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Integration of Corn and Cane for Ethanol Production: Effects of Lactobacilli Contamination on Fermentative Parameters and Use of Ionizing Radiation Treatment for Disinfection

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Abstract: Recently, in Brazil, corn ethanol industries are being installed and the integration with sugar/energy-cane has been proposed, using bagasse for cogeneration and the juice to dilute the corn. However, this integration may have some limitations, such as the quality of the cane juice and potential contamination by microorganisms brought with the cane from the field. In this article, we first tested the effects of mixing energy cane juice with corn on fermentative parameters. We also assessed the effects of Lactobacilli. contamination on organic acids produced during the fermentation and fermentation parameters and proposed the use of ionizing radiation to replace antibiotics as a disinfection control method. Our results showed that mixing energy cane juice with corn does not have any negative effect on fermentation parameters, including ethanol production. The contamination with Lactobacilli. considerably increased the production of acetic, lactic, and succinic acid, reducing the pH and ethanol content from 89.2 g L^{-1} in the sterilized treatment to 72.9 g L^{-1} in the contaminated treatment. Therefore, for the integration between corn and cane to be applied on an industrial scale, it is essential to have effective disinfection before fermentation. Ionizing radiation (20 kGy) virtually disinfected the wort, showing itself to be a promising technology; however, an economic viability study for adopting it in the industry should be carried out.

Keywords: biofuels; sustainability; biorefinery; biomass; antibiotics replacement

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1. Introduction

In Brazil, renewable energy represents more than two-thirds of the national energy matrix and sugarcane and its derived products account for almost 20% of that [1]. In this context, biofuel production in Brazil is expected to increase in the coming years due to RenovaBio, a federal government program creating a carbon credit market [2,3].

The United States is the world's largest ethanol producer and mainly uses corn as raw material [4], whereas Brazil is the second largest ethanol producer, mainly from sugarcane [5]. The sugarcane ethanol production process is energetically self-sufficient, with an energetic balance from 8.3 to 10.6, due to the use of bagasse as biomass in the cogeneration process [6,7]. In the case of corn ethanol, however, no vegetable biomass is available for that purpose and fossil fuels are commonly used to supply the plant energy demand, leading to a low energetic balance of 1.3 to 1.6 [8].

Brazil is the world's third-largest corn producer, with about more than half of the production concentrated in the Center-West, making use of the same area utilized to produce soybean in a cultivation system that allows having crops every year on the same land [9], facing logistical challenges in being transported to ports and being exported. As a consequence of that, corn prices are low in these regions, recently attracting corn ethanol plants [10]. In the Center-South/West of Brazil, December to March is the rainy season, which does not allow a sugarcane harvest and the ethanol industry stops. Thus, sugarcane ethanol industries in these regions are also expanding their ethanol production through the installation of industrial components that allow the production of corn throughout the year, or only during the off-season (December to March) [11]. In this context, this study is part of a broader project that aims to assess the possibilities and limitations of integrating the recently adopted production of corn ethanol into the already traditional production of sugarcane ethanol in Brazil. In a previous article, we assessed the possibility of using energy cane to replace the water in the corn dilution. We found that the energy cane juice provided nutrients to the wort (i.e., phosphate and nitrate), which were significantly correlated with the increased yeast fermentation efficiency [12].

Despite the promising results, some limitations and concerns regarding the use of energy cane were highlighted [12]: (i) it is still a new technology and has been struggling to be established on a commercial scale [13]; (ii) broth composition could vary significantly depending on the variety and harvest period [14], which would affect the dilution process and the fermentation efficiency; (iii) cane would possibly bring impurities and contaminants from the field, reducing the fermentation efficiency [15].

In the ethanol industry, *Lactobacillus* spp. are considered the main contaminant affecting the fermentation process. These microorganisms metabolize carbohydrates for growth and energy production, leading to the formation of organic acids, mainly, lactic acid and acetic acid. Thus, they cause direct and indirect losses in yeast's fermentative yield [16]. For decades, the most used method of control of these microorganisms has been the application of antibiotics, such as penicillin G, streptomycin, tetracycline [17,18], virginiamycin [19,20], or mixtures with a broad spectrum of action. However, due to the risk of emerging antibiotic-resistant bacteria, the use of antibiotics should be reduced [21]. In this context, ionizing radiation is a microbial control method of great potential as it reduces or eliminates the contamination in biotechnological processes [22,23] and does not affect the quality of the substrate [24]. This technology is already used in industrial-scale processes for cereal grains, wastewater, and sugary solutions [25–27]. Recently [28], different irradiation doses were tested on sugarcane wort and it was found that even at the lowest doses, 99.9% of the contaminants were removed. However, no studies were performed to assess the efficiency of ionizing radiation on disinfecting wort mixed with cane juice and corn.

Based on this background, in the context of the integration of corn and energy/sugarcane for ethanol production, we hypothesized that:

1. The composition of the energy cane juice would be a major factor affecting fermentative parameters (Study 1);
2. The contamination with *Lactobacillus* spp. would increase the synthesis of secondary compounds, affecting the quality of the wort and reducing the fermentation efficiency (Study 2, part I);
3. Ionizing radiation would be an effective technology to reduce/control the contaminants brought with the cane in the industrial process of corn ethanol production (Study 2, part II).

2. Materials and Methods

All the experiments were conducted in the Sugar and Alcohol laboratory of the Department of Agri-Food Industry, Food, and Nutrition of the College of Agriculture "Luiz de Queiroz", at the University of São Paulo (ESALQ/USP) campus, in Piracicaba and in the Department of Chemical Engineering at Escola Politécnica of the University of São Paulo.

The corn used in both studies was purchased from a local store, was composed of 71% of starch, and was ground by hammer milling (Marconi Laboratory Equipment, Piracicaba, Sao Paulo, Brazil) to obtain a fine powder (<2 mm). The energy cane used in study 1 was harvested from an experimental station located in Rio Claro (Sao Paulo), approximately 40 km from Piracicaba. More information about the corn and energy cane treatments, preparation, and processing can be found in [12]. For Study 2, the sugars from the cane source were derived from a concentrated juice that was diluted to 90 g of fermentable sugars per liter.

2.1. Study 1

2.1.1. Experimental Design

In Study 1, we assessed the effects of mixing energy cane juice with corn on fermentative parameters. Figure 1a (adapted from [12]) represents the processes performed for each treatment. For the treatment in which corn was the only carbohydrate source, the process was exactly the same as [12]. For the treatment with the mixture of corn and energy cane juice, the difference was that in this study, lower amounts of energy cane juice were applied and water was used to complete the due volume in the reactors. The composition of the energy cane juice used in study 1 can be seen in Table 1. Seven replicates were used for each treatment.

