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EXPLORING THE MICROBIAL ECOLOGY IN RUM PRODUCTION: QUAN-TITATION, DIVERSITY, AND SIGNIFICANCE

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Abstract. This study aims to characterize the microbial diversity associated with rum production with a focus on understanding the microbial factors contributing to variability in fermentation processes. Samples were systematically collected from critical process points including the molasses storage vessels, yeast propagation tanks, fermenters, water, and dunder across four months at Bundaberg Distilling Company (BDC). Using culture-dependent methodologies, the study quantified total bacterial load, lactic acid bacteria, and yeast populations to characterize microbial succession across the rum production cycle. The microbial populations exhibited variations across different stages and sampling points, highlighting the dynamic nature of microbial communities during rum production. Distinct microbial profiles were observed at different stages, underscoring the dynamic nature of microbial community structure in an industrial fermentation context. Molasses samples exhibited high bacterial diversity, prominently featuring Lac-tobacillus spp., while water and dunder maintained consistently low microbial loads, reflecting their importance in preserving process integrity. Quantitative analyses revealed significant Lactobacillus proliferation during early fermentation, followed by a progressive dominance of Saccharomyces cerevisiae, which reached peak populations of 7.8×10^7 CFU/mL. Multivariate statistical analyses, including principal coordinate analysis (PCoA), Shannon diversity indices, and Bray-Curtis dissimilarity metrics, re-vealed both temporal and spatial separation of microbial communities. A marked decline in alpha diversity (from 1.49 to 0.68) was observed as fermentation progressed, alongside increasing inter-sample dissimilarity, indicating ecological succession toward a less diverse but functionally specialized yeast-dominated community. The findings of this study advance the understanding of mi-crobial population dynamics in rum fermentation and provide a framework for optimizing production processes. Identifying the key microbial players and the succession patterns enables targeted manipulation of the microorganisms to improve batch consistency, fermentation efficiency, and ultimately, product quality.

Keywords: microbial diversity; rum; fermentation; Saccharomyces cerevisiae; Lactobacillus

Introduction. Formulation of the problem

Rum production is influenced by the complex and often underappreciated interplay of microbial communities that drive both the biochemical conversion of sugars into ethanol and the development of sensory attributes [1-4]. A thorough understanding of these microbial interactions is central to improving fermentation efficiency and ensuring consistent product quality. In particular, characterizing the indigenous microbiome across different stages of production can offer critical insights into process optimization [5].

Analysis of recent research and publications

The primary feedstock to rum production, sugarcane molasses, presents a challenging environment for microbial growth due to its high sugar content, low water activity, and moderately low pH [6-8]. Despite these restrictive conditions, molasses harbors a diverse population of microorganisms that play important roles throughout the rum manufacturing process [1, 9]. Native yeasts, bacteria, and fungi interact to carry out industrial rum fermentations [1, 10]. While *Saccharomyces cerevisiae* is intentionally propagated to produce alcohol from molasses-derived sugars via glycolysis, indigenous microbial communities also impact process dynamics and product quality [11]. Various wild yeast strains naturally occur in molasses and may contribute to higher alcohols and esters affecting rum flavour [12]. Lactic acid bacteria metabolize sugars to lactic acid, thereby modifying acidity [13]. Their activities along with other microbial transformations influence both fermentation performance and sensory attributes of the final spirit. Unregulated populations can impair yields or introduce off-flavours [2, 3, 5, 14, 15]. Conversely, selectively culturing certain wild yeast varieties offers possibilities to enhance consistency and tailor distinctive rum properties [1, 14]. Achieving a comprehensive understanding of rum's indigenous microbiota is thus essential to advance process control strategies.

Characterizing microbial community structures and dynamics across industrial operations provides insight needed for targeted microbiological management [16-18]. However, gaps still remain regarding succession patterns, functional roles, and variability impacts. This study aims to address such knowledge deficiencies through a broad survey of rum's industrial-scale microbiome. Tracking populations from the primary raw material (molasses) enables modelling of ecological transformations and elucidates relationships to fermentation performance [19, 20]. The concept of characterization not only broadens horizons but also paves the way for precision nutrient supplementation, finely tuned according to population-nutrient relationships.

