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Minireview

Microbial ecology applied to fuel ethanol production from sugarcane

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Abstract

The production of fuel ethanol in sugarcane biorefineries is a nonaseptic industrial operation, which employs cell recycling and the use of adapted *Saccharomyces cerevisiae* strains. Microbial contaminants are present and, depending on the conditions, may lead to process performance deterioration. Past studies have identified the main microbial species present in this environment, using culture-dependent techniques. A few recent studies started to deploy culture-independent techniques to better understand this microbiota and its dynamics. In both cases, lactic acid bacteria have been identified as the main contaminating microorganisms. Less than a handful of reports are available on the interactions between yeast and contaminating bacteria, using synthetic microbial communities, proposing that interactions are not necessarily always detrimental. The present mini-review aims at systematizing the current knowledge on the microbiota present in the alcoholic fermentation environment in sugarcane biorefineries and setting the ground and claiming the need for a microbial ecology perspective to be applied to this system, which in turn might lead to future process improvements.

Keywords: alcoholic fermentation; fermentation ecology; fermentation microbiome; lactic acid bacteria; *Saccharomyces cerevisiae*; sugarcane biorefinery

Introduction

Industrial biotechnology normally makes use of an axenic microbial culture to convert substrates into products of commercial interest, which requires an industrial plant equipped for fully aseptic operation. Such structures represent not only higher capital expenditure, but also higher operational expenditure, when compared to nonaseptic setups, due to special construction materials (e.g. high-quality stainless steel) and the constant need for sterilization operations and maintenance of accessories, such as special valves and filters (Schmidell et al. 2001). This implies that for low-value bioproducts, a fully aseptic industrial process is not an option, and other measures are put in place to favor the presence and growth of the desired microorganism, to the detriment of competing microbial species. Such is the case of the bioethanol industry, which worldwide delivers >100 million cubic meters of a liquid biofuel that can be either blended with or substitute gasoline (Jacobus et al. 2021), contributing to decrease CO_2 emissions by up to 86%, when compared to the use of gasoline alone (Jayswal et al. 2017).

In Brazil, where ∼30% of the world's bioethanol production takes place, ∼80% of the bioethanol is produced from sugarcane-based feedstocks, mainly sugarcane juice and sugarcane molasses, in ∼350 different industrial units, which are spread mainly in the Southeast, Centerwest, and Northeast regions of the country. In each of these biorefineries, different strains of the yeast *Saccharomyces cerevisiae* convert the sugars present in these feedstocks—predominantly sucrose, but also minor amounts of glucose and fructose—into ethanol, through alcoholic fermentation, in large-scale cylindrical vats made of carbon steel (typically hundreds of cubic meters) (Della-Bianca et al. 2013, Lopes et al. 2016, Jacobus et al. 2021, Rego-Costa et al. 2023).

Although strategies are used to hinder the growth of competing microbial species, the presence of contaminating microbes cannot be completely avoided. In practice, contamination is regularly monitored by bioethanol producers and considered acceptable up to a certain level (typically ∼10^7 rods/ml), without the need to take action (except for sulfuric acid treatment, which is applied in a prophylactic manner, during the cell recycling stage). When these levels increase by one or even two orders of magnitude, for reasons that are not yet fully understood, some measures are put in place, such as increasing the use of antimicrobials and other chemicals (e.g. antifoaming agents and dispersants), which contribute to in-

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creasing production costs and to decreasing the environmental sustainability of the whole business (Lopes et al. 2016).

The industrial production season starts every year when the sugarcane harvest begins (around April in the Southeast of Brazil) by inoculating the process with selected *S. cerevisiae* strains. A handful of such strains is commercially available (Jacobus et al. 2021). After production starts, the microbial populations present in the fermentation environment start to present dynamics, which differ not only from year to year in the same industrial unit but even more from one industrial unit to others (Rego-Costa et al. 2023). This is true both for *S. cerevisiae* and for contaminating strains, which are mostly composed of lactic acid bacteria (LAB) (Costa et al. 2015, Lopes et al. 2016, Ceccato-Antonini 2018).

It is possible to consider a microbiome for this habitat, similar to the description of other microbiomes, such as the oral (Dewhirst et al. 2010), the gut (Spragge et al. 2023), and the vaginal (France et al. 2022) microbiomes in the human body, as well as some food microbiomes, as those present in sourdough (Landis et al. 2021) and kefir (Bengoa et al. 2019). However, the sugarcane biorefinery microbiome has hitherto not been studied thoroughly, at least not from a microbial ecology perspective. Some studies conducted in the past decades report the prevalent microbial taxa, using culture-dependent methodologies, but many reports remain in the form of theses in Portuguese (Gallo 1989, Rosales 1989, de Oliva-Neto 1990, Cherubin 2003). There are only a few later studies that employed culture-independent techniques, which are known to be superior, at least in terms of the capability of identifying as many species as possible, due to the so-called great plate count anomaly (Razumov 1932). Only a few very recent studies attempted to investigate the relationships between yeast and contaminating bacteria, the first of them in the context of North American corn-based bioethanol (Rich et al. 2018, Lino et al. 2021, 2024).

This mini-review aims at systematizing the knowledge on the microbiota present in the alcoholic fermentation environment in sugarcane biorefineries and claiming the need for a microbial ecology perspective to be applied to this system, to better understand the microbial interactions in this environment and how they influence industrial performance. We believe this might lead to a new industrial paradigm, which consists of starting the production season every year by inoculating the process not only with *S. cerevisiae*, but with a tailor-made microbial community, consisting of yeast and some beneficial or innocuous bacteria.