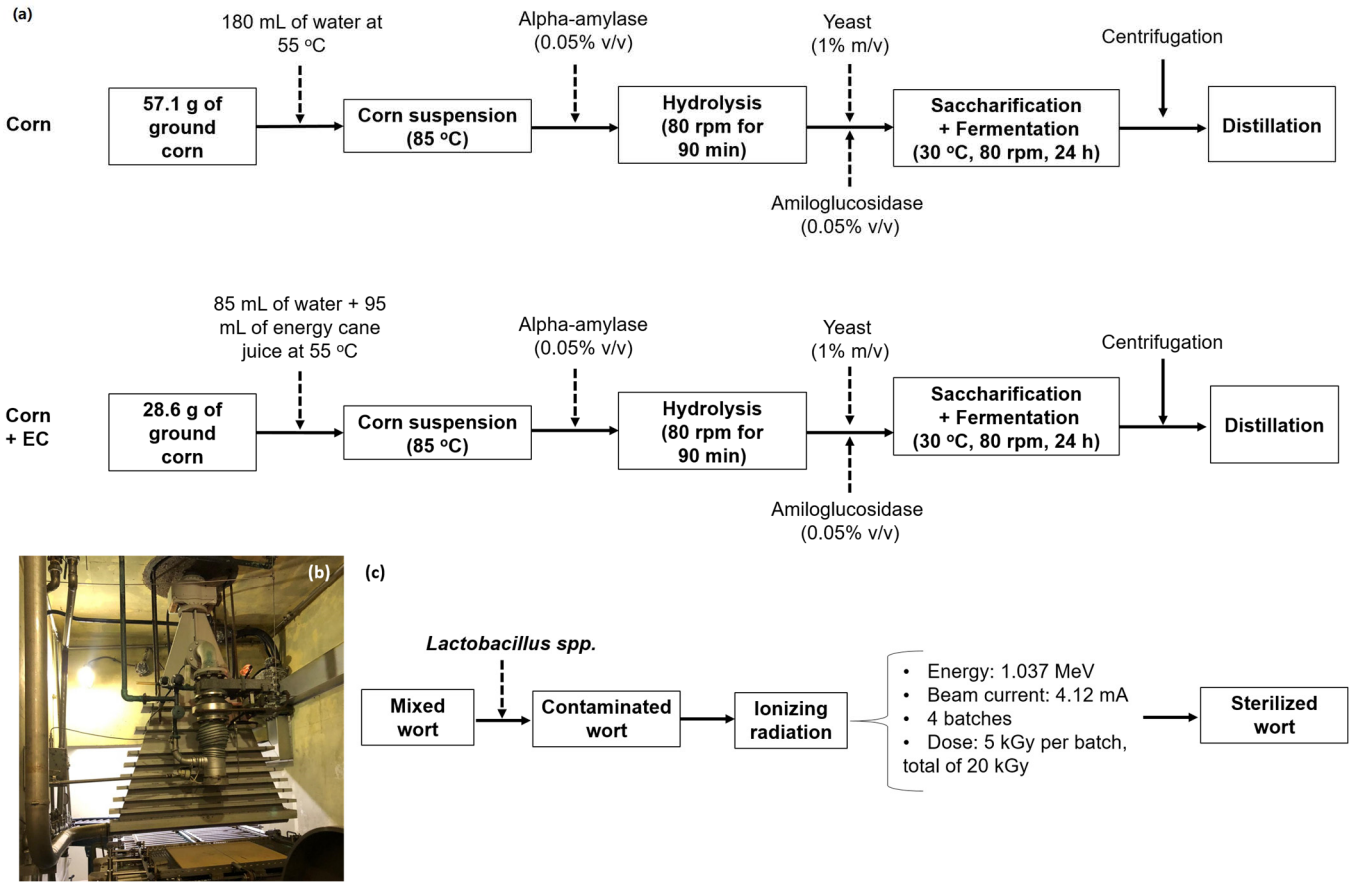

Figure 1. (**a**) Diagram demonstrating the processes used for both treatments used in study one (only corn and mixture of corn and energy cane as sources of carbohydrates for yest fermentation). (**b**) Industrial electron accelerator used in this study (Dynamitron—Job 188, model DC 1500/25/4) manufactured by RDIRadiation Dynamics Incorporation®. (**c**) Diagram with the steps done for the sterilization of mixed wort in this study.

Table 1. Composition of the energy cane juice used in Study 1.

	Sucrose	Fructose	Glucose	Mannitol	Glycerol
			g L^{-1}		
Energy cane	98.2	26.6	28.4	7.89	2.32

Determined by ion chromatography (930 Compact IC Flex, Metrohm) (Eith, 2006).

2.1.2. Analyses

After fermentation, the wine/beer was centrifuged, the supernatants were collected and the following parameters were determined: yeast cell viability, ethanol, acetic acid, lactic acid, glycerol, and residual sugars (sucrose, glucose, fructose) contents.

The determination of east cell viability was by the differential staining of living cells method. For that, the samples were diluted 20 times and 0.1% methylene blue solution was applied. Dead and live cells were counted by light microscopy [29,30]. More information about the method can be found in [12].

The ethanol content was determined by distilling the supernatant (micro-distiller MA 012/1, Marconi) and analyzing it in a digital densimeter (EDM 4000, Schmidt Haensch). Acetic acid, lactic acid, glycerol, and residual sugar contents were measured by ion chromatography (930 Compact IC Flex, Methrom). More detailed information about these measurements can be found in [12].

2.2. Study 2

2.2.1. Wort Preparation

After milling, the corn fine powder (<2 mm) was suspended with diluted cane syrup (90 g fermentable sugars per liter) at a rate of 444 g of corn per liter of diluted syrup. This suspension had the pH adjusted to 5.8 and then was subjected to heating in a hydrolysis reactor until it reached a temperature of 85 °C. In this condition, α-amylase enzyme (batch NWP00339, Novozymes) was added at a rate of 0.1% or 0.05% (*v*/*v*) (Figure 1), keeping the suspension under constant agitation at 80 rpm, at a temperature of 85 °C, for 150 min, until the complete dextrinization of the starch in the suspension. This suspension had the pH adjusted to 5.0 and cooled to 65 °C by a "cold water" heat exchanger. After that, amyloglucosidase (batch NAN10028, Novozymes) was added at a rate of 0.1% (*v*/*v*) or 0.05% (*v*/*v*) (Figure 1). The solution was kept under constant stirring at 80 rpm at 65 °C for 150 min. All hydrolyzed material was centrifuged to remove the solid fraction. The remaining liquid fraction was used to prepare the wort.