Overall, this article aims to uncover the complex microbial ecology underpinning rum production and its influence on fermentation outcomes. To achieve this, the study will first characterize and quantify the microbiota present in raw molasses and at various stages of the production process using cultivation-based methods. It will then investigate how microbial populations shift throughout the course of fermentation, providing insight into dynamic microbial successions. Particular emphasis will be placed on enumerating microbial groups known to influence rum fermentation, such as lactic acid bacteria and wild yeasts. Additionally, the article will explore variations in microbial composition between different fermenters to better understand factors contributing to fermentation variability and product inconsistency. The microbial community composition in rum production is expected to undergo predictable succession patterns shaped by process-specific environmental filters, with *Lactobacillus* spp. emerging as the dominant bacteria and *Saccharomyces cerevisiae* as the prevailing yeast in later stages.

Materials and methods.

Samples were collected from key process points at the Bundaberg Distilling Company (BDC; location coordinates 24.8591°S 152.3671°E) (Figure 1). Timepoint sampling was employed to capture the dynamic microbial populations during the substrate's transitions between various production environments.Enumeration and isolation of microorganisms was performed using culture-based techniques. Nutrient agar was used for the enumeration of total bacteria, while lactic acid bacteria were determined using deMan–Rogosa–Sharpe (MRS) media. Total yeast populations were enumerated on Sabouraud dextrose agar supplemented with oxytetracycline (100 µg/mL) to inhibit bacterial growth. Lysine media was used to differentiate wild and commercial yeast strains based on amino acid auxotrophies. Statistical testing established the significant differences in microbial abundances. Assessing diversity indices and dissimilarities between stages offered ecological context. This comprehensive sampling-analysis strategy systematically profiles the industrial rum fermentation microbiome. Capturing production-associated population fluctuations guides targeted process optimization leveraging microbial attributes for consistent, high-quality rum production.

Chemicals and reagents

Unless otherwise stated, all reagents used in this study were of analytical grade and were purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Molasses and associated production samples were obtained directly from Bundaberg Distilling Company. Aqueous solutions and culture media were prepared using micropure water generated from a Thermo Scientific Barnstead MicroPure water purification system (Scoresby, VIC, Australia). Media was sterilized by autoclaving at 121°C for 15 minutes or membrane filtration with 0.22 µm syringe filters (Millipore, MA, USA) for heat-sensitive components.

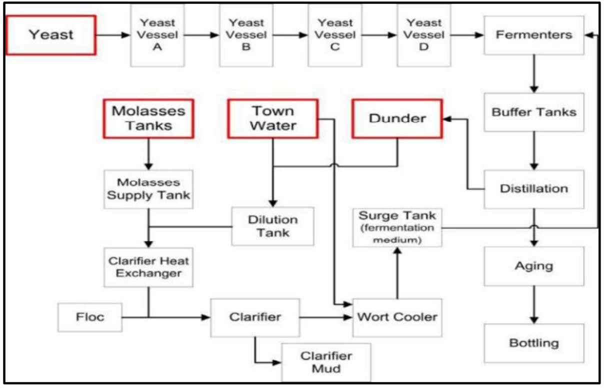

Fig. 1 Rum manufacturing process flow (Adapted from Green, 2015 *[1]*)

Process description and microbiological sampling strategy

The primary raw material in rum production, molasses, is sourced from an adjacent sugar mill (Millaquin Sugar Mill, Bundaberg) and transported to the distillery through pipelines at a high temperature (> 60°C). Upon arrival, it is stored in large concrete wells, serving as storage vessels ensuring a continuous supply for rum production. The molasses remains in these tanks for extended periods, typically up to 10 months or longer, with storage conditions being uncontrolled. The temperature of the molasses fluctuates with the ambient temperature, ranging from 20-40°C depending on the season. When required, molasses is pumped from the storage wells to an intermediary supply tank, then to a clarification vessel, where it is diluted with water and dunder to achieve a Brix value of 45-50°. Dunder is added to the fermentation medium at a final concentration of 7.5% of the volume. A flocculant (Kemira Superfloc C-573) is introduced through a venturi mixer at a ratio of 500 mL per 300 L to facilitate the sedimentation of mud and unwanted particulate matter. The mixture is heated to 70°C and allowed to stand for approximately 1 hour to enable solids sedimentation. Subsequently, the clarified molasses mixture passes through a heat exchanger to cool it to 35°C. The Brix is further reduced to 28 – 30° by dilution with potable water, and the resulting mixture, now known as the “wort”, is transferred to a surge tank. This wort, consisting of molasses, water, and dunder, is utilized for yeast propagation and the main rum fermentation process.

