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Fermentation of Dairy-Relevant Sugars by *Saccharomyces*, *Kluyveromyces*, and *Brettanomyces*: An Exploratory Study with Implications for the Utilization of Acid Whey, Part II

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Abstract: In Greek-style yogurt production, every kilogram of product yields 2 to 3 kg of acid whey (YAW); this coproduct's composition and low pH pose challenges for its proper valorization and reinsertion into the food supply chain. However, 240 mL of YAW contains over 9 g of lactose and represents a good source of minerals; these traits can be leveraged to develop nutritious fermented beverages. The purpose of this study is to investigate the aerobic fermentation of dairy sugars by different yeasts by characterizing these processes and their products. This will determine whether such methods provide viable options for the production of acetic-acid-containing beverages from YAW. To achieve this, yeast nitrogen base was used to prepare four growth media formulations, each supplemented with lactose, glucose, galactose, or a 1:1 mix of glucose and galactose (GLU:GAL), and each adjusted to a pH of 4.20. Fermentations were performed by pure cultures of *S. cerevisiae*, *K. marxianus*, *B. claussenii*, or *B. bruxellensis*, and were held at 25 °C with agitation at 185 rpm. For each treatment, density, pH, and microbial enumeration were measured over time to obtain process profiles, while ethanol, organic acids, and sugars were analyzed at the beginning and the end of each fermentation via HPLC, to determine resulting products. ANOVA and Tukey's honest significant difference test at a significance level of 0.05 were used to compare residual sugars and fermentation products. Variable rates of sugar consumption were observed for each species. In GLU:GAL, *B. claussenii* consumed all of the glucose, left behind most of the galactose, and produced a high concentration of acetic acid. These results suggest the potential to develop versatile processes that target glucose for acetic acid production, while leaving available galactose to confer products with prebiotic properties. The development of processes for the conversion of YAW into beverages with organic acids and other healthful components not only aligns with consumers' demands for better-for-you products, but also promotes the valorization of this otherwise underutilized dairy coproduct.

Keywords: acid whey valorization; acetic acid beverage

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1. Introduction

During the production of Greek-style yogurt, every kilogram of product yields 2 to 3 kg of acid whey (YAW) [1]. In the United States, Greek-style yogurt accounted for approximately 44% of yogurt sales in 2020 [2], translating into 1.8–2.7 billion kg of YAW [3]. For this reason, multiple strategies for the industrial utilization of YAW have been investigated over the years [1,4–7]. The present study focuses on strategies to take advantage of this dairy coproduct's nutritional characteristics by revalorizing it into novel beverages through its fermentation by yeasts. While in Part I of this project, Rivera Flores et al. [8] assessed the anaerobic fermentation of the sugars present in YAW by multiple yeasts, Part II, presented here, focuses on comparable fermentation processes under oxic conditions.

It has been reported that 240 mL of YAW contains over 9 g of lactose and represents a good source of various minerals considered essential for human nutrition [9,10]; these

attributes can be leveraged to develop nutritious beverages. Depending on its origin, YAW can contain approximately 30%, 24%, and 9% of the respective recommended daily intakes for calcium, phosphorus, and potassium per 240 mL [9,11]. Its vitamin profile includes pantothenic acid (0.36 mg/100 g), important in the production of hemoglobin; riboflavin (0.09 mg/100 g), beneficial for blood and brain health; and thiamine (0.08 mg/100 g), critical for nerve function [9,11]. Ferruzzi et al. [12] highlighted the role of beverages in the Dietary Guidelines for Americans; they emphasized the importance of dairy nutrients in the human diet, and the need for nutrient-dense, low-in-sugar beverage alternatives. Thus, developing beverages that take advantage of the natural nutrient profile of YAW could not only add value to this coproduct, but it could also benefit such beverages' potential consumers. These potential benefits become more attractive when combined with the increased sustainability of food systems, and the overall reduction in environmental impact that would result from redirecting YAW back into the food supply.

Functional beverages are rising in popularity within the beverage market, as consumers aim to stay healthy while keeping up with their hectic lives [13]. Moreover, nowadays, more consumers recognize fermented drinks as being functional [14], and are interested in low-alcohol products and products high in acetic acid such as kefir and kombucha.

Acetic-acid-containing beverages are good examples of functional products. They offer multiple benefits to consumers beyond satisfaction or nutrition [14]; acetic acid can decrease hepatic glucose production, increase insulin secretion, and promote weight reduction [15]. These recognized benefits have resulted in an increased interest in acetic-acid-containing beverages, and in the potential substrates and microbes that can be used to develop such drinks.

Researchers such as Marcus et al. [16] and Luo et al. [17] have studied various organisms that could be employed to metabolize the sugars present in dairy coproducts, with the greater aim of the development of unique fermented beverages. Their findings identified yeasts of the *Brettanomyces* and *Kluyveromyces* genera as potential candidates for this endeavor, due to the ability of some species within these genera to metabolize lactose and produce acetic acid and ethanol. In part I of this study, we discussed the rationale behind selecting *Brettanomyces claussenii*, *Kluyveromyces marxianus*, and *Saccharomyces cerevisiae* for our investigations [8]. In Part II, we also considered *Brettanomyces bruxellensis* and its potential for high acetic acid production, as demonstrated by multiple authors [7,18,19]. For instance, Freer [18] reported the production of 0.43 g acetate/g glucose by a strain of m in a basal medium with an initial concentration of 100 g glucose per liter; this level of production of acetate accounts for approximately 64% of the theoretical maximum, and highlights *B. bruxellensis* as a potentially valuable point of comparison with the other species investigated in the current study, with regard to acetic acid production.

The present work aims to assess the processes and products that result when different biotechnologically attractive yeasts metabolize dairy-relevant sugars in the presence of oxygen. Assessment will be conducted by means of the investigation of pertinent process parameters in order to ultimately explore the potential for manufacturing acetic-acid-containing beverages from YAW. A nonselective medium will be used for these investigations, as, although findings will inform the use of YAW as the ultimate intended substrate, YAW's more complex matrix may interfere with the clear observation of the yeasts' habits of sugar utilization [8]. Considerations such as density, pH, and microbial concentration profiles, as well as sugar consumption and produced compounds are discussed in order to characterize potential processes that could assist in taking advantage of YAW.

Part I of this study [1] explored anaerobic fermentation processes to propose alternatives for ethanol production and the development of alcohol-containing beverages from YAW. The present observations are intended to complement that set of conclusions, and further expand the body of knowledge regarding potential for manufacturing fermented beverages. With these two publications, we hope to provide valuable insights for producers of dairy, food, and beverages; to promote the application of fermentation as a novel means

to revalorize YAW; and to propose biotechnological processes that food manufacturers can also consider for the reutilization of other dairy coproducts.