2.2.2. Contamination

The wort was contaminated with different strains of bacteria before the ionizing radiation treatment (Figure 1c). The contaminants used were bacteria from the genus *Lactobacillus*, belonging to the library of microorganisms at BELa-Bioprocess Engineering Lab/USP: *Lactiplantibacillus plantarum* (code: ESALQ 4), *Limosilactobacillus fermentum* (code: ESALQ 3), *Lacticaseibacillus paracasei* (code: LAB. 4), and *Limosilactobacillus reuteri* (code: ATC23272). These microorganisms were chosen due to the fact that they are representatives of the groups' contaminants in the industrial fermentation process [31].

2.2.3. Treatment with Ionizing Radiation

The treatment with ionizing radiation of part of the wort was performed at the Technological Radiation Center (CTR), of the Institute of Energy and Nuclear Research (IPEN—CNEN SP). The equipment used was an industrial electron accelerator (Dynamitron—Job 188, model DC 1500/25/4) manufactured by RDIRadiation Dynamics Incorporation® (Figure 1b). The wort was irradiated in four batches in Pyrex® containers and conditioned so that the thickness of the treated wort layer was 4 mm, which was controlled by the volume of samples that were exposed to radiation. The following parameters were used:

energy of 1.037 MeV with a beam current of 4.12 mA, and 5 kGy per batch, with a total of 20 kGY (Figure 1c). The total radiation was selected based on [28].

Samples from the untreated and ionizing radiation-treated wort were collected and inoculated in Petri dishes containing MRS media (Man Rogosa & Sharpe). The MRS is a media developed to favor the growth, isolation, and counting of lactobacilli. More information about its formulation and use can be found in [32,33].

2.2.4. Fermentation: Experimental Design

In Study 2, for the fermentation experiment, two treatments were used: ion radiation-treated (IRT) and contaminated untreated (CU). The yeast used in this study is a strain commonly used by the ethanol industry (Thermosacc, Lallemand Biofuels & Distilled Spirits). Both worts (IRT and CU) were diluted to 150 g of fermentable sugars per liter and rehydrated yeast was applied at a rate of 1.5% (w/v). The fermentation was conducted in 24-liters bioreactors (model Labfors 5, Infors AG, Switzerland). In the beginning, bioreactors were filled in a batch of 850 mL of wort. After four hours, the bioreactors were constantly fed with wort at a rate of 12.5 g of fermentable sugars per hour for 24 h. The fermentation was conducted for another 17 h, in a total of 45 h of fermentation.

2.2.5. Fermentation: Analyses

Samples were collected from the reactors at 20 min (0.3 h), 8 h, 17 h, 25 h, 39 h, and 45 h after the beginning of the fermentation. The samples were centrifuged to remove the yeast and the following parameters of the supernatant were analyzed by HPLC: succinate, lactate, acetate, ethanol, and fermentable sugars (glucose, fructose, and sucrose) contents. For that, 100 µL of sample was diluted with 900 µL of ultra-pure water and injected in an ion exchange column HPX-87H (Bio-Rad) at 60 °C, with 5 mM H_2SO_4 as the mobile phase and a flow rate of 0.6 mL min^{-1}. The pH was measured by a benchtop pHmeter. Fermentation yield was calculated based on the theoretical stoichiometric yield (64.75 mL of ethanol per 100 g of fermentable sugars); for more detailed information, see [12].

2.3. Statistical Analyses

The collected data were digitalized and initially processed in Excel®. A summary spreadsheet was input into IBM SPSS Statistics 28.0 to perform statistical analyses. For Study 1, one-way ANOVA was performed for all the parameters and Student's *t*-test (<0.05) was used to compare the means of both treatments. Graphs were made on Sigma Plot 14.0.

3. Results and Discussion

3.1. Study 1

In Study 1, we assessed the effects of mixing energy cane juice and corn on fermentative parameters. There was no significant difference between treatments on final ethanol content, yeast cell viability, glycerol, acetic acid, sucrose, and glucose contents. The fructose content was significantly higher in the corn treatment when compared to the mixture of corn and energy cane (Table 2).

As can be seen in Figure S1 (Supplementary materials), the CO_2 release from the reactors with the mixture (corn + EC) was higher than in the reactors with only corn in the first 7 h and it was equalized at 20 h.

Sica et al. (2021) fully replaced the water with energy cane juice and found that it significantly increased the alcohol content and the fermentation yield and efficiency. They performed a principal component analysis (PCA) and their results indicated that the fermentation efficiency was highly correlated with nitrate (0.80) and phosphate (0.99) contents in the wort. In the industry, whereas the sugarcane wort does not need to be supplemented with nutrients [34], corn is usually supplemented with nutrients (i.e., urea) to promote yeast growth and stimulate fermentation [16]. Dias et al. (2022) used a mixture of corn and cane. They assessed the effects of supplementation of different nutrients to the mixed wort and found an increase in ethanol content when nitrogen was supplied and

a reduction in residual sugars. They confirmed that the supplementation with nitrogen increased yeast biomass production [35]. We found that the mixed wort had lower residual sugars when compared to the treatment with only corn.

Table 2. Ethanol (vv^{-1}), glycerol (wv^{-1}), and acetic acid (wv^{-1}) contents, yeast cells viability, and residual sugars after 24 h of fermentation obtained in Study 1 using corn and mixture of corn and energy cane as a source of carbohydrates for fermentation.

		Study 1	
		Corn	**Mixture**
Ethanolcontent	%	10.6 ± 0.7	10.3 ± 0.2
Cell viability		90.2 ± 1.9	93.5 ± 1.1
Glycerol	g L^{-1}	1.49 ± 0.2	1.54 ± 0.07
Acetic acid		0.47 ± 0.02	0.48 ± 0.01
		Residual sugars	
Sucrose	mg L^{-1}	n.d.	n.d.
Glucose		n.d.	n.d.
Fructose *		634 ± 62 a	382 ± 58 b

* Different letters in the same line indicate a significant difference between treatments (<0.05) using Student's *t*-test. Values after ± indicate standard deviation.

The composition of energy cane biomass and juice quality is highly influenced by the harvest date [14], environment, weather conditions, cultivar, year, and other factors [36]. In this study, the energy cane juice had higher fermentable sugars (~150 g L^{-1}) when compared to [12] (105 g L^{-1}) and, for this reason, we only added around half of the volume of energy cane juice and complemented it with water. This could have diluted the nutrient contents and eliminated the positive effects of adding energy cane juice to the corn, indicating that the composition of the energy cane and the mixing rate will play an important role in the fermentation efficiency. Therefore, for this technology to be applied, from an industry perspective, it would be needed to monitor the quality of the juice from energy cane harvested and make a real-time decision on the dilution rates. Another possibility would be to integrate corn into a sugarcane industry during the rainy season to supplement it with syrup or molasses.