Yeast propagation is conducted to acclimate the yeast strain (*Saccharomyces cerevisiae*) to the rum wort and increase biomass for large-scale fermentation. The propagation process involves sequential inoculations in vessels labelled A to D. Initially, yeast biomass from a yeast slope is used to inoculate 250 mL of sterilised molasses wort (heated at 121°C for 20 minutes at 150 kPa) (28 – 30° Brix), followed by incubation at 30-32°C for 24 hours. This culture is then transferred to a 1600 mL propagation vessel (referred to as a “billy”, a small-scale, intermediate fermentation container used in microbial or yeast propagation) with sterilised molasses wort (28 – 30° Brix) and incubated for an additional 24 hours. The culture from this stage is used in a four-stage propagation process, where it is sequentially transferred to vessels A, B, C, and D. All the contents from the propagation billy are aseptically transferred into yeast vessel A, which contains 200 L of wort, and the mixture is incubated for 12-14 hours at 32°C. The entire content from vessel A is then transferred to vessel B, which contains 400 L of sterilized molasses wort (28 – 30° Brix) and incubated for 2-3 hours at 32°C. Following this, the entire content of vessel B is transferred to vessel C, which holds 3400 L of sterilized molasses wort (28 – 30° Brix). Incubation in vessel C lasts for 1 hour at 30°C, after which half of the volume (1700 L) is transferred to vessel D (total volume 3400 L), and incubated under identical conditions to vessel C. Following an additional hour of incubation in vessel D, the entire volume is transferred to a fermenter. The remaining volume (1700 L) in vessel C will be topped up with fresh sterilized wort to restore the working volume. Depending on production requirements, this may be used for inoculating an additional fermenter.

There are 8 fermenters at Bundaberg Distilling Company, 2 tanks with a 41000 L capacity and 6 tanks with a 79000 L capacity. The fermentation tanks are filled with non-sterilised wort having a Brix value of 28 – 30°. During the filling stage, which lasts up to 6 hours, the wort is mildly agitated as it is distributed from the top by pipes set against the tank walls, creating a vortex-like flow, and providing sufficient aeration for the rapid initiation of fermentation. Fermentation is conducted at 35°C for 36 hours without aeration.

Cleaning and sanitation procedures at the Bundaberg Distilling Company primarily involve clean-in-place (CIP) methods. Physical cleaning techniques, such as scrubbing, are conducted during alcohol free operations (i.e., when production is not running). At these times, the plant is shut down, usually due to the exhaustion of molasses supply. Subsequently, the supply tank, clarifier, and fermenters are empty, and a full plant wide cleaning operation can be conducted. The cleaning-in-place (CIP) procedures are controlled by computers, involve highly pressurized nitric acid, caustic, sanitizers, and water rinses. The acid and caustic clean, occurring at approximately 74-77°C, are interspersed with rinses, resulting in a total cleaning time of around 2 hours and 45 minutes. There are cleaning schedules for various equipment and areas, including daily, weekly, monthly, or 'as required' cleans. The yeast vessels are cleaned after each propagation step, with the associated pipework also sterilized with steam before and after transfer. Each yeast vessel has an isolated CIP circuit, allowing for individual cleaning. Fermenters and associated pipes are cleaned between fermentations, typically every 36-40 hours.

Microbiological sampling was conducted at various points in the rum production process including molasses tanks (storage wells), yeast vessels (propagation tanks), and fermenters. Sampling points have been strategically placed throughout the production process to gather relevant data. The sampling points, along with their respective sample states, sample volumes, and sampling ports, are noted in Table 1. These sampling points have been carefully selected to capture important data at various stages of the rum production process, allowing for analysis and monitoring of key parameters. The sample states, volumes, and sampling ports are specified to ensure accurate and representative samples for analysis. Purposive sampling was employed to comprehensively explore the range of microorganisms involved in rum production by collecting samples from various points and processes within the production process at the Bundaberg Distilling Company.

Molasses samples were collected from all three storage wells and the supply tank, whilst dunder and town water samples were taken at the point of addition/dilution. After diluting the molasses with town water and mixing with dunder the wort was transferred to the surge tank, at which point another set of samples was collected. Using a heat exchanger, the wort is cooled and then directed to either the yeast tanks for yeast propagation or one of the fermenters. Samples were aseptically collected from all four yeast tanks using the available sampling tap to assess the presence of microorganisms.