The habitat

The alcoholic fermentation environment in Brazilian sugarcane biorefineries consists of cylindrical vessels (vats) made from carbon steel with conical bottoms, the fermentors. Because cell recycling with acid-washing is performed, there are additional vessels for cell treatment, normally open to the environment. In addition, holding tanks and centrifuges that separate the yeast slurry from the fermented broth (*wine*) are also part of the habitat. Configurations vary among industrial units, but typically there are between 6 and 12 fermentors, each with a capacity of some hundreds to a thousand cubic meters (Basso et al. 2011, Della-Bianca et al. 2013, Lopes et al. 2016, Bermejo et al. 2021). Vats may be open or closed at the top (an option that allows for recovering ethanol that is dragged with CO_2) and neighboring vats may or may not be connected by pipes at the bottom. Fermentations are performed in fed-batch mode (Schmidell et al. 2001), starting with a highly concentrated yeast suspension. Fermentation medium (*must*) with ~200 $g.l^{-1}$ total reducing sugars (TRS) is fed into each vat during the first 4 to 6 h, ensuring that TRS concentrations in the fermentor are kept lower than those found in the *must*, which typically range around 200 g $TRS.l^{-1}$, which could eventually inhibit yeast performance. After the vat has reached its maximum holding capacity, it takes another ~4–6 h to finish fermentation, at which point the fermented broth (*wine*) is transferred to a continuous centrifuge that separates the supernatant (processed further via distillation, to recover ethanol) from the yeast slurry, which is subjected to a treatment operation where sulphuric acid and eventually other chemicals, such as antimicrobials, are added to act against contaminating bacteria and to avoid yeast cell aggregation. Dispersants are added during this step as a preventative measure to control foam formation. Antifoaming agents may also be added directly into fermentors, when and if foam reaches a foam sensor (Nielsen et al. 2017). More details on the process can be found in previous works (Basso et al. 2011, Amorim et al. 2011, Della-Bianca et al. 2013, Lopes et al. 2016, Bermejo et al. 2021, Jacobus et al. 2021, Rego-Costa et al. 2023).

This alcoholic fermentation environment is not carbon-limited since sugars, such as sucrose, glucose, and fructose, are abundant in the incoming *must* stream and during the whole fermentation run (Bermejo et al. 2021). However, the environment can be nitrogen-limited and, depending on how the *must* is prepared, it might also be limited by other nutrients, such as vitamins (Eliodório et al. 2023a), trace elements, and some macronutrients (de Souza et al. 2015). Since a combination of sugarcane juice, molasses, and water is typically used to prepare the *must*, nutrient composition varies considerably throughout the production season and between different industrial units. Usually, sugarcane juice has some nutritional deficiencies as compared to molasses, whereas the latter is more toxic due to the presence of high concentrations of inorganic elements (de Souza et al. 2015), as well as inhibitory compounds from sugar processing, such as furans and Maillard reaction products (Eliodório et al. 2023b, Huang et al. 2023). On the other hand, molasses has a strong buffering capacity, in contrast to juice, which may impact the acid-washing step (Basso et al. 2011, Jacobus et al. 2021).

Along the process, temperatures range between 30°C and 37°C, which per se affects the growth of different microbial species to different extents. Temperatures in the range of 30°C–35°C are optimal for the metabolic activities of the *S. cerevisiae* strains used in this process, and above 37°C can be detrimental or inhibitory (Thomas et al. 1993). However, for contaminating lactobacilli, optimal temperatures sit in the range of 37°C or even above (Pot et al. 2014, Bosma et al. 2017, Ceccato-Antonini 2018). There is recent evidence that temperature increases in the process (above 37°C) lead to the enrichment of some heterofermentative bacteria in two independent industrial bioethanol plants (Lino et al. 2024).

There are no data regarding the oxygen availability along the process, but it is certainly extremely limited in the fermentation vessels due to the substantial CO_2 evolution throughout the fermentation process, the large volumes involved, meaning low surface area-to-volume ratios, hindering mass transfer phenomena (da Costa et al. 2018, 2019). Fermentors are not aerated and generally not mechanically stirred. The lack of oxygen prevents several microbial species from thriving in this environment (da Costa et al. 2019). On the other hand, some dissolved oxygen might be available during the acid-wash step due to the open nature of the tanks (Della-Bianca et al. 2013). In addition to their fermentative metabolism, many contaminating LAB species are expected to respire if heme and menaquinone are provided exogenously (Pedersen et al. 2012).

There are some growth-limiting factors in this habitat, which prevent many species from thriving. pH values are typically below 5.0 throughout the fermentation cycle, and during the yeast slurry treatment step, the use of sulfuric acid leads to a pH decrease to values between 1.5 and 2.5 for ∼1–2 h (Basso et al. 2011). At the end of each fermentation, ethanol concentrations reach values around 10% (v/v), which is restrictive for most known microbial species, particularly when combined with temperature increases (Basso et al. 2011). Nutrient limitation can be a problem, especially when sugarcane juice makes most of the fermentation *must*, because it has very low nitrogen concentrations and may be deficient in some important minerals such as magnesium and zinc (Walker 2004). As mentioned above, sugarcane juice displays a weak buffering capacity (Basso et al. 2011, Della-Bianca et al. 2013, de Souza et al. 2015), which leads to severe acidic conditions (de Souza et al. 2015). In addition, due to the fastidious nature of lactobacilli and the lack of diverse organic nitrogen sources, such as specific amino acids and other organic micronutrients, such as vitamins, this habitat is certainly nutrient-limited to these bacteria (Pot et al. 2014, Ponomarova et al. 2017). Inhibition caused by some minerals can be an issue when molasses is mostly used to formulate the *must*, because potassium, aluminium, and other metals' concentrations can be high (Basso, 2008). Inhibitory compounds derived from the degradation of sugar and nitrogen compounds are frequent in molasses-based *musts* (Eliodório et al. 2023b).

The sugarcane biorefinery alcoholic fermentation environment has characteristics of natural, man-made and even laboratory environments (Kirchman 2018). Considering it is open, containing diverse microbial communities, and the low growth rates involved (<100 yeast doublings occur on average during an ∼8-month production season; Rego-Costa et al. 2023), it resembles natural environments. Considering it is not organic carbon-limited, it has similarities with laboratory cultures. Finally, considering that it would not occur spontaneously at the scale it is performed, it can be characterized as a man-made environment.

Microbial contamination during fuel ethanol production

It is common in this industrial process that the starter *S. cerevisiae* strain(s) is(are) replaced by different invading *S. cerevisiae* strains (da Silva-Filho et al. 2005, Basso et al. 2008, Rego-Costa et al. 2023). Strain succession and co-existence may occur, and it has been recently demonstrated that the invading *S. cerevisiae* strains most probably come from the sugarcane environment itself (plantation and/or biorefinery), meaning they are not introduced from other environments by vectors such as insects or birds (Rego-Costa et al. 2023).