3.2. Study 2

3.2.1. Effects of Bacterial Contamination on Fermentative Parameters

In this study, the ethanol production was the same in both treatments until 7 h. After 10 h, the IRT treatment showed a considerable increase in ethanol content when compared to the control treatment contaminated with Lactobacilli (Figure 2). These differences could be related to the production of organic acids by the contaminating bacteria and/or by the yeast due to physiological responses to the sugar and nutrient consumption by bacteria and the production of organic acids. The industry considers an exceptional fermentative yield, when it is equivalent to 90 to 92% of the stoichiometric yield, with a small deviation of sugars for cell multiplication and formation of by-products. According to [37], bacterial contamination at 10^8 and 10^9 can reduce fermentation yield from 14% to 90%.

In the scientific literature, not many studies on the effects of organic acids on ethanol production from corn hydrolysate are found. The few studies that used corn hydrolyzate showed a protective effect of the more complex medium due to the increased buffering capacity, mitigating the antagonistic effects of *Lactobacillus* spp. contamination but yet significantly reducing the yeast's ethanol production [38–40]. In this study, we found that the contamination with lactobacilli reduced by 18% the ethanol production (Figure 2). Similarly, [16] found that inoculating *Lactobacillus* spp. 24 h before the fermentation with *S. cerevisiae* reduced by 22% the ethanol production, and it was concluded that bacterial growth, production of acetic acid or lactic acid, or increasing glycerol synthesis could not fully explain this reduction.

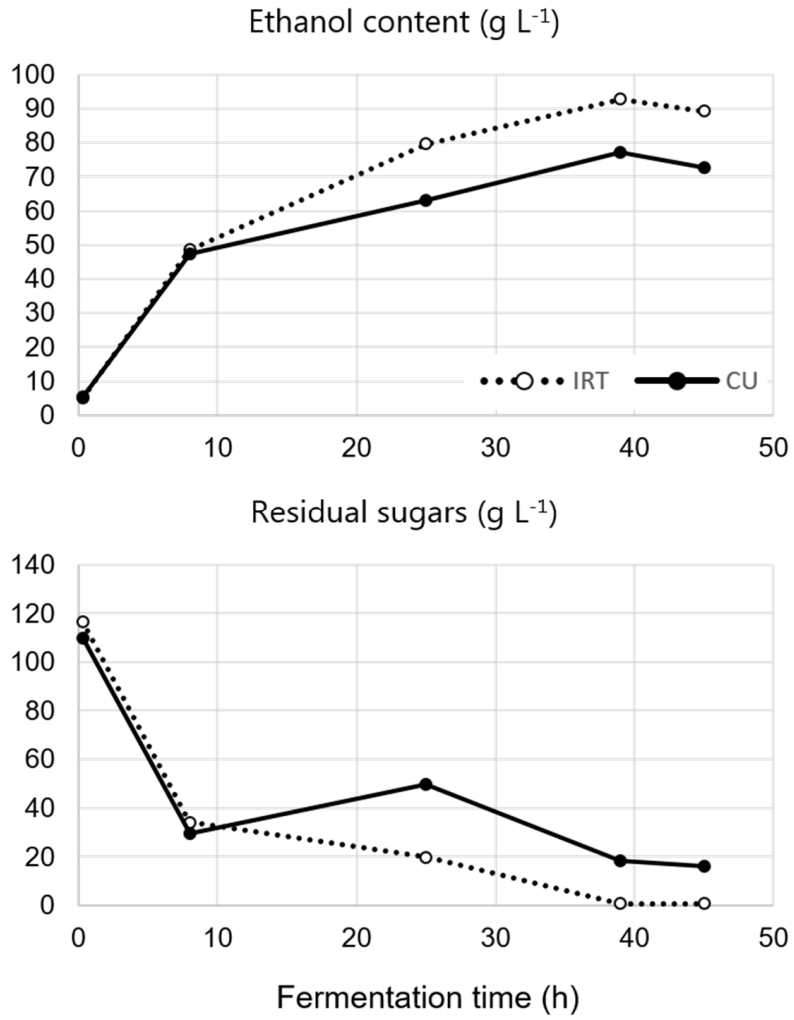

Figure 2. Ethanol content and residual sugars for both treatments (IRT—ionizing radiation treated; CU—contaminated untreated) at different fermentation times in Study 2.

3.2.2. Effects of Bacterial Contamination on Organic Acid Synthesis and pH

In this study, contamination with lactobacilli considerably increased the production of acetate and lactate already after 8 h of fermentation. The IRT treatment did not show a considerable change on lactate content over the fermentation period, whereas the acetate content increased, reaching around 1 g L^{-1} at around 40 h of fermentation (Figure 3).

These results indicates that the lactate and acetate were mainly synthesized by the contaminants, but the acetate could have also been partially synthesized by the yeast in the contaminated wort. The acetic acid is well known to be a physiological product of yeast fermentation [41] and is related to *S. cerevisiae* cell aging [42] and death [43].

It has been known that yeasts can be active in acidic environments, coexisting with contaminating bacteria, which are the main producers of lactic and acetic acid in this context [44]. On the other hand, the production of secondary compounds creates an antagonistic relationship between bacteria and yeast [45]. The production of organic acids—such as lactate and acetate—by contaminating bacteria and the competition for nutrients are the main factors reducing ethanol production by yeast; however, several researchers have reported that these factors cannot fully explain the ethanol production inhibition [38,39,46].