2. Materials and Methods

2.1. Materials

Yeast nitrogen base with amino acids, D-glucose, D-galactose, D-lactose monohydrate, lactic acid (90%), and chloramphenicol were all acquired from Sigma-Aldrich (St. Louis, MO, USA). The commercial yeasts *B. claussenii* and *B. bruxellensis* (products OYL-201 and OYL-202, respectively) were each purchased from Omega Yeast Labs (Chicago, IL, USA) in the form of a slurry; these were subcultured and cryopreserved at −80 °C. Dehydrated *S. cerevisiae* (IOC BE FRUITS) was purchased from Lallemand Oenology (Edwardstown, South Australia), and *K. marxianus* (FSL B9-0008) was obtained from the Food Safety Laboratory at Cornell University; the latter organism had been isolated from a dairy sample [20] and had been cryopreserved at −80 °C.

2.2. Fermentation Media

Yeast nitrogen base with amino acids was used to obtain a base medium in which the types and concentrations of sugars available for fermentation could be individualized for different treatments. The dehydrated medium was reconstituted to a concentration of 1× using water that had been filtered through a Milli-Q Advantage A10 system (MilliporeSigma, Burlington, MA, USA), and 40 g/L of either glucose (GLU), galactose (GAL), lactose (LAC), or a 1:1 mixture of glucose and galactose (GLU:GAL) was used to obtain four different media formulations, each with a different source of fermentable carbon. These distinct carbon sources were aimed at representing all the possible types of fermentable sugars present in YAW at the total sugar concentration typically observed in this coproduct [8]. These preparations were adjusted to a pH of 4.2000 ± 0.0050 with a solution of lactic acid (22.5% *v*/*v*), using an ICinac analyzer (AMS Alliance, Rome, Italy); the final concentration of acid per treatment after this adjustment was approximately 0.10 g/L. Lastly, these media were filter-sterilized using 0.45 µm polyethersulfone vacuum membrane filters (VWR International, Radnor, PA, USA), and were stored at 4 °C until use. Each biological replicate was performed in freshly prepared media.

2.3. Yeast Cultures

Resuscitation of frozen isolates was performed by streaking the cultures onto potato dextrose agar (Hardy Diagnostics, Santa Maria, CA, USA) supplemented with chloramphenicol at a concentration of 25 mg/L. Isolates were incubated at 30 °C for 2 days in the case of *K. marxianus*, and 6 days for both *B. claussenii* and *B. bruxellensis*. Single colonies of each isolate were then subcultured in test tubes each containing 5 mL of a sterile 12% (*w*/*v*) suspension of commercial dry malt extract (Briess Malt and Ingredients Company, Chilton, WI, USA). They were incubated for 48 h at 30 °C with constant agitation (200 rpm). A subsequent propagation step was then performed, in which these cultures were each transferred to an individual flask containing 50 mL of additional sterile dry malt extract medium. They were then incubated at the same temperature and agitation until reaching a cell concentration greater than 3.2×10^8 cfu/mL (approximately 2 days for all species). Cell concentrations were monitored daily by counting 1/20 dilutions of each culture using a hemocytometer and a microscope. These dilutions were prepared by first combining each culture with 1× phosphate buffered saline for a 1/10 dilution, and then combining that dilution with a 0.1% (*v*/*v*) methylene blue solution (Ward's Natural Science, Rochester, NY, USA), at a 1:1 ratio; the methylene blue also served for assessment of cell viability [21]. New cultures were propagated for each biological replicate. Preparation of the *S. cerevisiae* culture was conducted according to the manufacturer's recommendations, as explained in detail below.

2.4. Fermentation Setup

Each yeast species investigated was paired with each type of medium as a unique treatment, for a total of 16 fermentations. Each vessel received a volume of the appropriate culture, the concentration of which allowed for an inoculation level of 4×10^6 cfu/mL. To prepare the tubes that would serve as the inocula, two processes were followed. The yeast cells in the cultures grown in dry malt extract suspension—i.e., *B. bruxellensis*, *B. claussenii*, and *K. marxianus*—were separated from this medium by centrifugation at 3220× *g* for 2 min, then cell pellets were resuspended in 10 mL of sterile Milli-Q water. Alternatively, with regard to the *S. cerevisiae* culture, 200 mg of active dried yeast was transferred to each of four inoculation tubes, and each aliquot of culture was rehydrated in sterile Milli-Q water for 30 min prior to the inoculation; the resulting inocula provided the desired cell concentration level for each appropriate flask. Each fermentation was carried out in 500 mL of medium in a 1-L Erlenmeyer flask topped with sterile aluminum foil. The treatments were incubated at 25 °C, with a shaking at 185 rpm, which produced vigorous agitation of the liquid. For each biological replicate of this experiment, the respective fermentation treatments were initially established on a Monday. The durations of the fermentations varied between species, as they were each defined by the time point at which the density measurements became constant (see Section 3.1.1).

2.5. Data Collection

2.5.1. Density, pH, and Microbial Enumeration

As stated, each biological replicate of this experiment was initially set up on a Monday; samples of each fermentate were taken each day Monday through Friday, until each treatment's fermentation ceased, as discussed below. All such samples were measured in duplicate. On each sampling day, a total volume of 11.5 mL was collected from each fermentate for these analyses, and this sample was distributed in the following way: 7 mL for density measurements, 4 mL for pH measurements, and 0.5 mL for microbial enumeration. Density samples were sonicated for 20 min using an ultrasonic bath (VWR, Radnor, PA, USA) in order to remove any gas resulting from the fermentation; they were then analyzed with a DMA 35 density meter (Anton Paar, Graz, Austria) at a temperature of 20 °C. For pH measurements, samples were dispensed into test tubes measuring 16 × 125 mm, and were measured using the ICinac analyzer. For microbial enumeration, the spread plate technique was used: following serial dilutions with 1× phosphate buffered saline solution, samples were plated onto potato dextrose agar that was supplemented with chloramphenicol at a concentration of 25 mg/L. The plates were incubated at 30 °C for 144 h in the case of the *Brettanomyces* species, 24 h in the case of *K. marxianus*, and 48 h in the case of *S. cerevisiae*. After these respective incubation periods, microbial counts, in cfu/mL, were obtained using a Color Q-Count model 530 (Advanced Instruments Inc., Norwood, MA, USA).

