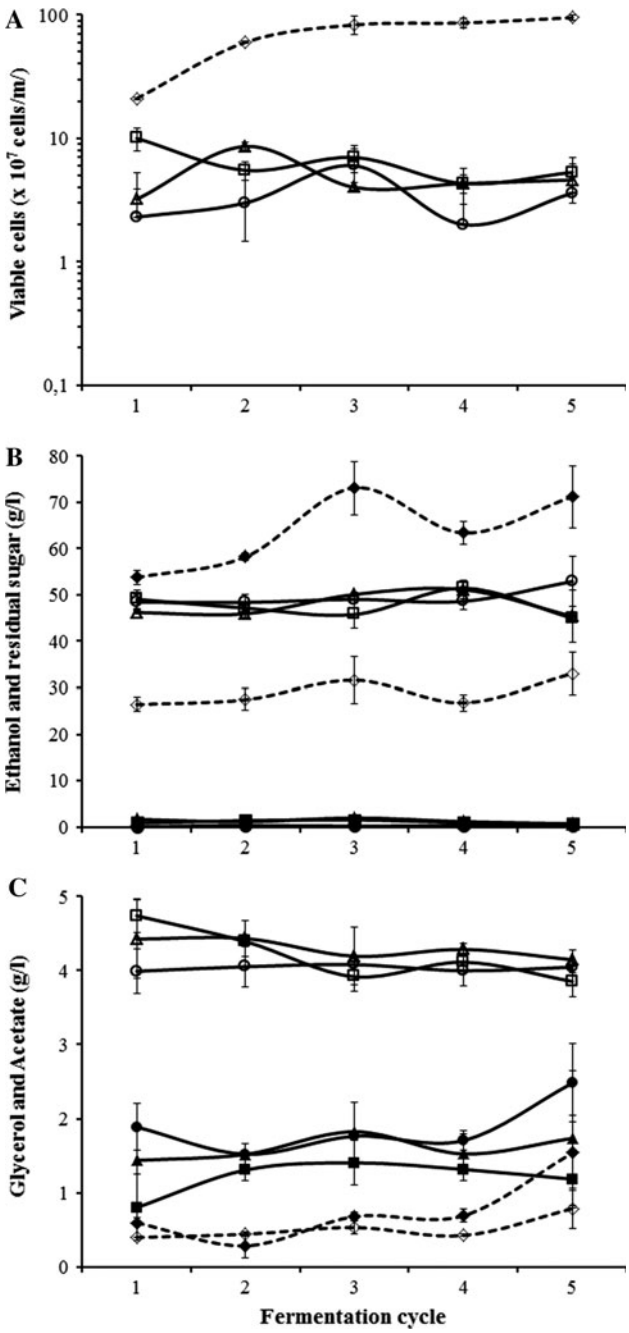

Fig. 3 Mixed cell fermentations (with recycling) using sugarcane juice with 120 g sugar/l as substrate. Mix 1 contained *S. cerevisiae* and *D. bruxellensis* (*circle*), mix 2 contained *S. cerevisiae* and *L. vini* (*square*), mix 3 contained *D. bruxellensis* and *L. vini* (*dotted line*), and mix 4 contained all three microorganisms (*triangle*). **a** Cell viability. **b** Ethanol (*open symbols*) and residual sugar (*closed symbols*). **c** Glycerol (*open symbols*) and acetate (*closed symbols*)

recycles, which did not affected ethanol production by *S. cerevisiae* cells (Fig. 3b). The ethanol yield fell to 0.32 and 0.21 g/g when recycled fermentations were deliberately contaminated with *L. fermentum* [4] and *L. paracasei* [3], respectively. Ethanol concentration of 29 g/l was

observed in the mix 3, with a huge accumulation of sugar in the fermented wort (Fig. 3b). This represented an ethanol yield of 0.40 (±0.01) g/g detected here, much higher than the 0.22 (±0.08) g/g of pure cultures of GDB 248 cells [11].

Sucrose was detected as the sole residual sugar in mix 3, unlike the fructose accumulation in the pure *D. bruxellensis* fermentation [11]. Glycerol and acetate were mostly produced by *S. cerevisiae*, and were not affected by the presence of *L. vini* (Fig. 3c). No lactate was observed in the fermented wort (data not shown), although this had been expected from the lactate homofermentative metabolism of *L. vini* [12]. In contrast, lactate concentration reached 20 g/l in *S. cerevisiae/L. paracasei* continuous mixed fermentations, which might affect the *S. cerevisiae* physiology [3].

It can be concluded that *L. vini* did not affect *S. cerevisiae* by any kind of antagonistic effects under stable fermentative conditions. In previous studies, the presence of Lactobacilli, especially *L. fermentum* (a heterofermentative bacterium), was shown to be detrimental to the fermentation because it reduced the ethanol yield and *S. cerevisiae* viability and increased the production of glycerol and lactic acid [6, 9, 16]. In contrast, exactly the opposite effects were observed in the present study with *L. vini* (a homofermentative bacterium). This suggests that heterofermentative Lactobacilli such as *L. fermentum* are more harmful to ethanol fermentation than homofermentative species such as *L. vini* (used in the present work) and *L. plantarum* (in a study by L.C. Basso—personal communication). In addition, the presence of *L. vini* increased the fermentative activity of a *D. bruxellensis* population as seen in Fig. 3b. This stimulation, whatever it is, could explain some ethanol production in fermentation processes where *D. bruxellensis* is predominant (Fig. 1 and Ref. [11]). Further studies will address the physiological factors that are responsible for this stimulatory effect on *D. bruxellensis*.

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