For the fermenters, samples were taken at 0, 12, 24, and 36 hours after fermentation to capture the changes in microbial composition over time. These additional sampling points allowed for a more detailed analysis of the microbial ecology of the rum production process, providing insights into the different stages of rum production and their impact on the microbial community. Sampling was conducted over a 12-month period during normal distillery operations.

From each sample of molasses, 1 gram was precisely weighed and transferred aseptically into a 15 mL centrifuge tube. A 9 mL volume of sterile Ringer’s solution was added to each tube and vigorously vortex mixed using a Scientific Industries VM-3000 vortex mixer (Biosan, Latvia) set to maximum to facilitate homogenization and subsequent serial dilutions.

3,500 rpm setting for 60 seconds to separate bacterial cells from heavier particulate matter from which the liquid phase was decanted. This created a stock suspension from which serial decimal dilutions could be prepared.

Table 1 – Sampling points used during microbiological analysis.

Sampling point	Sample state	Sample volume	Sampling port
Front molasses well	Viscous liquid	250 mL	Tap
Middle molasses well	Viscous liquid	250 mL	Tap
Far molasses well	Viscous liquid	250 mL	Tap
Molasses supply tank	Viscous liquid	250 mL	Fixed tube
Town water	Liquid	10 mL	Tap
Clarifier	Liquid	10 mL	Tap
Wort surge tank in	Liquid	10 mL	Fixed port
Wort surge tank out	Liquid	10 mL	Fixed port
Post wort cooler	Liquid	10 mL	Fixed port
Yeast propagation billy	Liquid	10 mL	Direct sampling
Yeast propagation tank A	Liquid	10 mL	Fixed port
Yeast propagation tank B	Liquid	10 mL	Fixed port
Yeast propagation tank C	Liquid	10 mL	Fixed port
Yeast propagation tank D	Liquid	10 mL	Fixed port
Fermenter (0 hours)	Liquid	10 mL	Tap
Fermenter (6 hours)	Liquid	10 mL	Tap
Fermenter (12 hours)	Liquid	10 mL	Tap
Fermenter (18 hours)	Liquid	10 mL	Tap
Fermenter (24 hours)	Liquid	10 mL	Tap
Fermenter (36 hours)	Liquid	10 mL	Tap
Dunder	Liquid	10 mL	Tap
Mud	Semi-solid	250 mL	Tap
Caustic tank	Liquid	10 mL	Tap
Acid tank	Liquid	10 mL	Tap
Sanitizer tank	Liquid	10 mL	Tap

Aliquots (1 mL) from each stock suspension were transferred into 9 mL Ringer's solution generating a 10^{-1} dilution. The process was repeated to obtain subsequent dilutions up to 10^{-6}. From this serial dilution, 0.1 mL aliquots were aseptically pipetted onto pre-poured Nutrient agar plates, which were then incubated at 30 °C for 48 hours.

Enumeration of lactic acid bacteria

deMan-Rogosa-Sharpe (MRS) agar was used for selective isolation and enumeration of lactic acid bacteria (LAB). These media formulations incorporate carbohydrates, pH buffers, and supplements that restrict growth of non-LAB, allowing accurate identification and counting of this bacterial group.

For each sample, 1 gram was weighed aseptically and transferred to a sterile 15 mL centrifuge tube containing 9 mL pre-prepared Ringer's solution. Samples were vortex mixed at maximum speed for 60 seconds using a Scientific Industries VM-3000 mixer to obtain a homogeneous stock suspension. Serial decimal dilutions from 10^{-1} to 10^{-6} were prepared by transferring 1 mL aliquots from the stock to subsequent dilution tubes. From appropriate rows, 0.1 mL was plated in onto MRS agar plates. Samples were spread evenly across the dried surfaces using a sterile L-shaped cell spreader. Plating was conducted under aseptic conditions inside a Class II biosafety cabinet. Inoculated MRS plates were closed and inverted before incubation at 30°C for 48 hours, the optimal growth temperature for LAB.

Distinct brown/white colonies were enumerated visually using a colony counter. Colony counts were multiplied by respective dilution factors to determine LAB concentration per gram of sample. This protocol selectively isolated and enumerated lactic acid bacteria abundances.