2.5.2. Analyses of Sugars, Organic Acids, and Ethanol

With regard to each fermentate, a total of 20 mL was collected at both the beginning and the end of the fermentation to measure the concentrations of sugars (lactose, glucose, and galactose, in g/L); organic acids (lactic and acetic acids, in g/L); and ethanol (% *v*/*v*). These analyses were performed by the Wine Analytical Laboratory (Cornell Agritech, Geneva, NY, USA) according to the same procedure previously described by Rivera Flores et al. [8].

2.6. Statistical Analysis

2.6.1. General Analyses

All statistical analyses were performed using JMP software version 16.0.0 (SAS Institute, Cary, NC, USA), with an established significance level of 0.05. The comparisons of means of samples taken at the same time point were made via Analysis of Variance (ANOVA) and Tuckey's honest significant difference test. The "matched pairs" function was used for dependent data points in time series analyses. Except for the treatments fermented by *K. marxianus*—for which a fourth replicate was executed—all fermentations

were carried out in biological triplicates. Unless stated otherwise, as presented in all graphs and tables, each data point represents the mean among these replicates; error bars represent standard deviations. Microbial concentration measurements were log-transformed and analyzed using a linear scale.

2.6.2. Nonlinear Density Modeling

Nonlinear modeling was carried out as proposed by Rivera Flores et al. [8] using the Fit Curve function in JMP. The data were segregated by species, using the average density as the response, time as the regressor, and carbon source as the group. All density profiles were fitted using a total of four models: logistic models of three and four parameters, and exponential models of two and three parameters. The best fit for each species was selected using the model comparison tool in the Fit Curve platform: for the *Brettanomyces* species, this was a logistic four-parameter model; for *K. marxianus* and *S. cerevisiae*, an exponential three-parameter model. Table S1 in the Supplemental Materials presents the predictive equation for each model, and Table S2 presents the estimated parameters for these equations, as regards each treatment.

After all of the density curves corresponding to the fermentations of an individual species were successfully fitted, the growth rate parameters of these curves were compared across treatments via analysis of means; this methodology was made available by the compare parameter estimates function. The upper and lower decision limits of this analysis were computed using a significance level of 0.05, and they served to identify significant differences between treatments fermented by the same species.

3. Results and Discussion

3.1. Fermentation Characterization

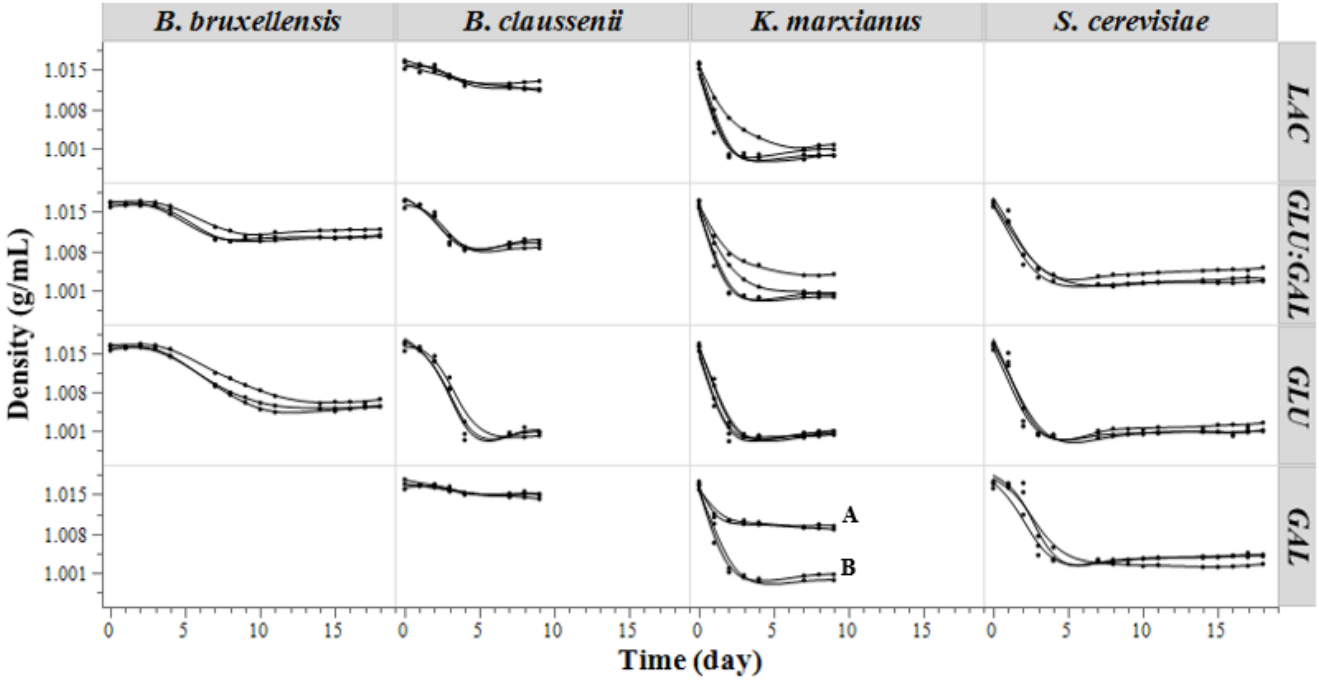
3.1.1. Density

The density of each treatment was measured on a regular basis throughout the course of the fermentations, in order to acquire real-time information regarding the fermentation pattern of each species in each medium, specifically its rate of sugar consumption. Figure 1 presents the densities measured during the aerobic fermentations of the 4 YNB media modified to provide sugar profiles representative of the respective sugars associated with YAW. The table below the graph provides the duration, in days, of each fermentation, defined applying the method proposed by Rivera Flores et al. [8] and using the density measurements of 3 consecutive sampling days.

The treatments excluded from Figure 1 (*B. Bruxellensis*/LAC; *B. bruxellensis*/GAL; *S. cerevisiae*/LAC) consistently showed signs of the growth of contaminant organisms, and were therefore disregarded in the current analysis. Incubation under oxic conditions was challenging for these treatments due to the continuous presence of simple sugars in the media, in the absence of any predominant organism able to consume them. While the species *B. bruxellensis* exhibits a strain-dependent ability to metabolize lactose and galactose [7], the strain included in this study cannot consume either sugar [16,17]; it is also established that *S. cerevisiae* cannot metabolize lactose [22]. These factors, consequently, provided favorable conditions for other organisms to grow.