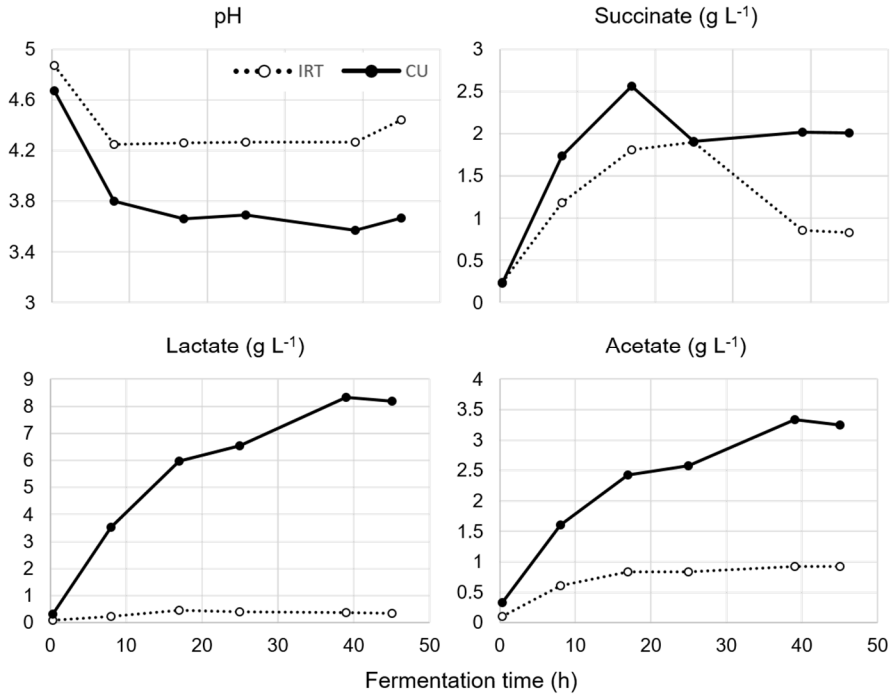

Figure 3. pH, succinate, lactate, and acetate contents for both treatments (IRT—ionizing radiation treated; CU—contaminated untreated) at different fermentation times in Study 2.

According to [47], at a range from 10 to 40 g L^{-1} of acetic acid, the ethanol production by yeast can be inhibited, reducing up to 80% the ethanol production [47]. In this study, the acetic acid content of the contaminated treatment reached a maximum of 3.5 g L^{-1} (Figure 3), which was already enough to reduce the ethanol production (Figure 2). Ref. [38] demonstrated the effects of acetic acid at pH 4, reaching a maximum ethanol content of 4% at a concentration of 2.36% of acid. Another study showed that contamination with *Lactobacillus* spp. reduced yeast growth by 50% [48]. Based on these arguments, it can be assumed that acetic acid, lactic acid, can interact and affect the metabolism and growth of yeasts, directly reflecting on the decrease in ethanol yield [49].

In the first 17 h, both treatments synthesized increased contents of succinate, with the contaminated always having higher contents. At 24 h, the succinate content was the same for both treatments and after that, the contaminated content remained and did not show considerable changes, whereas the IRT succinate content was reduced (Figure 3). This is corroborated by Heerde & Radler (1978), who found that succinic acid is the main organic acid produced by yeast and can reach up to 1.7 g L^{-1} [50]. These results indicates that the synthesis of succinate was mainly from the yeast and it is influenced by the contaminants. According to [51], there is no evidence of any physiological function for the large amount of succinic acid excreted by the yeast. Nevertheless, an ecological function can be attributed, as its production makes the yeast more competitive in an industrial fermentation environment [51].

The CU treatment pH was considerably lower than IRT through the fermentation process (Figure 3). This sharper reduction in pH values during fermentation can be attributed to bacteria contaminating the process, due to the formation of organic acids [40,52,53]. In the first 7 h of fermentation, the pH of IRT and CU were reduced to 4.2 and 3.8, respectively. This was also the same period of time at which the succinic acid showed the highest increase in both treatments (Figure 3). The succinic acid is one of the most important factors related to the increase of the titratable acidity during fermentation [54], as 1.23 g of succinic acid L^{-1} may increase it by 50% [55].

It is known that acetic acid in its undissociated form diffuses through the plasma membrane into the yeast cell. In the cytosol, the pH values higher than in the external

environment lead to the dissociation of acid species from their molecular form to the ionic form, releasing protons that cause a decrease in the cytosolic pH, consequently, and may cause damage to the cell, caused by acidification of the cytoplasm. These alterations in cell physiology also directly reflect on the fermentation yield for ethanol production [56–59]. To mitigate the acidification of the cytosolic pH, caused by the dissociation of acid inside the cell, two proteins are essential: plasma membrane ATPases (PM-ATPase) and vacuolar ATPases (V-ATPase), both responsible for removing H^+ from the cytoplasm. However, it may cause ATP consumption by the cell [56,60]. Consequently, fewer ATP molecules will be available for the formation of biomass, resulting in cells with less vitality to carry out fermentation, thus increasing fermentation time, decreasing yeast viability, and reducing ethanol productivity.

Relatively high concentrations of H^+ inside the cell promote high demands for ATP, which may not let the cell avoid acidification of the cytosolic pH, generating subsequent cell damage. This includes disturbance of the electrochemical gradient, decrease in DNA and RNA synthesis, and, as previously discussed, programmed cell death [41,43]. In addition to the harmful effects of changing intracellular pH, excess acetate can also negatively interfere with cell metabolism, inhibiting the activity of the enolase enzyme in the glycolytic pathway, increasing turgor pressure, and generating oxidative stress [61,62]. As the pH increases, the dissociation of weak acids occurs, which leads to a decrease in the concentration of the undissociated form of the acid and increases the concentration of the conjugate base. Theoretically, the toxicity caused by acetic acid is greater at a lower pH, when the number of undissociated molecules is greater than at a high pH [63].

3.2.3. Disinfection with Ionizing Radiation

In Figure 4, we compared the wort contamination before (a) and after (b) the treatment with ionizing radiation. We used an electron accelerator at a total ionizing dose of radiation of 20 kGy, which was shown to be efficient in reducing (or virtually eliminating) the wort microbial contamination.

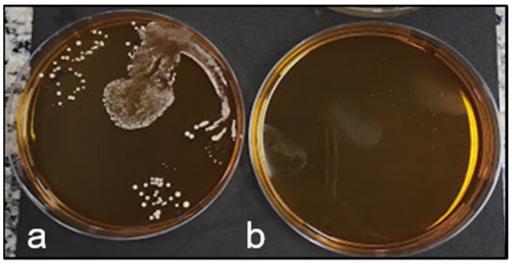

Figure 4. MRS media in petri dishes inoculated with wort before (**a**) and after (**b**) ionizing radiation treatment (20 kGy) in Study 2 (10 times dilution).

Contamination brought by the cane from the field and its negative effects on the fermentative parameters is one of the biggest challenges to adopting the integration of corn and energy or sugarcane during the fermentation on an industrial scale. Currently, in the sugarcane industry, antibiotics are used to control bacterial contamination [64]; however, it might represent a high risk in the short and long term. In the short term, antibiotics are found in yeast, which is used as animal feed and can cause health issues in animals [65]. The use of antibiotics in corn ethanol production could also be problematic and cause a risk to animals, as the DDGS for the animal feed is one of the main revenues for corn ethanol industry and the presence of antibiotics would affect the quality of the product. In the long term, the excessive use of antibiotics will lead to antimicrobial resistance in bacterial pathogens and be a threat to human health [21].