We might speculate that the microbial contaminants also form an endemic community to this environment, as proposed by Skinner and Leathers (2004) for corn ethanol production in the USA. In fact, most of the knowledge we have on bacterial contamination during ethanol production processes was obtained from the North American, corn-based process, as reported in several works (Bischoff et al. 2009, Beckner et al. 2011, Rich et al. 2011, 2015, 2018, 2020, Li et al. 2016, Firmino et al. 2020; and older references cited in these works). However, there are several differences between this process and Brazilian sugarcane ethanol production (Table 1). It should also be noted that continuous yeast propagation systems are used during corn ethanol production, which might represent an important source of contaminating microbes (Bischoff et al. 2009), while sugarcane ethanol facilities only propagate yeast at the beginning of the sugarcane crushing season, due to the use of cell recycling (Lopes et al. 2016).

The fermentation yield is claimed to be the most important parameter in bioethanol production (Gombert and Van Maris 2015). It has been reported that yields around 90% of the theoretical maximum (0.511 g of ethanol per gram of hexose-equivalent) have been achieved since the beginning of the 1990s (Della-Bianca et al. 2013). However, yields are difficult to be calculated in industrial settings, and some recent works have disputed this level, suggesting that values between 85–90% may be more realistic (Pereira et al. 2018, Bermejo et al. 2021). In any situation, even small increases in the yield would represent enormous economic and environmental gains, due to the large volumes of ethanol produced every year.

Another crucial aspect to the economic and environmental sustainability of ethanol production is the use of chemical supplies, which relates directly to microbial contamination. Typical cell counts in the fermentation medium are ∼10^8 cells/ml for *S. cerevisiae* and ∼10^6 cells/ml for contaminating bacterial species, under normal processing conditions. In order to guarantee such values, the prophylactic use of antibiotics and/or other chemical supplies is applied (Lopes et al. 2016). During acute contamination episodes, also named bacterial blooms, when the rod counts may reach ∼10^8 cells/ml or even higher, the use of chemicals is intensified. The presence of contaminating bacteria is industrially monitored by methods such as rod counting by microscopy and/or HPLC or enzymatic analysis of reporter molecules, such as acetic and lactic acids.

The reasons for the increase in the levels of bacterial contaminants are not yet fully understood, and this is an important avenue for research, so that we can better understand the dynamics of the alcoholic fermentation microbiome (AFM) and thus design better strategies for process intervention or even to prevent such undesired situations. Under conditions of high contamination levels, changes in the process are observed. Among them, there is a greater aggregation of yeast cells, known as flocculation, which leads to a decrease in process productivity. There is a chronic cycle of bacteria returning to the process due to cell recycling, since flocculated yeasts are not suitable for proper yeast concentration in the centrifugation step. This significantly increases the return of the *wine* and, consequently, the bacteria with the acids they produce. In these situations, companies begin to increase the use of chemicals, which is done mainly during the acid treatment stage used in the preparation of the inoculum to be used in subsequent fermentations. These inputs include antibiotics against Gram-positive bacteria and, less frequently, for Gram-negative bacteria (monensin, tetracycline, penicillins, etc.) or antimicrobials such as chlorine dioxide, hops acids or even hydrogen peroxide, antifoams, antiflocculants, dispersants, besides sulfuric acid. The choice of antibiotics is carried out by some companies by a rapid sensitivity test of contaminants to different antibiotic options to choose the most suitable one. In this process, the microbiological population present in the *wine*, such as bacteria and yeast, is exposed to fermentation in the laboratory with different antimicrobial molecules. This makes it possible to verify which is most effective for that specific population and to identify the resistance of bacteria to certain antimicrobials. However, in spite of all these efforts, it is hardly possible to fully counteract contamination and to quickly restore the process to normal operating conditions. In some cases, the process may even be halted, causing severe financial losses. Information in this paragraph was based on information exchanged with various professionals from different companies, since it is not available in written references.

Table 1. Main differences between sugarcane and corn bioethanol production.

Characteristic	Brazil (sugarcane)	North America (corn)
Cell recycling	Yes	No
Solids in suspension	<1%	>30%
Fermentation yield	90%–92%	85%–90%
Fermentation time	6–12 h	45–60 h
Initial yeast concentration	8%–12%	3%–4%
Final ethanol titer[a]	7%–12% (v/v)	12%–18%
Days of operation per year[b]	200–240	345
Main by-products[c]	Vinasse, yeast biomass	DDGS, corn oil
Predominant yeast	Non-GMO	GMO

[a]Higher titers can be used in corn ethanol production, since no cell recycling is used; thus cell viability at the end of fermentation is not crucial.
[b]More days of operation are possible in corn ethanol production, since corn can be stored; sugarcane has to be processed immediately after harvest and during the rainy season/days harvesting is not possible.
[c]DDGS has an added-value similar to ethanol and is used for animal feed; Vinasse is used for fertirrigation and, more recently, for biomethane production by some companies; yeast biomass is currently being evaluated for new markets, so that its added value increases.
Adapted from Lopes et al. (2016)

Contaminating microbes are typically LAB, which belong to the Firmicutes phylum, as reported in several studies that employed either culture-dependent (Gallo 1989, Rosales 1989, de Oliva-Neto 1990, Lucena et al. 2010) or, more recently, culture-independent (Costa et al. 2015, Bonatelli et al. 2017) approaches. The most frequent genus encountered in this environment is the former *Lactobacillus* genus, which has recently been reclassified into several new genera, since it was verified that the former taxon did not form a monophyletic clade (Zheng et al. 2020). Another important aspect is that, as shown by Bischoff et al. (2009), different strains from the same species, in this case *Lactobacillus fermentum*, may have rather different susceptibilities to the same antibiotic. Thus, studies on the effect of antimicrobial treatments should be performed taking the strain level into account.