Overall, only half of the treatments had final densities at levels similar to those observed for otherwise similar treatments held under anoxic conditions during our previous study [8], in which the maximum density decrease was approximately 0.016 g/mL for all treatments except for *S. cerevisiae* in LAC and *B. claussenii* in GLU:GAL. In the current study, of the profiles that did not achieve this maximum density decrease, some fermentations became stalled, such as those of *B. bruxellensis* and *B. claussenii* in GLU:GAL; some were stagnant, as seen with *B. claussenii* in GAL; and one treatment, *K. marxianus* in GAL, exhibited a pronounced divergence in density between biological replicates. Fermentations carried out by *B. bruxellensis* in both GLU:GAL and GLU resulted in substantially lesser decreases in density, as compared to those observed with other cultures. In GLU:GAL, the density dropped 0.005 g/mL in 9 days, potentially indicating the incomplete consumption

of either glucose or galactose. Fourteen days into GLU fermentation, the overall decrease in density was exactly twice as much as that seen in GLU:GAL, suggesting galactose remained unconsumed. Nevertheless, the change in density over time associated with *B. bruxellensis* in GLU was slower than the more rapid consumption by other species. Thus, under the conditions studied, *B. bruxellensis* seemed to metabolize glucose much more slowly than did the other organisms that were investigated. It has been reported that this species generally requires longer fermentation times, thus being better suited for inclusion in sequential multi-species fermentations [23,24].



Lactose	-	Lactose	9	Lactose	4	Lactose	-
Glucose + Galactose	9	Glucose + Galactose	7	Glucose + Galactose	4	Glucose + Galactose	8
Glucose	14	Glucose	8	Glucose	3	Glucose	4
Galactose	-	Galactose	8	Galactose (A), (B)	4, 3	Galactose	7

Figure 1. Density measurements of fermentates containing sugars associated with acid whey; fermentates were held at 25 °C and subjected to shaking at 185 rpm. Investigated sugars, supplemented at an initial concentration of 40 g/L: glucose (GLU); galactose (GAL); 1:1 ratio of glucose and galactose (GLU:GAL); and lactose (LAC). Three biological replicates are presented for each treatment, with the exception of those involving *K. marxianus*, for which a fourth biological replicate was executed and presented. Below the graph is a table which indicates the number of days necessary for each fermentation to reach a stable density, which has been defined as the end of the fermentation. Three treatments (*B. bruxellensis*/LAC; *B. bruxellensis*/GAL; *S. cerevisiae*/LAC) have been excluded here and in all subsequent figures and tables, as those treatments consistently showed signs of contamination. The results of *K. marxianus* in GAL have been subcategorized into treatments GAL(A) and GAL(B), based on the different trends observed in this substrate.

B. claussenii generated distinct density profiles for the various carbon sources present in the media. In GLU, it reduced the density to its nadir by day 8; in both LAC and GAL it caused a very minimal decrease; and in GLU:GAL, it caused a more substantial but still incomplete reduction in density. In the last treatment, this species produced a density curve similar to the one observed in Part I of this study, in which the total density decrease was approximately half of that seen in GLU [8]. In that paper, the authors determined that the unique density curve for *B. claussenii* in GLU:GAL resulted from the incomplete consumption of that treatment's sugars; specifically, almost all of the galactose remained

unconsumed at the end of the fermentation. Refer to Section 3.2 for the initial and final sugar concentrations of the various treatments.

In general, treatments fermented by *K. marxianus* exhibited rapid and complete decreases in density, each taking no longer than 4 days to display the maximum expected decrease. However, in GAL, among the density trends of the initial three biological replicates performed, one curve diverged significantly from the other two (these trends are presented in Figure 1 as curves A and B, respectively). For that reason, a fourth replicate was executed to determine if these dissimilar results could be reproduced; this fourth replicate generated a density curve that followed the same pattern as that of trend A. In spite of these results being unanticipated, they do correlate with the concentrations of residual sugars observed in both sets of replicates; at the ends of these fermentations, GAL(A) retained a mean concentration of 19 g/L of residual galactose, while the GAL(B) replicates showed no evidence of residual galactose (see Section 3.2).

Finally, *S. cerevisiae* brought about a rapid drop in density in all of its treatments, although the overall time taken for the various density curves of the four media to reach their respective lowest points and stabilize was more than that observed under anoxic conditions (8 vs. 6 days for GLU:GAL, 4 vs. 3 for GLU, and 7 vs. 4 for GAL, comparing aerobic and anaerobic growth conditions, respectively) [8].

Nonlinear Density Modeling

Density curves were also analyzed using a nonlinear modeling approach, which served as a more formal methodology to determine any differences between individual fermentation rates. For each species, all density curves were fitted using the same model, thus allowing for comparisons of the equation parameters of the various investigated carbon sources, as fermented by the same organism.

Based on our data, the experimental curves pertaining to the treatments fermented by the *Brettanomyces* species were best fitted with a logistic model with four parameters: lower asymptote (a), upper asymptote (b), growth rate (c), and inflection point (d). On the other hand, the fermentations of *K. marxianus* and *S. cerevisiae* were best modeled by an exponential equation with three parameters: asymptote (a), scale (b), and growth rate (c). Each of these equation parameters estimates a specific fermentation characteristic described previously by Rivera Flores et al. [8].

The indicated asymptotes represent the delimiting density values either at the beginning (highest density, upper asymptote) or at the end (lowest density, lower asymptote) of each fermentation, as expressed in g/mL. The growth rate represents the change in density units per unit of time (g mL^{-1} day^{-1})—an estimation of the fermentation speed during the phase of active fermentation. The inflection point represents the moment at which each fermentation reached its maximum speed and after which its activity gradually decreased, and is expressed in days. Details concerning these equations can be found in Tables S1 and S2, as well as in Figure S1, all in the Supplementary Materials.

The analysis of means for the growth rates of the density curves associated with different carbon sources fermented by the same species revealed no significant differences for both species of *Brettanomyces*, nor for *K. marxianus* (Figure S2). Statistically speaking, the only significantly superior growth rate estimate was that of *S. cerevisiae* in GLU. In this treatment, glucose was consumed significantly faster than were the other investigated carbon sources in their respective media; moreover, GLU also exhibited a lower "a" value estimate (lower asymptote), indicating a larger density decrease in overall less time.

Parts I and II of the present study propose a methodology for the real-time assessment of the rate of reduction in density for each of multiple fermentations; these density measurements are used as a surrogate for the consumption of sugars over time. For the current study, the realization of this methodology was slightly hindered due to staffing limitations, which necessitated gaps in the sampling schedule that resulted in the omission of data points that may have brought greater clarity to our analyses. Thus, more frequent and consistent sampling, as well as a larger number of replicates, would help to minimize

the standard errors of the equations' estimates, and improve the descriptive power of each model. To the best of our knowledge, no other study has compared the estimates of nonlinear model parameters of density curves using analysis of means.