In a recent study, ref. [28] assessed ionizing radiation as an alternative to antibiotics in sugarcane ethanol production. They tested different ionizing doses and found that even at lower doses (10 kGy) it caused a reduction of 99.9% of the total bacteria in the substrate. In addition to that, all the wort treated with ionizing radiation had significantly higher yields and productivities when compared to the contaminated control and a relatively low variable cost, USD 0.128 per cubic meter of wort for a radiation dose of 20 kGy [28]. This is a relatively low cost considering the considerable impacts of contaminant microorganisms on the fermentation yield. One should also consider the investment and maintenance cost of adopting ionizing radiation in the ethanol industry and also the long-term cost and negative impacts of potential antibiotic resistance (up to one trillion US dollars worldwide per year in 2050, according to the World Bank [66]).

4. Conclusions

In study 1, our results showed that the integration of corn and energy cane did not have negative effects on the fermentative parameters, including final ethanol content. However, energy cane juice quality changes depending on the variety and harvest date. Therefore, it is essential to analyze and properly calculate the mixing rates.

In study 2, contamination with *Lactabacillus* spp. can cause considerable losses during the fermentation, and ionizing radiation was shown to be a promising technology to reduce contamination, replacing antibiotics. However, a study to test lower irradiation doses should be performed and the economic viability should be assessed in order to be applied on the industrial scale.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9020089/s1, Figure S1: Average accumulated carbon dioxide release from the reactors at different fermentation time in Study 1.

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Data Availability Statement: The authors will make the data available, if necessary.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could appear to influence the work reported in this article.

References

1. Ministry of Mines and Energy. *Brazilian Energy Balance-Summary Report of 2020*; Ministry of Mines and Energy: Brasília, Brazil, 2021.
2. Grassi, M.C.B.; Pereira, G.A.G. Energy-Cane and RenovaBio: Brazilian Vectors to Boost the Development of Biofuels. *Ind. Crop. Prod.* **2019**, *129*, 201–205. [CrossRef]
3. Ministry of Mines and Energy Brazilian Ten-Year Energy Expansion Plan. Available online: http://www.epe.gov.br/sites-pt/publicacoes-dados-abertos/publicacoes/Documents/PDE2027_aprovado_OFICIAL.pdf (accessed on 5 February 2020).
4. RFA (Renewable Fuels Association) 2020 Ethanol Industry Outlook. Available online: www.eenergyadams.com (accessed on 7 February 2021).
5. UNICA Production and Milling History-Total Ethanol Production, 2019/2020. Available online: https://observatoriodacana.com.br/historico-de-producao-e-moagem.php?idMn=31&tipoHistorico=2&acao=visualizar&idTabela=2448&produto=etanol_total&safraIni=2019%2F2020&safraFim=2019%2F2020&estado=SP&idioma=2 (accessed on 18 January 2021).
6. Goldemberg, J. Ethanol for a Sustainable Energy Future. *Sci. AAAS* **2007**, *315*, 808–810. [CrossRef] [PubMed]
7. Macedo, I.C.; Seabra, J.E.A.; Silva, J.E.A.R. Green House Gases Emissions in the Production and Use of Ethanol from Sugarcane in Brazil: The 2005/2006 Averages and a Prediction for 2020. *Biomass Bioenergy* **2008**, *32*, 582–595. [CrossRef]