The current paradigm is that microbial contaminants negatively affect the process by competing with yeast for nutrients, by excreting organic acids that inhibit yeast growth and by causing yeast cell aggregation, leading to lower ethanol productivities and poor centrifugation performance (Basso et al. 2011). It has been proposed that bacterial concentrations around 10^8 cells.ml^{-1} may lead to a decrease of 10 000–30 000 l ethanol.day^{-1} in a biorefinery producing ~10^6 l ethanol.day^{-1} (Amorim et al. 2011), which means 1%–3% loss, but more studies are needed on this issue.

Microbial ecology applied to bioethanol production from sugarcane

A brief historical record of key studies aiming at understanding the microbial ecology of alcoholic fermentations used to produce bioethanol from sugarcane is presented in Table 2.

Early studies on the microbial ecology of alcoholic fermentations carried out in Brazilian sugarcane biorefineries used classical culture-dependent approaches to describe the composition of the microbiota present in this environment. These works were, in great part, not published in scientific articles and remain available only in Ph.D. theses in Portuguese. Not all of them can be directly found on the internet. Gallo (1989) collected samples from one industrial unit, during the production season of 1986, from four different process points. Among the 334 bacterial isolates obtained, the genera with at least 2% frequencies were: *Lactobacillus* (~60%), *Bacillus* (~27%), and *Staphylococcus* (~9%). The three main species were *L. fermentum, Lactobacillus helveticus,* and *Lactobacillus plantarum*. The author also observed that the sulfuric acid treatment, for 2 h at pH 2.5, caused a 44.6% decrease in the bacterial diversity.

Rosales (1989) collected nine samples from one industrial unit in the year of 1985. The number of isolates obtained was 222, which were classified into 13 genera and 21 species. The three main species of *Lactobacillus* found were: *L. fermentum, Lactobacillus brevis,* and *Lactobacillus confusus*.

De Oliva-Neto (1990) sampled the yeast cream (before sulfuric acid treatment) from two industrial units; one of them presented microbiological problems, and the other operated normally. Among a total of 44 isolates, the main bacterial species encountered were: *L. fermentum* (62%), *Lactobacillus murinus* (9%) and *L. plantarum* (2%). It was also seen that 85% of the *Lactobacilli* were obligately heterofermentative. Later, the same author co-cultured *S. cerevisiae* (a baker's strain) and *L. fermentum* CCT 1407, using high-test molasses, in a system that mimics industrial bioethanol production. It was observed that along 17 fermentation cycles, there was an increase in the bacterial population, together with increased acidity and decreased pH; yeast cell viability, sugar consumption and ethanol production dropped (de Oliva-Neto and Yokoya 1994). The same *L. fermentum* strain (CCT 1407) was used by Cherubin (2003) to show that the negative effects caused by this bacterium are less pronounced on *S. cerevisiae* PE-2, a bioethanol strain, when compared to a baker's yeast strain. The author also observed increased glycerol formation by yeast and higher viability connected to a higher trehalose content in the yeast cells (Basso et al. 2008), in the co-cultures. Later, Ludwig et al. (2001) showed that *L. fermentum* CCT 1396 causes flocculation of *S. cerevisiae* (a baker's strain) cells when the bacterial cell concentration is above a certain threshold (~1.4 g/l) and at pH values above ~3.0. In a later report, Reis et al. (2018) co-cultured *L. fermentum* CCT 0559 with *S. cerevisiae* PE-2 and a wild *S. cerevisiae* isolate displaying rough colony morphology, showing that the negative effects on the fermentation, such as unused sugar and decreased productivity, are enhanced when the wild yeast is present.

The first work that employed molecular methods to investigate the alcoholic fermentation microbiota was reported by Lucena et al. (2010), who obtained 498 LAB isolates from four industrial units in the Northeast region of Brazil, in 2007 and 2008. Their methodology was still culture-dependent, however, the identification of strains was not based on biochemical, physiological, or morphological properties, as had been done hitherto, but on the rRNA operon restriction enzyme profiles and sequencing of 16S rRNA. The authors observed that the main species found belonged to the *Lactobacillus* genus, that the diversity of species decreases along the production season, and that the predominant species towards the end of the season were *L. fermentum* and *Lactobacillus vini*.

Table 2. Reports on the microbial ecology of ethanol production from sugarcane.

Date	Type of study	References	Comments
<1990	Identification of bacterial contaminants using culture-dependent approaches	Gallo (1989), Rosales (1989), de Oliva-Neto (1990)	Most studies are Ph.D. theses in Portuguese
1990–2003	Interactions between bacteria and yeast	de Oliva-Neto and Yokoya (1994, 2001), Ludwig et al. (2001), Cherubin (2003)	Only one bacterial strain was co-cultured with one yeast strain
2010	Identification of contaminating bacteria by molecular methods	Lucena et al. (2010)	Still a culture-dependent approach
2014	Effect of homo- and heterofermentative LAB on yeast	Basso et al. (2014)	First work on the physiology of hetero- and homo-fermentative LAB
2015	Transcriptional reprogramming of yeast in the presence of acute contamination	Carvalho-Netto et al. (2015)	Employed RNA-seq
2015	First study using a culture-independent approach to evaluate microbial community composition	Costa et al. (2015)	Several process points were sampled, but not the fermentor itself
2017	First demonstration of a scaled-down system that mimics the real industrial process	Raghavendran et al. (2017)	Validated with the physiology of the *S. cerevisiae* PE-2 strain
2018	Aim was to investigate the influence of a bacterial contaminant and a wild yeast on process performance	Reis et al. (2018)	Co-culture of three strains: a bioethanol yeast, a wild yeast strain, and *L. fermentum*
>2021	First studies using synthetic microbial communities to evaluate the impact on fermentation yield	Lino et al. (2021, 2024)	First studies to look into higher-order interactions; strain-specific effects identified; beneficial effect of *Lactobacillus amylovorus* on ethanol yield observed