3.1.2. pH

The pH measurements obtained during the investigated yeasts' aerobic fermentations of dairy-relevant sugars under conditions similar to those of YAW are shown in Figure 2. In general, an exponential decrease in the initial pH (4.2) was observed, followed by stabilization near pH 2.4. This general phenomenon was observed, with some slight level of variation, for each species, and it held true even for treatments that failed to undergo a complete decrease in density, except in the cases of *B. claussenii* in LAC and GAL, respectively, in which final pH values above 2.6 were observed.

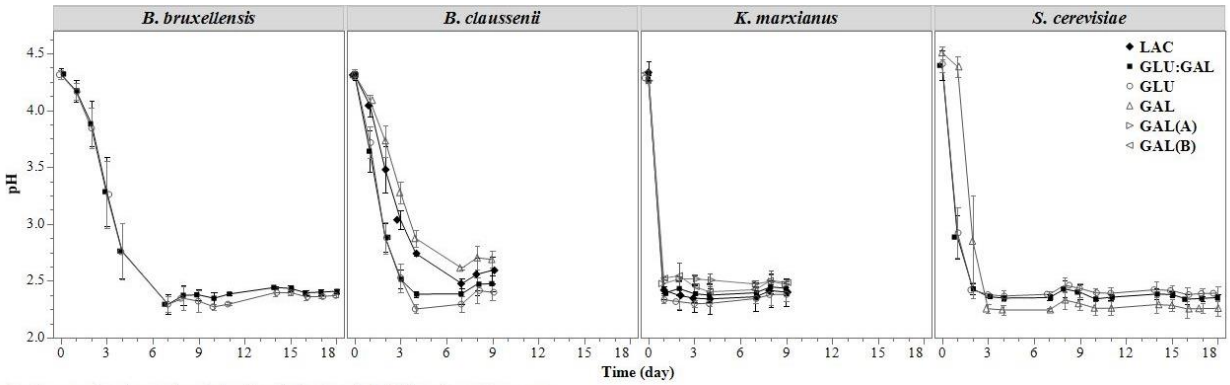


Figure 2. pH measurements of fermentates containing sugars associated with acid whey; fermentates were held at 25 °C and subjected to shaking at 185 rpm. Investigated sugars, supplemented at an initial concentration of 40 g/L: glucose (GLU); galactose (GAL); 1:1 ratio of glucose and galactose (GLU:GAL); and lactose (LAC). Error bars represent the standard deviations of three biological replicates, with the exception of *K. marxianus*, for which a fourth replicate was included. The results of *K. marxianus* in GAL have been subcategorized into treatments GAL(A) and GAL(B), based on the trends observed in this treatment's density results.

It is known that the production of organic acids during aerobic metabolism by yeasts leads to a reduction of the extracellular pH. Although the current study was primarily concerned with monitoring the concentrations of lactic acid and acetic acid, additional organic acids are produced during yeast metabolism, and those acids appeared to play a role in the changes in pH observed here [25]. Such is the case for the *K. marxianus* treatments, in which very low levels of both lactic and acetic acid were observed at the end of each respective fermentation (see Section 3.3.2); nevertheless, these fermentates' pH values did not significantly differ from those seen in the media fermented by other species. Lukondeh et al. [26] reported the presence of acids such as pyruvic, malic, acetic, citric, propionic, and fumaric acids in lactose media that had been batch cultured with *K. marxianus* FII 510700.

3.1.3. Microbial Concentration

Changes in microbial concentration during the fermentation process are presented in Figure 3. The maximum cell concentration achieved by each yeast was approximately 8 log cfu/mL, which represented an increase of 2 log cfu/mL compared to the concentration at the time of each fermentate's initial inoculation. The maximum concentration was seen toward the end of the exponential segment of each density curve.

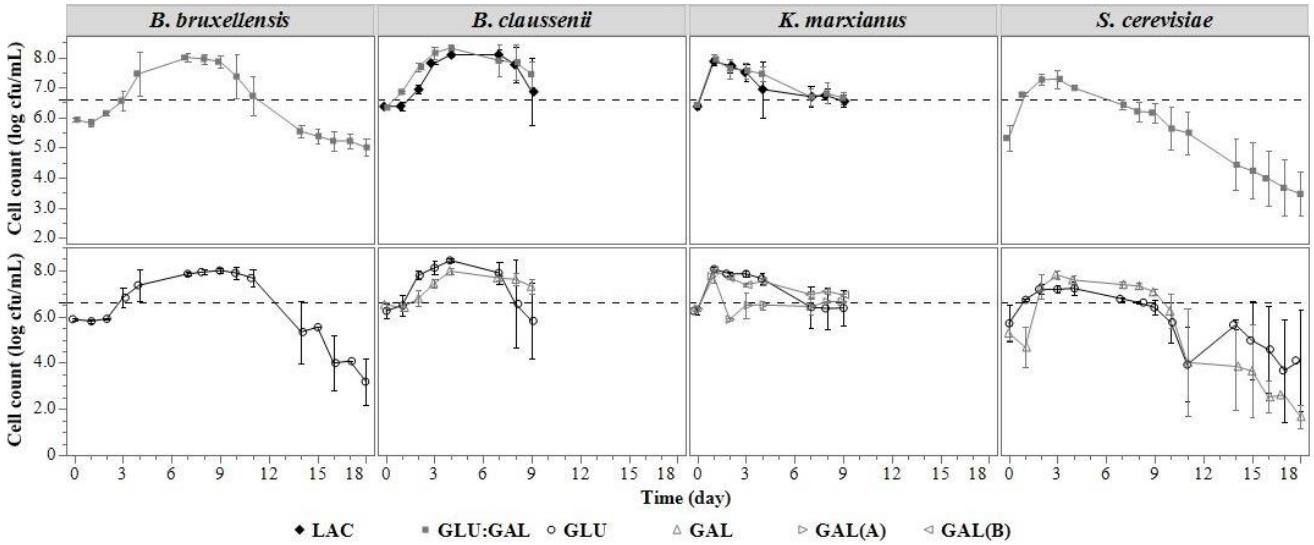


Figure 3. Cell concentrations of fermentates containing sugars associated with acid whey, as obtained by plating on potato dextrose agar; fermentates were held at 25 °C and subjected to shaking at 185 rpm. Top: lactose (LAC); 1:1 ratio of glucose and galactose (GLU:GAL). Bottom: glucose (GLU); galactose (GAL). Dashed lines indicate the intended cell concentration of each fermentate at the moment of inoculation. Error bars represent the standard deviations of three biological replicates, with the exception of *K. marxianus*, for which a fourth replicate was included. The results of *K. marxianus* in GAL have been subcategorized into treatments GAL(A) and GAL(B), based on the trends observed in this treatment's density results.