8. Hill, J. Environmental Costs and Benefits of Transportation Biofuel Production from Food- and Lignocellulose-Based Energy Crops. A Review. In *Sustainable Agriculture*; Lichtfouse, E., Navarrete, M., Debaeke, P., Véronique, S., Alberola, C., Eds.; Springer: Dordrecht, The Netherlands, 2009; Volume 27, pp. 125–139.
9. FAO FAOSTAT. Available online: http://www.fao.org/faostat/en/#data/QC (accessed on 10 November 2019).
10. Barros, S.; Woody, K. *Corn Ethanol Production Booms in Brazil*; United States Deparment of Agriculture (USDA): Brasilia, Brazil, 2020.
11. CerradinhoBIO Cerradinho Bio Creates Business Division for Products Derived from Corn. Available online: https://www.cerradinhobio.com.br/en/news/cerradinho-bio-creates-business-division-for-products-derived-from-corn/ (accessed on 19 December 2022).
12. Sica, P.; Prado, L.M.L.M.; Granja, P.; de Carvalho, E.M.; Mattos, E.d.C.; Calegari, R.P.; Silverio, M.; Martins, B.C.; Baptista, A.S. Effects of Energy Cane (*Saccharum* spp.) Juice on Corn Ethanol (Zea Mays) Fermentation Efficiency: Integration towards a More Sustainable Production. *Fermentation* **2021**, *7*, 30. [CrossRef]
13. Sica, P. Sugarcane Breeding for Enhanced Fiber and Its Impacts on Industrial Processes. In *Sugarcane-Biotechnology for Biofuels*; Khan, M.S., Ed.; IntechOpen: London, UK, 2021; p. 16.
14. Knoll, J.E.; Anderson, W.F.; Richard, E.P.; Doran-Peterson, J.; Baldwin, B.; Hale, A.L.; Viator, R.P. Harvest Date Effects on Biomass Quality and Ethanol Yield of New Energycane (Saccharum Hyb.) Genotypes in the Southeast USA. *Biomass Bioenergy* **2013**, *56*, 147–156. [CrossRef]
15. Ceccato-Antonini, S.R.; Bassi, A.P.G.; Paraluppi, A.L.; dos Santos, E.G.D.; Matsuoka, S. Deterioration and Fermentability of Energy Cane Juice. *Cienc. Rural.* **2017**, *47*, e20160860. [CrossRef]
16. Thomas, K.C.; Hynes, S.H.; Ingledew, W.M. Effect of Lactobacilli on Yeast Growth, Viability and Batch and Semi-Continuous Alcoholic Fermentation of Corn Mash. *J. Appl. Microbiol.* **2001**, *90*, 819–828. [CrossRef] [PubMed]
17. Aquarone, E. Penicillin and Tetracycline as Contamination Control Agents in Alcoholic Fermentation of Sugar Cane Molasses. *Appl. Microbiol.* **1959**, *8*, 263–268. [CrossRef]
18. Day, W.H.; Serjak, W.C.; Stratton, J.R.; Stone, L. Antibiotics as Contamination-Control Agents In Grain Alcohol Fermentations. *J. Agric. Food Chem.* **1954**, *2*, 252–258. [CrossRef]
19. Hynes, S.H.; Kjarsgaard, D.M.; Thomas, K.C.; Ingledew, W.M. Use of Virginiamycin to Control the Growth of Lactic Acid Bacteria during Alcohol Fermentation. *J. Ind. Microbiol. Biotechnol.* **1997**, *18*, 284–291. [CrossRef] [PubMed]
20. Islam, M.; Toledo, R.; Hamdy, M.K. Stability of Virginiamycin and Penicillin during Alcohol Fermentation. *J. Biosci. Bioenergy* **1999**, *17*, 369–376. [CrossRef]
21. Frieri, M.; Kumar, K.; Boutin, A. Antibiotic Resistance. *J. Infect. Public Health* **2017**, *10*, 369–378. [CrossRef]
22. Al-Assaf, S.; Coqueret, X.; Dahlan, K.Z.H.M.; Sen, M.; Ulanski, P. *The Radiation Chemistry of Polysaccharides*; International Atomic Energy Agency: Vienna, Austria, 2016; Volume 124, ISBN 978-92-0-101516-7.
23. Molins, R.A. *Food Irradiation: Principles and Applications*, 1st ed.; John Wiley & Sons: Hoboken, NJ, USA, 2001; ISBN 0471356344.
24. Bhat, R.; Karim Alias, A.; Paliyath, G. Use of Electron Beams in Food Preservation. In *Progress in Food Preservation*; Bhat, R., Abd Karim Alias, G.P., Eds.; Wiley-Blackwell: Oxford, UK, 2012; Volume 17, pp. 343–372.
25. Borrely, S.I.; Del Mastro, N.L.; Sampa, M.H.O. Improvement of Municipal Wastewaters by Electron Beam Accelerator in Brazil. *Radiat. Phys. Chem.* **1998**, *52*, 333–337. [CrossRef]
26. Podadera, P. Estudo Das Propriedades Do Açúcar Líquido Invertido Processado Com Radiação Gama e Feixe de Elétrons. Ph.D. Thesis, University of Sao Paulo, Sao Paulo, Brazil, 2007.
27. Rela, P.R. Desenvolvimento De Dispositivo De Irradiação Para Tratamento De Efluentes Industriais Com Feixe De Elétrons. Ph.D. Thesis, University of Sao Paulo, Sao Paulo, Brazil, 2003.
28. Calegari, R.P.; da Silva, E.A.; da Silva, A.P.M.; Gomes, M.P.; Mota, L.A.; Arthur, V.; Baptista, A.S. Wort Disinfection Treatment with Electron Beam for Bioethanol Production. *Sci. Agric.* **2023**, *80*. [CrossRef]
29. Pierce, J.S. Institute of Brewing: Analysis Committee Measurement of Yeast Viability. *J. Inst. Brew.* **1970**, *76*, 442–443. [CrossRef]
30. Oliveira, A.; Gallo, C.; Alcarde, V.; Godoy, A.; Amorim, H. *Methods for Microbiological Control in Sugar and Alcohol Production*, 1st ed.; FERMENTEC: Piracicaba, Brazil; ESALQ: Piracicaba, Brazil; FEALQ: Piracicaba, Brazil, 1996; p. 89. (In Portuguese)
31. Firmino, F.C.; Porcellato, D.; Cox, M.; Suen, G.; Broadbent, J.R.; Steele, J.L. Characterization of Microbial Communities in Ethanol Biorefineries. *J. Ind. Microbiol. Biotechnol.* **2020**, *47*, 183–195. [CrossRef] [PubMed]
32. Roy, D. Media for the Isolation and Enumeration of Bifidobacteria in Dairy Products. *Int. J. Food Microbiol.* **2001**, *69*, 167–182. [CrossRef] [PubMed]
33. LarborClin MRS Agar Lactobacilus. Available online: https://www.laborclin.com.br/wp-content/uploads/2019/06/MRS_Agar_Lactobacilus.pdf (accessed on 14 December 2022).
34. Prado, R.D.M.; Caione, G.; Campos, C.N.S. Filter Cake and Vinasse as Fertilizers Contributing to Conservation Agriculture. *Appl. Environ. Soil Sci.* **2013**, *2013*, 581984. [CrossRef]
35. Silva, N.D.; Alvim, M.R.; Rosario, C.G.A.; Espinosa, D.C.R.; Baltazar, M. dos P.G. Nutrients' Supplementation Impacts on Alcholoic Fermentation of Corn and Sugarcane Mixed Wort. *Res. Sq. Prepriting* **2022**, *1*, 1–26. [CrossRef]
36. Knoll, J.E.; Anderson, W.F.; Missaoui, A.; Hale, A.; Hanna, W.W. Biomass Production and Stability of Five Energycane Cultivars at Two Latitudes in Georgia. *Agrosystems Geosci. Environ.* **2021**, *4*, 1–14. [CrossRef]