Basso et al. (2014) showed that heterofermentative *L. fermentum* FT-230B has a more detrimental effect on alcoholic fermentation by *S. cerevisiae* CAT-1 in a mimicked industrial process setting, when compared to the effect caused by homofermentative *L. plantarum* FT-025B. Bischoff et al. (2009) proposed that acetic acid is a major factor behind decreased ethanol production. It was shown that during alcoholic fermentations carried out using corn mash, ethanol production decreased under the presence of lactic and acetic acids, which act synergistically, also with the increase of temperature, meaning that their combined effect is stronger than the sum of each component's individual effect (Graves et al. 2007). However, as observed in several works, these mechanisms alone do not explain the detrimental effect of lactobacilli on ethanol production (Beckner et al. 2011). Carvalho-Netto et al. (2015) showed that *S. cerevisiae* co-aggregates with *L. fermentum* cells in samples taken directly from the industrial process, in which cell aggregates were visually observed. They also showed that the transcriptome of *S. cerevisiae* PE-2 is different in this situation, when compared with normal process conditions (without cell aggregates). One of their observations was that *S. cerevisiae* PE-2 downregulated the expression of seven cell wall-related genes under the flocculated condition, in response to the presence of organic acids, which might contribute to increasing its tolerance to the condition.

The first paper to describe the microbial communities in ethanol production using culture-independent methods revealed that these communities are dynamic and highly variable, according to the production stage. These changes were related to temperature alterations, and the number of microbial species (i.e. richness) was dramatically reduced after evaporation and, especially, fermentation. While the number of fungal taxa was higher during the juice and mixed juice stages, with a smaller presence of archaea, bacteria alone dominated the remaining steps (Bacilli from Firmicutes above all) (Costa et al. 2015). Bacterial taxa capable of producing biofilms and spores were predominant in high-temperature stages. Lino et al. (2021), using a metagenomics approach, also revealed the presence of nonbacterial contaminants in two biorefineries. Besides yeasts, most sequences came from Bacteria, followed by viruses and Archaea.

Sampling one continuous process (CPD) and one fed-batch distillery (FBD), Bonatelli et al. (2017) found that Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes were the most abundant bacterial phyla in ethanol-producing systems (although more taxa were detected in the fermentation tanks). Different production models selected different microbial groups (Weissella and Pediococcus were more abundant in FBD, and Acetobacter and Anaeosporobacter in CPD), and even though the genus *Lactobacillus* was most predominant in both tanks, a higher number of species of the group was observed in CPD. These taxonomic differences are reflected in the predicted functional potential of the communities, with 20 distinct gene families (from a total of 29) among treatments.

Similar to Costa et al. (2015), Queiroz et al. (2020) evaluated the microbial communities at different stages of the production process. Communities differed among sampling times, and communities at the fermentation stage were especially different. However, the last production steps had a higher richness than previously observed (Costa et al. 2015), and the overall taxonomic profile presented a higher diversity of other microbial groups in comparison with the other studies (although *Lactobacillus* still dominated) (Costa et al. 2015, Bonatelli et al. 2017).

Several factors can influence the composition and abundance of microbial communities in different ethanol production steps, such as temperature, pH, and synthesis of ethanol and organic acids (Queiroz et al. 2020). However, evaluating these alterations, as well as differences among model systems, depends on our ability to collect and share proper metadata, such as process performance indicators and general process variables, which are not easily available. In addition, to date, very few works have been performed on Brazilian refineries using metagenomics (Rego-Costa et al. 2023, Lino et al. 2024), so a broader assessment of microbial communities, without amplification bias, and the evaluation of their functional roles is still lacking.

Several studies aimed at investigating the effects of different antimicrobial treatments. De Oliva-Neto and Yokoya (2001) compared the Minimum Inhibitory Concentration of different antibiotics and other biocides on some strains of *S. cerevisiae, L. fermentum,* and *L. mesenteroides*. The results indicate that several antimicrobial options, except for the antibiotics, affect yeast as much as the bacteria and thus are not suitable for industrial use. There are well-known drawbacks of using antibiotics in this process (Braga et al. 2017), and therefore, there have been continuous efforts to identify alternatives to commonly used penicillin, streptomycin, tetracycline, monensin, and virginiamycin. Some non-conventional antimicrobial strategies include the use of ClO_2, hop acids, and even ethanol itself combined with sulfuric acid, as well as other options not yet employed industrially (Ceccato-Antonini 2018, Costa et al. 2018).

Decreasing vinasse volumes has several economic and environmental advantages, and one way of achieving this goal is by decreasing/controlling bacterial contamination in the process, since this will enable yeast to reach and cope with higher ethanol titers (Lopes et al. 2016). Although there have been reports on the presence of non-*Saccharomyces* yeasts as contaminants in sugarcane biorefineries (De Souza Liberal et al. 2007), we will here focus on bacteria as contaminants, since our recent metagenomic analyses in samples taken from several biorefineries in the Southeast region of Brazil yielded no signs of non-*Saccharomyces* yeast strains (data not shown).

Less than a decade ago, Rich et al. (2018) presented the first proposal of using beneficial bacteria to create a more stable fermentation environment in the bioethanol industry, as an alternative to the commonly used antibiotic treatment. The authors established a model "stuck" fermentation, based on the presence of the harmful *L. fermentum* 0315–1 strain, and challenged this system individually with many different other bacterial strains, several of which managed to counteract or mitigate the deleterious effect caused by *L. fermentum*. In their previous work (Bischoff et al. 2009), a dose-response relationship had been observed, i.e. the higher the presence of the harmful bacterial strain, the lower the final ethanol level achieved and the higher the amount of lactic and acetic acids, as well as of residual glucose. However, in their later study (Rich et al. 2018), it was shown that the presence of acetic or lactic acids, in mixed cultures, does not correlate directly with decreased ethanol levels. According to the authors, "this begins to highlight the complexity of mixed culture systems, and certainly could raise questions about the utility of monitoring organic acid concentrations as an indicator of problematic bacterial infections." For this reason, more studies on the interactions between yeast and contaminating bacteria, as well as among the contaminating bacteria themselves, are necessary to better understand the stability of the microbial community and the final outcome of the industrial process. The authors conclude their work by stating that routine inoculation of beneficial bacteria could be a means of obtaining a stable and high ethanol-yielding process, due to the capacity of these strains to counteract the harmful effect of other bacterial strains that invade and persist in the process. It should be noted that these studies were carried out in the context of North American corn ethanol, which presents several differences from Brazilian sugarcane ethanol (Table 1).