It is worth noting that the beginning of the stationary phase of growth for each of the *Brettanomyces* sp. exhibited a close relation to the inflexion point of its density curve. The estimates for the timing of these inflection points were days 4 and 7 for *B. bruxellensis* in GLU:GAL and GLU, respectively, and around day 3 for *B. claussenii* in all treatments (Table S2). The ability to draw comparisons between the points at which the *Brettanomyces* cell concentration curves plateaued and the inflection points of their density curves was made possible due to the logistic model fitted to this genus's density data; such comparisons were not possible for *Kluyveromyces* and *Saccharomyces*, each of which was fitted with an exponential model that did not include inflection point as a parameter. Despite this shortcoming of the exponential model, it is logical to speculate that for all investigated species, the arrest of cell replication caused the fermentation rate to start declining.

Microbial counts for *K. marxianus* exhibited similar trends for all media, except for GAL(A), in which a sudden and considerable decrease in cell concentration was seen on day 2. This decrease caused a difference between the counts of the two GAL trends that was statistically significant between days 2 and 4 ($p < 0.003$), and it may explain the lower sugar uptake in GAL(A). Interestingly, the cell concentrations in all other media fermented by *K. marxianus* remained higher than or equal to those at the time of the initial inoculation, remaining so even several days after the end of the fermentations, a phenomenon which was not seen with the other yeasts of the present study. A comprehensive study needs to be conducted regarding the survival of yeasts under the conditions inherent to YAW, to further assess the feasibility of the utilization of these organisms in order to achieve biotechnological conversions of this coproduct.

3.2. Sugar Concentration

While density profiles were used in this study to characterize the fermentation in real time, sugar analysis was used to reveal the actual consumption of the fermentable carbon sources under investigation. The respective density curves of the various treatments, representing a broad diversity of fermentation profiles, demonstrated a close relationship to the concentrations of sugars left unconsumed by each species at the end of this process; data

for sugar concentrations at the beginning and end of each fermentation are presented in Table 1. For *K. marxianus* in GAL, the two observed trends are presented as separate results.

Table 1. Initial and final concentrations of fermentable carbon sources in fermentates containing sugars associated with acid whey; fermentates were held at 25 °C and subjected to shaking at 185 rpm. Means and standard deviations are provided, representing biological triplicates, with the exception of values associated with *K. marxianus*, for which a fourth replicate was included in the experiment.

		Supplemented Medium				
		Lactose *	**Glucose + Galactose**		**Glucose**	**Galactose**
Species	**Timepoint**	**Lactose (g/L)**	**Glucose (g/L)**	**Galactose (g/L)**	**Glucose (g/L)**	**Galactose (g/L)**
B. bruxellensis	Day 0	-	18.96 ± 0.13	18.77 ± 0.22	38.16 ± 0.54	-
	Day 18	-	ND	18.94 ± 0.61	7.26 ± 0.50	-
B. claussenii	Day 0	34.84 ± 1.20	18.74 ± 0.56	18.57 ± 0.76	38.08 ± 0.87	37.21 ± 0.42
	Day 9	19.38 ± 4.18	ND	14.79 ± 0.60	ND	28.26 ± 1.60
K. marxianus	Day 0	32.76 ± 3.22	18.94 ± 0.14	18.73 ± 0.69	38.04 ± 0.92	36.94 ± 0.81
	Day 9	0.43 ± 0.75	ND	0.6 ± 1.04	ND	A: 18.61 ± 0.93 B: ND
S. cerevisiae	Day 0	-	18.55 ± 0.57	18.46 ± 0.69	37.6 ± 1.27	35.89 ± 1.74
	Day 18	-	ND	3.24 ± 2.21	ND	2.09 ± 0.86

* Any glucose or galactose that could have resulted from this treatment was not detected in any sample. ND: nondetectable. Limits of detection, in g/L: lactose 0.003, glucose 0.004, and galactose 0.005.

In GLU:GAL, *B. bruxellensis* consumed all of the glucose but did not engage in galactose uptake, a result which is in agreement with those of previous studies involving this strain [16,17]; however, residual glucose was seen at the end of this organism's fermentation of GLU. Incomplete consumption of glucose by this organism under oxic conditions has been observed before. Aguilar Uscanga et al. [27] reported observing this phenomenon with strain IHEM n. 6037 in minimal medium at pH 4.0, and attributed it to an inhibitory role played by acetic acid under oxic conditions. These researchers observed that 7% of the glucose initially present in the medium was left untouched in finished fermentates in which the acetic acid level had reached 7 g/L [27]. Other researchers have reported that acetic acid inhibits glucose consumption in various yeast species under both oxic and anoxic conditions [28–30]; that inhibition was seen at a cellular level, at which the undissociated form of the acid caused changes in the intracellular pH [28].

In the treatments fermented by *B. claussenii*, glucose was the only sugar that was consumed in its entirety (both in GLU and GLU:GAL), while the treatments containing either lactose or galactose exhibited residual levels of their respective sugars even after their densities had stabilized. Residual lactose in LAC (19.4 g/L) was an unanticipated finding, given that Lawton [31] reported full lactose consumption by the same strain of this organism in a buffered medium (pH 4.4) under similar fermentation conditions. This incongruity suggests that the considerably lower extracellular pH seen in *B. claussenii*'s LAC treatment in this study (pH 2.5) may have been a contributing factor in a premature arrest of this fermentation. Further, for industrial use of this strain, medium buffering may be required for complete sugar utilization; if *B. claussenii* is utilized for applications involving YAW, such buffering may not be necessary, as seen by Luo et al. [17].

Galactose utilization in *B. claussenii*'s GAL treatment was also notably low (<25%). The density curve representing this treatment suggests that in this fermentate, galactose uptake was almost entirely confined to the first 4 days of the fermentation, after which the concentration of this sugar remained stable. In GLU:GAL, the consumption of galactose was minimal, even after the point at which the medium's glucose had been effectively depleted. These results are comparable to those observed under anoxic conditions in Part I of the present study [8] and suggest that the aerobic fermentation of YAW by *B. claussenii* is another option for developing beverages with residual galactose and its prebiotic properties [32].

If this trend continues to be observed in other dairy streams, such as sweet whey and permeates, multiple novel fermented beverages could be developed, each of which could provide attractive opportunities for the valorization of these coproducts.

Analyses of the different media fermented by *S. cerevisiae* revealed that this organism consumed all of the glucose in its entirety in all treatments that contained it, and left behind only low concentrations of galactose in the treatments that contained that sugar; this is within the range of normal behavior for this species.