37. Alterthum, F.; Cruz, M.R.M.; Vairo, M.L.R.; Gambassi, D.M. Effect of Contaminating Microorganisms of Alcoholic Fermentation on Microdistilleries (in Portuguese). *STAB. Açúcar, Álcool Subprodutos* **1984**, *3*, 42–49.
38. Graves, T.; Narendranath, N.V.; Dawson, K.; Power, R. Effect of PH and Lactic or Acetic Acid on Ethanol Productivity by Saccharomyces Cerevisiae in Corn Mash. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 469–474. [CrossRef] [PubMed]
39. Graves, T.; Narendranath, N.V.; Dawson, K.; Power, R. Interaction Effects of Lactic Acid and Acetic Acid at Different Temperatures on Ethanol Production by Saccharomyces Cerevisiae in Corn Mash. *Appl. Microbiol. Biotechnol.* **2007**, *73*, 1190–1196. [CrossRef] [PubMed]
40. Thomas, K.C.; Hynes, S.H.; Ingledew, W.M. Influence of Medium Buffering Capacity on Inhibition of Saccharomyces Cerevisiae Growth by Acetic and Lactic Acids. *Appl. Environ. Microbiol.* **2002**, *68*, 1616–1623. [CrossRef] [PubMed]
41. Giannattasio, S.; Guaragnella, N.; Ždralević, M.; Marra, E. Molecular Mechanisms of Saccharomyces Cerevisiae Stress Adaptation and Programmed Cell Death in Response to Acetic Acid. *Front. Microbiol.* **2013**, *4*, 1–7. [CrossRef]
42. Burtner, C.R.; Murakami, C.J.; Kennedy, B.K.; Kaeberlein, M. A Molecular Mechanism of Chronological Aging in Yeast. *Cell Cycle* **2009**, *8*, 1256–1270. [CrossRef]
43. Ludovico, P.; Sousa, M.J.; Silva, M.T.; Leão, C.; Côrte-Real, M. Saccharomyces Cerevisiae Commits to a Programmed Cell Death Process in Response to Acetic Acid. *Microbiology* **2001**, *147*, 2409–2415. [CrossRef]
44. Boraam, F.; Faid, M.; Larpent, J.P.; Breton, A. Lactic Acid Bacteria and Yeast Associated with Traditional Moroccan Sour-Dough Bread Fermentation. *Sci. Aliment.* **1993**, *13*, 501–509.
45. Walksman, S.A. Microbial Antagonism and Antibiotic Subastances. *Oxford Univ. Press* **1945**, *129*, 531–533.
46. Narendranath, N.V.; Hynes, S.H.; Thomas, K.C.; Ingledew, W.M. Effects of Lactobacilli on Yeast-Catalyzed Ethanol Fermentations. *Appl. Environ. Microbiol.* **1997**, *63*, 4158–4163. [CrossRef]
47. Maiorella, B.L.; Blanch, H.W.; Wilke, C.R. Economic Evaluation of Alternative Ethanol Fermentation Processes. *Biotechnol. Bioeng.* **1984**, *26*, 1003–1025. [CrossRef] [PubMed]
48. Ngang, J.J.E.; Letourneau, F.; Wolniewicz, E.; Villa, P. Applied Microbiology Biotechnology Inhibition of Beet Molasses Alcoholic Fermentation by Lactobacilli. *Appl. Microbiol. Biotechnol.* **1990**, *33*, 490–493.
49. Cherubin, R.A. Efeitos Da Viabilidade Da Levedura e Da Contaminação Bacteriana Na Fermentação Alcoólica. Ph.D. Thesis, University of Sao Paulo, Sao Paulo, Brazil, 2003.
50. Heerde, E.; Radler, F. Metabolism of the Anaerobic Formation of Succinic Acid by Saccharomyces Cerevisiae. *Arch. Microbiol.* **1978**, *117*, 269–276. [CrossRef]
51. Basso, L.C.; Alves, D.M.G.; Amorim, H.V. The Antibacterial Action of Succinic Acid Formation by Yeast during Fermentation. *Rev. Microbiol.* **1997**, *28*, 77–82.
52. Bayrock, D.P.; Ingledew, W.M. Inhibition of Yeast by Lactic Acid Bacteria in Continuous Culture: Nutrient Depletion and/or Acid Toxicity? *J. Ind. Microbiol. Biotechnol.* **2004**, *31*, 362–368. [CrossRef] [PubMed]
53. Bayrock, D.P.; Michael Ingledew, W. Application of Multistage Continuous Fermentation for Production of Fuel Alcohol by Very-High-Gravity Fermentation Technology. *J. Ind. Microbiol. Biotechnol.* **2001**, *27*, 87–93. [CrossRef]
54. Song, H.; Lee, S.Y. Production of Succinic Acid by Bacterial Fermentation. *Enzyme Microb. Technol.* **2006**, *39*, 352–361. [CrossRef]
55. Bertolini, L.; Zambonelli, C.; Giudici, P.; Castellari, L. Higher Alcohol Production by Cryotolerant Saccharomyces Strains. *Am. J. Enol. Vitic. J. Enol. Vitic.* **1996**, *47*, 343–345.
56. Casey, E.; Sedlak, M.; Ho, N.W.Y.; Mosier, N.S. Effect of Acetic Acid and PH on the Cofermentation of Glucose and Xylose to Ethanol by a Genetically Engineered Strain of Saccharomyces Cerevisiae. *FEMS Yeast Res.* **2010**, *10*, 385–393. [CrossRef]
57. Jönsson, L.J.; Alriksson, B.; Nilvebrant, N.-O. New Developments in Microwave Histoprocessing. *Am. Biotechnol. Lab.* **2003**, *21*, 40.
58. Jönsson, L.J.; Martín, C. Pretreatment of Lignocellulose: Formation of Inhibitory by-Products and Strategies for Minimizing Their Effects. *Bioresour. Technol.* **2016**, *199*, 103–112. [CrossRef] [PubMed]
59. Palmqvist, E.; Hahn-Hägerdal, B. Fermentation of Lignocellulosic Hydrolysates. II: Inhibitors and Mechanisms of Inhibition. *Bioresour. Technol.* **2000**, *74*, 25–33. [CrossRef]
60. Geng, P.; Zhang, L.; Shi, G.Y. Omics Analysis of Acetic Acid Tolerance in Saccharomyces Cerevisiae. *World J. Microbiol. Biotechnol.* **2017**, *33*, 1–8. [CrossRef] [PubMed]
61. Bellissimi, E.; Van Dijken, J.P.; Pronk, J.T.; Van Maris, A.J.A. Effects of Acetic Acid on the Kinetics of Xylose Fermentation by an Engineered, Xylose-Isomerase-Based Saccharomyces Cerevisiae Strain. *FEMS Yeast Res.* **2009**, *9*, 358–364. [CrossRef] [PubMed]
62. Swinnen, S.; Fernández-Niño, M.; González-Ramos, D.; van Maris, A.J.A.; Nevoigt, E. The Fraction of Cells That Resume Growth after Acetic Acid Addition Is a Strain-Dependent Parameter of Acetic Acid Tolerance in Saccharomyces Cerevisiae. *FEMS Yeast Res.* **2014**, *14*, 642–653. [CrossRef]
63. HENTGES, D.J. Influence of PH on the Inhibitory Activity of Formic and Acetic Acids for Shigella. *J. Bacteriol.* **1967**, *93*, 2029–2030. [CrossRef]
64. Maia, N.J.L.; Corrêa, J.A.F.; Rigotti, R.T.; da Silva Junior, A.A.; Luciano, F.B. Combination of Natural Antimicrobials for Contamination Control in Ethanol Production. *World J. Microbiol. Biotechnol.* **2019**, *35*, 158. [CrossRef]

65. Hoff, R.B.; Molognoni, L.; Deolindo, C.T.P.; de Oliveira, T.; Mattos, J.L.S.; de Oliveira, L.V.A.; Daguer, H.; Mattos, J.L.S. Residues of Antibiotics in Yeasts from Ethanol Production: A Possible Contamination Route for Feedingstuffs. *J. Environ. Sci. Health Part B* **2021**, *56*, 307–312. [CrossRef]
66. World Bank. *Drug-Resistant Infections: A Threat to Our Economic Future*; World Bank: Washington, DC, USA, 2017.