As shown by Bischoff et al. (2009), the use of complex laboratory YP-based media leads to completely different results, in terms of process performance, when compared to real industrial media (corn mash), both in cultures challenged and unchallenged with contaminating bacteria. Since real industrial media are highly unstable and variable, challenging research reproducibility and the interpretation of results, we recently developed and validated a synthetic sugarcane molasses medium (Eliodorio et al. 2023a). It will be interesting to combine this fully defined medium with the scaled-down system reported by Raghavendran et al. (2017) in future studies with mixed cultures of yeast and bacteria.

Recently, Lino et al. (2021) performed functional landscape analysis on a synthetic microbial community involving 80% of the diversity encountered in a sugarcane biorefinery. They observed that higher-order interactions counterbalance the effects observed in pairwise interactions. They also show evidence that a beneficial interaction between *L. amylovorus* and *S. cerevisiae* occurs mediated by crossfeeding acetaldehyde. Although interesting, the results presented by these authors are restricted to a 100 : 1 yeast-to-bacteria ratio, thus rather emulating chronic than acute contamination episodes, and microbial isolates were obtained from a single industrial unit, presumably (not clearly described in the methods section). Later, the same authors (Lino et al. 2024) investigated the AFM using samples taken directly from two industrial units in the production year of 2017. They show evidence that the presence of some *L. fermentum* strains, which is stimulated by temperature increases, is detrimental to the process, but the same was not observed for other strains of the same species, similarly to what had been shown by Rich et al. (2018), highlighting the importance of performing studies at the strain level. They also observed that the presence of *Lactobacillus amylovorus* is correlated with better process performance. Contrasting the previous literature, a single recent report claims that *L. fermentum* might improve ethanol yields, when in co-culture with a particular strain of *S. cerevisiae* used to produce cachaça (Brexó et al. 2025).

Interactions between LAB and the yeast *S. cerevisiae*

There is a predominance of LAB, especially of the (former) genus *Lactobacillus*, in the yeast-dominated, alcoholic fermentation environment—an association commonly observed in other ecological settings and industrial processes (Table 3). However, in the context of sugarcane ethanol production, several questions remain. Why are LAB predominant in this environment? What characteristics do they have that make them so adapted to fermenta-

Table 3. Processes/environments (habitat) in which yeast and LAB coexist.

Process/environment	Prevailing conditions	Major species/genera apart from *S. cerevisiae*	References
Sugarcane biorefinery ethanolic fermentation	Anoxia or low oxygen availability, combined with low pH, high ethanol titers and sugar abundance	BACTERIA *Enterococcus, Limosilactobacillus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Weissella* YEAST *Dekkera bruxellensis* (species–species interactions between *D. bruxellensis* and *S. cerevisiae* are still poorly understood)	Costa et al. (2008), Dellias et al. (2018), Bassi et al. (2018), Lino et al. (2021)
Corn biorefinery ethanolic fermentation	Anoxia or low oxygen availability, combined with low pH, high ethanol titers and sugar abundance	BACTERIA *Lactobacillus, Pediococcus* YEAST *D. bruxellensis* (species–species interactions between *D. bruxellensis* and *S. cerevisiae* are still poorly understood)	Passoth et al. (2007), Beckner et al. (2011)
Kefir	Organic acids, aldehydes, amino acids, ethanol, bioactive peptides, bacteriocins, proteins, vitamins, minerals, sugars, lipids, and exopolysaccharides	BACTERIA LAB, acetic acid bacteria FUNGI yeasts and other fungal species	Liu et al. (2022)
Sourdough	Low pH, high carbohydrate concentrations, oxygen limitation, and higher cell counts of LAB compared to yeasts	BACTERIA (former) *Lactobacillus* species, especially heterofermentative ones (*L. fermentum, L. paralimentarius, L. plantarum, and Lactobacillus sanfranciscensis*), dominate the sourdough environment YEAST *Candida humilis* and *Kazachstania exigua* are typical sourdough yeast species	De Vuyst et al. (2014)
Wine fermentations	Initial sugar concentration ranges from 160 to 240 g/L. Assimilable nitrogen concentration varies from 50 to 500 mg N/L, corresponding to concentrations of ammoniacal nitrogen from 15 to 150 mg N/L and free alpha-amino nitrogen from 30 to 300 mg N/L	BACTERIA Proteobacteria decline during fermentation, and Firmicutes increase (*Lactobacillales*) YEAST Yeasts during early fermentation stages (*Torulaspora delbrueckii*, *Lachancea thermotolerans*, and *Starmerella bacillaris*) and *S. cerevisiae* during late fermentation stages	Bely et al. (1990), Conacher et al. (2021)
Vaginal microbiome	pH ~3.5–4.5;, very low oxygen availability	BACTERIA *Lactobacillus*-dominates vaginal microbiota (*L. crispatus*, *L. iners*, *L. gasseri*, and *L. jensenii*); Other types of vaginal microbiota (diverse array of facultative and strictly anaerobic microorganisms, such as *Atopobium*, *Corynebacterium*, *Anaerococcus*, *Peptoniphilus*, and *Prevotella* spp.) YEAST Candidal spores and fungal hyphae (only in Bacterial vaginosis)	Ma et al. (2012)
Whiskey production		YEAST BACTERIA LAB	Liu et al. (2023)

tion conditions? Is there some close interaction between them and *S. cerevisiae* that results in this intimate co-existence?

LAB are Gram-positive, nonspore forming, catalase-negative rods that are incapable of producing ATP through a proton-gradient mechanism due to the absence of cytochromes and porphyrins. Thus, ATP production occurs predominantly through sugar fermentation via homofermentative or heterofermentative pathways. In homofermentative LAB, classical glycolysis (the Embden–Meyerhof–Parnas pathway) occurs, resulting in lactic acid as the main product. Heterofermentative LAB use the pentose phosphoketolase pathway, generating lactic acid, ethanol, and carbon dioxide. Other LAB characteristics include rapid growth, optimal growth temperatures ranging from 30°C to 45°C, tolerance to low pH, auxotrophy for many amino acids and a preference for carbohydrate-rich environments. Although they do not use oxygen, LAB can grow in aerobic environments due to the presence of peroxidases that protect them from reactive oxygen

species (Novik et al. 2017, Ponomarova et al. 2017, Sionek et al. 2024).