Regarding *K. marxianus*, our results show the full consumption of sugars in all treatments except for GAL, for which divergent results were obtained. The final galactose concentration in GAL(A) (18.6 g/L) was approximately half of its initial level, while GAL(B) contained no residual sugar at the end of its fermentation. As brought up before, these results correlate highly with their respective density curves, which depict a drop in density in GAL(A) which is less than half of that seen in GAL(B).

On the heterogeneity of *K. marxianus*. No indication of the presence of genotypic subpopulations of *K. marxianus* FSL B9-0008 was found with regard to the isolate used in the present study. We investigated this possibility by streaking this isolate out on rich medium and then generating replica plates on the following media: yeast extract peptone dextrose (used as a control rich medium); yeast extract peptone galactose (to identify colonies' differential growth when dextrose is replaced with galactose as the sole fermentable carbon source); yeast extract peptone glycerol ethanol (to identify colonies' differences in respiratory growth); and synthetic dextrose and synthetic galactose (to identify colonies that represent auxotrophs). All colonies grew similarly on all of these media. Based on this observation, it appears that the data collected for GAL(A) were the result of an instance of nongenetic heterogeneity—a phenomenon in which genetically identical cells exposed to the same environmental conditions exhibit differences in their behavior and cellular structures [33]. A documented example of such a phenomenon is the inheritance of a prion state in cells of *S. cerevisiae*; this prion state causes changes in the cell's phenotype in the absence of changes to the nucleotide sequence of any of its nuclear genes [34]. These prions can be considered epigenetic determinants that modify cellular processes as a result of changes in the environment, without the introduction of changes in the sequence and the function of the genome [34]. For example, the [GAR+] prion state converts *S. cerevisiae* from metabolic specialists into generalists able to consume alternate carbon sources in the presence of glucose [35]. In another example of nongenetic heterogeneity, Dawson et al. [36] have discussed bacterial and fungal subpopulations capable of surviving antimicrobial treatments; it has been determined that these subpopulations, labeled "persister cells," are phenotypic variants that have originated from genetically identical cells [37,38]. While the mechanisms involved in this phenomenon are still under investigation, in a documented instance with *S. cerevisiae*, the nutrient environment was found to influence the development of a persister-like state that affects regulation of metabolism and glucose sensing/signaling [39,40].

3.3. Production of Ethanol and Organic Acids

3.3.1. Ethanol

Table 2 presents final ethanol concentrations generated by the investigated yeasts during their respective fermentations of four different media, all adjusted to the pH of YAW and containing dairy-relevant sugars. No ethanol was detected in any treatment on day 0. Although it was beyond the scope of this study to provide a deep formal analysis of differences in ethanol production displayed by different species within the same medium, lowercase superscripts have been provided to indicate instances of significant difference. The maximum yields of ethanol, by volume, were seen in treatments fermented by *K. marxianus*, which were approximately 1.50% (*v*/*v*) ethanol for all fermentable carbon sources, except in the case of GAL(A).

Table 2. Final concentrations of ethanol (% *v*/*v*) in fermentates containing sugars associated with acid whey; fermentates were held at 25 °C and subjected to shaking at 185 rpm. Means and standard deviations are provided, representing biological triplicates, with the exception of values associated with *K. marxianus*, for which a fourth replicate was included in the experiment.

		Ethanol (% *v*/*v*)			
Species	**Timepoint**	**Lactose**	**Glucose + Galactose**	**Glucose**	**Galactose**
B. bruxellensis	Day 18	-	0.20 ± 0.02^{Bc}	0.59 ± 0.05^{Ac}	-
B. claussenii	Day 9	0.11 ± 0.08^{Bb}	0.42 ± 0.13^{Bbc}	1.47 ± 0.18^{Aa}	ND
K. marxianus	Day 9	1.48 ± 0.32^{Aa}	1.52 ± 0.19^{Aa}	1.43 ± 0.09^{Aa}	A: 0.600 ± 0.033^{Bb} B: 1.576 ± 0.922^{Aa}
S. cerevisiae	Day 18	-	0.63 ± 0.08^{Ab}	0.95 ± 0.19^{Ab}	0.62 ± 0.35^{Ab}

ND: nondetectable. Limit of detection: 0.009% *v*/*v*. Different superscripts indicate significant differences ($p < 0.05$) in ethanol concentration: uppercase, within each row, across media fermented by the same species; lowercase, within each column, across species that fermented the same medium.

In each *B. bruxellensis* treatment presented, the phenomenon of ethanol production appears to be in conformity with the amount of sugar(s) consumed; i.e., a significantly higher amount of ethanol was produced in GLU, compared to the concentration in GLU:GAL. Aside from a lower level of consumption of glucose in GLU:GAL, the lower ethanol concentration may also be a consequence of the prodigious production of acetic acid by *B. bruxellensis* in this treatment (see Section 3.3.2); the acetic acid may have been synthesized at the expense of ethanol. Freer [18] studied the conversion of both glucose and ethanol into acetic acid by several *Brettanomyces* species; the majority of investigated species could produce acetic acid when ethanol was used as an energy source, with *B. bruxellensis* NRRL Y-17525 yielding acetic acid concentrations as high as 33 g/L.

In a similar way, B. claussenii exhibited more efficient production of ethanol in GLU than it did in GLU:GAL, which was in part balanced by a higher acetic acid yield in the latter treatment. Additionally, very minimal to no ethanol production was seen with this organism in LAC and GAL, as was expected based on the minimal uptake of sugar seen in each of these treatments.

As mentioned before, the treatments fermented by *K. marxianus* had the highest ethanol concentrations at the ends of their respective fermentations, with the exception of GAL(A). Although this species is less associated with ethanol production under oxic conditions as compared to anoxic environments, its ability to synthesize this product in the presence of oxygen has been reported before [41,42]. *K. marxianus* and its effective ethanol production even during aerobic growth served as a reference for the amount of ethanol that could be produced from lactose under the conditions of the present study.

Regarding *S. cerevisiae*, overall ethanol concentrations on day 18 were low in all treatments. This product could have been used as a respiratory carbon source close to the ends of the fermentations, when preferred fermentable sugars were almost completely depleted.

3.3.2. Organic Acids

Table 3 shows the concentrations of lactic and acetic acid resulting from each treatment. Although it was beyond the scope of this study to provide a deep formal analysis of differences in production of acetic acid displayed by different species within the same medium, lowercase superscripts have been provided for this fermentation product to indicate instances of significant difference at timepoints representing the ends of respective treatments' fermentations. Our results suggest that no species produced lactic acid; in fact, both *B. claussenii* and *K. marxianus* seem to have exhausted it completely. Regarding acetic acid, some species produced it at greater concentrations than did others, on average producing it in the following order, from highest to lowest: *B. bruxellensis*, *B. claussenii*, *S. cerevisiae*, and *K. marxianus*.