LAB are capable of producing a variety of compounds with antimicrobial activity, including lactic and acetic acids, fatty acids, reuterin, volatiles and cyclic peptides, bacteriocins, and β-glucanase (Bayrock and Ingledew 2004, Beckner et al. 2011, Liu et al. 2017, Siedler et al. 2019), many of which act synergistically or additively, enhancing overall effectiveness (Siedler et al. 2019). Interestingly, yeast can also inhibit the growth of *Lactobacillus* by releasing medium-chain fatty acids into the culture medium. The conditions that favor inhibition are low pH and the presence of ethanol (Alexandre et al. 2004)., Sieuwerts et al. (2018) verified the interactions between *L. plantarum* and *S. cerevisiae* in a chemically defined medium suitable for lactobacilli. Bacterial growth was stimulated by yeast, but only in the presence of specific C-sources, such as fructose (1–20 g/l) and glucose (above 2 g/l). When *Lactobacillus sanfranciscensis* was used, the stimulatory effect occurred regardless of the C-source. On the other hand, *S. cerevisiae* growth was stimulated by *L. plantarum* only when the C-source was lactose. As the yeast is not capable of using lactose, it must have benefited from the lactic acid produced by *L. plantarum* and traces of glucose and galactose released by the bacterial β-galactosidase activity. A similar result was obtained by Ponomarova et al. (2017) with *Lactococcus lactis* and *S. cerevisiae* in a mutualistic relationship in which the bacteria depended on the yeast for nitrogen and the yeast depended on the bacteria for carbon due to the inability of metabolizing lactose.

Kapetanakis et al. (2023) found that the *S. cerevisiae* Ethanol Red strain benefited the growth of *L. fermentum* by secreting sufficient amounts of 13 essential amino acids. This secretion is mediated by the Drug: H^+ Antiporter family 1 proteins, such as Aqr1, Qdr2, and Qdr3. Deletion of *QDR* genes in the industrial strain reduced the yeast's ability to favor LAB propagation and could be an interesting strategy for obtaining a GMO with the ability to limit bacterial contamination during bioethanol production. In N-rich environments, *S. cerevisiae* secreted amino acids and thus allowed the growth of *L. plantarum*. The secretion of metabolic substances beneficial to LAB is conserved among *S. cerevisiae* yeasts, as described in winemaking and kefir isolates (Ponomarova et al. 2017).

Among three species of *Lactobacillus* (*L. fermentum, Lactobacillus acidophilus*, and *L. plantarum*), only *L. ferme ntum* produced ethanol from glucose, at almost half the production of *S. cerevisiae*. In a medium with fructose, *L. fermentum* produced mannitol mainly due to the expression of the enzyme mannitol dehydrogenase, which allows the regeneration of $NAD(P)^+$ by reducing fructose directly to mannitol (Wisselink et al. 2002). In sugarcane *must*, which contains sucrose, glucose, and fructose, the presence of *L. fermentum* can decrease the amount of sugar for ethanol production by yeast due to this conversion of fructose into mannitol (Basso et al. 2014).

Another characteristic of LAB that may be related to their predominance in sugarcane *must* is the ability to metabolize phenolic compounds. In the gut microbiome, the metabolization of phenolic compounds by LAB generated by the ingestion of fruits and other vegetables results in the production of organic acids that can inhibit the growth of pathogenic microorganisms. The use of phenolic compounds in the gut by LAB results in the production of new metabolites, increased bacterial biomass, and a stimulatory effect on other metabolic pathways. The main species studied were *L. plantarum* and *L. brevis*, both possessing the phenolic acid decarboxylase and reductase enzymes for the degradation of phenolic compounds, mainly in the form of hydroxycinnamic acids (Ozcan and Ekinci 2016). Filannino et al. (2015) showed that *L. fermentum* is capable of metabolizing quinic acid, which replaces fructose and pyruvate as hydrogen acceptors, providing an energetic advantage for this heterofermentative bacterium. The presence of glucose can induce the metabolism of phenolic compounds. Molasses and sugarcane juice are rich in phenolic compounds such as phenolic acids, flavonoids, and different glycosides (Duarte-Almeida et al. 2006, Payet et al. 2006, Singh et al. 2015, Silva et al. 2018). This biosynthetic capacity of LAB can contribute to their frequency and predominance in the fermentative environment, not directly competing for the sugar present in the *must*, but ensuring their survival through the possibility of metabolizing other substrates. The metabolites produced may also have an effect on interspecific competition (Yu et al. 2024), and may interfere with yeast's metabolism, depending on the LAB species and the specific compounds involved.

Since LAB are fastidious organisms, it is expected that the coexistence with *S. cerevisiae* allows the creation of a metabolic niche with a lasting ecological effect for LAB. Nitrogen overflow metabolism appears to be one of the forces driving these interspecific relationships (Ponomarova et al. 2017), but it is probably not the only mechanism. He et al. (2021) show results indicating that the presence of *L. plantarum* enhances ethanol tolerance in *S. cerevisiae* S288c. In a study that employed synthetic microbial communities, yeast growth rate and ethanol yields were improved with acetaldehyde production by *L. amylovorus* inoculated in the fermentation *must*. Acetaldehyde is used by yeast to balance the NAD^+/NADH cytosolic pool, resulting in lower glycerol formation and consequently higher ethanol yields (Lino et al. 2021).

In terms of the alcoholic fermentation environment in sugarcane biorefineries, considering the knowledge acquired from studies related to other environments where yeast and LAB flourish, we envisage that the role of LAB as a probiotic or as a contaminant microorganism will depend on the biosynthetic capacity of the bacteria, the ratio between yeast and bacterial cells, their ability to produce yeast-inhibiting substances in addition to lactic acid and acetic acid, and the composition of the *must*. This *S. cerevisiae*—LAB symbiotic interaction may reveal that the survival of an individual species is intrinsically linked to the metabolic activities of the other.