Table 3. Initial and final concentrations of lactic acid and acetic acid in fermentates containing sugars associated with acid whey; fermentates were held at 25 °C and subjected to shaking at 185 rpm. Means and standard deviations are provided, representing biological triplicates, with the exception of values associated with *K. marxianus*, for which a fourth replicate was included in the experiment.

		Lactic Acid (g/L)			
Species	**Timepoint**	**Lactose**	**Glucose + Galactose**	**Glucose**	**Galactose**
B. bruxellensis	Day 0	-	0.109 ± 0.001	0.108 ± 0.002	-
	Day 18	-	0.076 ± 0.004 ^{A}	0.082 ± 0.006 ^{A}	-
B. claussenii	Day 0	0.103 ± 0.002	0.107 ± 0.002	0.084 ± 0.056	0.109 ± 0.001
	Day 9	ND	ND	ND	0.020 ± 0.035
K. marxianus	Day 0	0.102 ± 0.002	0.106 ± 0.004	0.108 ± 0.001	0.109 ± 0.001
	Day 9	ND	0.018 ± 0.035	ND	A: ND B: ND
S. cerevisiae	Day 0	-	0.104 ± 0.003	0.103 ± 0.009	0.102 ± 0.008
	Day 18	-	0.104 ± 0.011 ^{A}	0.091 ± 0.009 ^{AB}	0.076 ± 0.005 ^{B}
		Acetic Acid (g/L)			
Species	**Timepoint**	**Lactose**	**Glucose + Galactose**	**Glucose**	**Galactose**
B. bruxellensis	Day 0	-	ND	ND	-
	Day 18	-	6.069 ± 0.275 ^{Ba}	7.696 ± 0.275 ^{Aa}	-
B. claussenii	Day 0	ND	ND	ND	ND
	Day 9	4.672 ± 0.500 ^{Aa}	4.575 ± 1.911 ^{Aa}	4.029 ± 0.72 ^{Ab}	2.803 ± 0.421 ^{Aa}
K. marxianus	Day 0	ND	ND	ND	ND
	Day 9	0.021 ± 0.042 ^{Ab}	0.022 ± 0.026 ^{Ab}	0.156 ± 0.271 ^{Ad}	A: 0.062 ± 0.036 ^{Ab} B: 0.023 ± 0.026 ^{Ab}
S. cerevisiae	Day 0	-	ND	ND	ND
	Day 18	-	1.612 ± 0.155 ^{Bb}	1.893 ± 0.309 ^{Bc}	2.586 ± 0.261 ^{Aa}

ND: nondetectable. Limits of detection: 0.091 g/L (lactic acid) and 0.001 g/L (acetic acid). Different superscripts indicate significant differences ($p < 0.05$) in acid concentration: uppercase, within each row, across media fermented by the same species; lowercase, within each column of acetic acid, across species that fermented the same medium.

As mentioned in the previous section, *B. bruxellensis* exhibited favorable acetic acid production. This phenomenon was significantly pronounced in GLU, which had an average total concentration of 7.7 g/L at the end of the fermentation. The efficacy exhibited by this species in terms of acetic acid synthesis has caught the attention of acetic acid manufacturers [7,43]. However, under the conditions studied, it took 14 days for the fermentation of GLU to be completed (Figure 1), suggesting that the productivity of this process is low.

Acetic acid production by *B. claussenii* was also favorable. Although the final concentrations of this acid were lower overall than those seen with *B. bruxellensis*—especially in GLU—the utilization of *B. claussenii* may still prove advantageous in certain industrial processes, such as ones that target the production of a combination of ethanol and acetic acid. Moreover, our results suggest that this organism can complete fermentations in less time (Figure 1), indicating an advantage over its fellow *Brettanomyces* species. On the other hand, it is interesting to highlight that there were no significant differences between the amounts of acetic acid produced by *B. claussenii* in the various media, even in LAC and GAL, in which significantly lower amounts of sugars were consumed; this may indicate a tendency to produce acetic acid from the metabolism of lactose and galactose.

In treatments fermented by *K. marxianus*, exceptionally low final acetic acid concentrations were seen. This is the result of its tendency toward ethanol production. In fact, on average, the order in which the species of this study produced acetic acid was fairly the inverse of that seen for ethanol, placing *K. marxianus* in last place in the former, and in first place in the latter.

Finally, *S. cerevisiae* produced higher concentrations of acetic acid when compared to *K. marxianus*, but not as high as those produced by the *Brettanomyces* species. The only *S. cerevisiae* treatment with a final acetic acid concentration comparable to any of those of the

Brettanomyces species was GAL (~2.6 g/L), which was comparable to that of *B. claussenii* in the same medium (~2.8 g/L); however, *S. cerevisiae* consumed substantially more galactose than *B. claussenii*.

4. Conclusions

The main goal of this study was to assess the aerobic fermentation of dairy-relevant sugars by different yeasts, in order to explore the manufacture of acetic-acid-containing beverages using YAW as an input, as a strategy to add value to this dairy coproduct. Our results show that *B. bruxellensis* produced acetic acid at favorable levels, but exhibited a sluggish fermentation profile. On the other hand, *B. claussenii* proved to metabolize sugars faster, with satisfactory acetic acid production, and a more versatile metabolism that can also be directed toward ethanol production, or toward products containing residual galactose for added prebiotic properties. A time-series analysis focused on acetic acid production by these two species would help to more clearly determine the suitability of either or both for industrial processes aimed at generating acetic acid from dairy coproducts like YAW. Overall, the anaerobic and aerobic fermentations observed in the first and second parts of this study suggest promising applications for the biotechnological conversion of residual sugars in YAW, in order to revalorize this dairy coproduct.

Supplementary Materials: The following are available online at https://doi.org/10.7298/f38g-ge82, Table S1: Prediction models for density curves of aerobic fermentations by different yeasts, in yeast nitrogen base with amino acids supplemented with lactose, glucose, and galactose; Table S2: Estimated parameters of fitted models for density curves of aerobic fermentations by different yeasts; Figure S1: Model plots for density curves of aerobic fermentations by different yeasts, in nonselective medium supplemented with sugars commonly found in acid whey; Figure S2: Analysis of mean (ANOM) of growth rate parameters obtained per carbon source for each yeast species.

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Conflicts of Interest: Sam Alcaine, the head researcher of this investigation, is the cofounder of Norwhey Brewing, a startup company that produces hard seltzers from yogurt whey.

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