Future directions

As discussed in the previous section, interactions between *S. cerevisiae* and LAB occur in different types of environments, but especially in man-made ones. The alcoholic fermentation environment in sugarcane biorefineries is rather understudied when compared to these other environments, meaning that we can learn from studies carried out in these other contexts. For instance, the cross-kingdom mechanism revealed by Jarosz et al. (2014) and later verified for LAB and yeast in the context of sake production (Watanabe 2024) has not yet been explored in the context of sugarcane bioethanol. Thus, it is of utmost importance that new studies are conducted, including both bottom-up (using synthetic microbial communities) and top-down assessments. The latter can be performed by bringing microbial communities from real biorefineries into the laboratory and conducting microcosm experiments—for instance, by subjecting the community to different challenges to observe how biodiversity fluctuates over time.

Due to the fastidious nature of lactobacilli, especially in terms of amino acid requirements, one of the main tasks that need to be undertaken is to confirm how the LAB found in this environment obtain their nitrogen supply, whether it arises from amino

acids secreted by yeast, as suggested by former studies in other environments, or could it be that cell death, both involving yeast and bacteria, provide LAB with the necessary nitrogen? We know that *S. cerevisiae* can grow in axenic cultures in sugarcane-based media. However, we need studies investigating whether this also holds true for the different bacterial contaminants found in these environments. If it can be demonstrated that they do not grow in this form, it would mean that they are obligatory symbionts with other species that also inhabit the same environment.

Another important question to be addressed is whether the microbial community structures of closely located biorefineries are more similar than those of more distantly related industrial units, the so-called distance-decay relationship (Kirchman 2018). This could bring insights into how far the microbiota of a particular industrial plant is influenced (or not) by neighboring biorefineries, which could mean that there is some mechanism in place that transfers microbial strains from one unit to another one, e.g. via animal vectors. In a recent work, it was demonstrated that, at least in terms of *S. cerevisiae* strains, there is a higher divergence when two industrial units were compared to each other, than when the same industrial unit is compared along two consecutive production seasons (Rego-Costa et al. 2023). In this work, it was also shown that the genome sequences of the ∼150 *S. cerevisiae* strains analysed clustered with other bioethanol and cachaça strains in a phylogenetic tree encompassing >1000 strains from the 1008-Yeast Genome Project (Peter et al. 2018). However, how far these observations hold for the non-*S. cerevisiae* organisms present in these communities, and for all microbial species present in the remaining ∼350 industrial units, is not yet known. Considering microbial diversity, it will also be interesting to compare this environment with other known microbial communities, such as those found in wastewater treatment plants, the human gut, and soils, in terms of indices such as alpha- and beta-diversity, to verify how they compare in terms of richness, evenness and other know microbial diversity measures. The recovery of metagenome assembled genomes can also be harnessed to uncover the functional potential of these communities.

It is well known that the *S. cerevisiae* strains that inhabit this environment are different from those used the production of wine, beer, sake, and other alcoholic beverages, as well as from baker's and wild strains. It will be interesting to better understand how the non-*Saccharomyces* species/strains, mainly bacterial ones, from the sugarcane biorefinery environment, compare to their counterparts in these other environments. It is envisaged that they will be different strains. There has been no evidence yet for the significant presence of protists or even DNA from other organisms (animals, plants, viruses, archaea) in this environment. How far that is true or simply the result of biased/targeted analyses carried out so far, is not yet known. In this context, viruses have the potential to be used to control bacterial contamination (Silva and Sauvageau 2014, Worley-Morse et al. 2015, Liu et al. 2015).

It will also be interesting to sequence the genomes of all these microbial strains to verify whether there are any indications of horizontal gene transfer, which could eventually have happened during co-existence over decades of industrial operation, which started in the 1970s on a large scale, or new genes of interest. Studies involving metatranscriptomics, metaproteomics, and metabolomics could also aid in revealing which metabolic pathways are active and which products are made under industrial conditions, eventually leading to the unraveling of symbiotic relationships, opening the possibility to engineer individual strains or even microbial communities to favor certain metabolic patterns. Process conditions could also be improved, as a result of such studies.

The research done so far makes it clear that properly identifying contaminants at the strain level is critical. Most of the studies performed hitherto have only evaluated communities at the genus or species level, or have only identified a subset of all the strains present in this fermentation environment. In addition, the use of amplicon sequencing solely targeting the bacterial 16S rRNA limits our understanding of nonbacterial contaminants (including other fungi, archaea, and viruses) and the functional potential of that community. More in-depth studies are crucial to evaluate the complexity of these microbial interactions and their drivers, and how they can affect process performance.

Studies that have unraveled the microbial communities during ethanol production using sequencing approaches confirmed that different bacterial phyla can be found throughout the process (ranging from 3 to 21 phyla) (Costa et al. 2015, Bonatelli et al. 2017, Queiroz et al. 2020). According to the ribosomal RNA database SILVA, 88 bacterial phyla have been described to date (v. 138, Quast et al. 2013), while the Genome Taxonomy Database—an initiative to establish a standardized microbial taxonomy based on genome phylogeny—indicates a higher number: 161 (08-RS214, Parks et al. 2022). Compared with several natural environments, alcoholic fermentations seem to harbor a small part of the total bacterial diversity. For example, a recent survey found more than 25 000 bacterial species in soils from 237 locations worldwide (Delgado-Baquerizo et al. 2018). Dominant soil species represent only about 2% of the total, yet they encompass 12 phyla. However, even with reduced diversity, it is clear that our knowledge about the microbial communities of alcoholic fermentation is still limited.

Unlike yeast, which are now inoculated every year in each industrial unit at the beginning of the production season, bacteria are not inoculated, and therefore, there may be stochastic factors involved in the assembly of the microbial community of each industrial plant. If we were to inoculate a bacterial community together with yeasts, would the structure and dynamics of the contaminant population follow a more predictable pattern than it does today? If yes, that would open the way to the use of probiotic microbes in this process, contributing to increasing its economic and environmental sustainability. Interestingly, this possibility was not mentioned in a recent review on potential technologies for bioethanol production (Attfield et al. 2025).

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