Microbial analysis of moulds and yeast populations

For each sample, 1 gram of molasses or 1 mL of liquid sample was aseptically transferred to a 15 mL centrifuge tube containing 9 mL of sterile Ringer's solution. Samples were mixed thoroughly using a vortex mixer at maximum speed for 60 seconds to obtain a homogeneous solution. Serial 10-fold dilutions from 10^{-1} to 10^{-6} were prepared by transferring 1 mL aliquots between dilution tubes. From appropriate rows, 0.1 mL was spread plated onto Sabouraud dextrose agar (SDA) supplemented with 100 µg/mL oxytetracycline to inhibit bacterial growth.

Sabouraud dextrose agar (SDA) was used as general yeast growth media to isolate and enumerate total yeast counts. Lysine agar differentiation was based on wild and commercial yeast strains possessing defective lysine biosynthesis pathways. Inoculated plates were closed, inverted, and incubated at 30 °C for 48 hours in an electrically heated incubator, the optimal temperature range for rum-associated yeasts. Yeast colonies were enumerated visually using a colony counter and recorded as CFU/gram and/or CFU/mL. Dominant colonial morphologies were observed.

Statistical analysis

Microbial count data exhibited non-normal distributions and heterogeneity of variances; thus, a log transformation was applied prior to statistical testing to stabilize variances and normalize data. To determine if there were significant differences in mean microbial abundances between the rum production stages, one-way analysis of variance (ANOVA) was performed using R Studio (Version 4.3.2). Separate one-way ANOVAs were conducted for each microbial group (total bacteria, lactic acid bacteria, total yeasts, wild yeasts) with stage as the categorical independent variable and log transformed counts as the continuous dependent variable. Conducting separate one-way ANOVAs for each microbial group allowed testing whether abundances significantly differed between production stages while accounting for different growth behaviours and ecological functions between taxa. Stage was an appropriate categorical independent variable as different process points represent distinct environments. If the overall ANOVA F-test yielded a significant p-value ($p < 0.05$), post-hoc multiple comparisons were conducted using Tukey's honest significant difference (HSD) test.

Microbial diversity was assessed using the Shannon diversity index (H′) and Bray-Curtis (BC) dissimilarity computed in R version 4.3.2 using the vegan package (version 2.6-4). The Shannon index considers richness and evenness, with higher values indicating greater diversity. It was calculated as $H' = -\Sigma pi(\ln pi)$ where pi is the proportion of each taxa. Bray-Curtis dissimilarity quantifies compositional differences between samples on a 0-1 scale, with higher values meaning more dissimilarity. It was derived using the formula $BC = 1 - 2C/(A+B)$ where A and B are the total abundances and C is the sum of lesser abundances for each sample pair.

Results of the research and their discussion

Microbial Composition in Rum Production Stages

Total bacterial count was relatively low compared to the total yeast/moulds count, consistent with a predominance of yeast populations in rum production (Table 2).

Table 2 – Microbiological Analysis at Various Stages of Rum Production: Bacterial and Yeast/Mould Counts, Lactic Acid Bacteria, and Wild Yeast Varieties

Stage/sampling point	Total bacterial count CFU/mL (Nutrient agar)		Total yeast/moulds count CFU/mL (SDA)		Lactic acid bacteria CFU/mL (MRS)		Wild yeast varieties CFU/mL (Lysine media)	
Front molasses well	2.0×10^2		3.4×10^4		2.0×10^1		8.2×10^3	
Middle molasses well	1.1×10^3		6.1×10^4		2.0×10^2		7.8×10^3	
Far molasses well	1.8×10^2		2.3×10^3		2.0×10^1		9.6×10^2	
Molasses supply tank	2.7×10^2		3.8×10^3		2.0×10^1		1.8×10^3	
Town water	nd		nd		nd		nd	
Clarifier	1.6×10^2		8.0×10^3		nd		nd	
Wort surge tank in	nd		nd		nd		nd	
Wort surge tank out	nd		nd		nd		nd	
Post wort cooler	nd		nd		nd		nd	
Yeast propagation billy	nd		2.3×10^8		nd		nd	
Yeast propagation tank A	3.6×10^1		1.2×10^4		1.4×10^1		nd	
Yeast propagation tank B	9.2×10^4		3.3×10^7		4.8×10^4		nd	
Yeast propagation tank C	2.3×10^6		4.6×10^8		8.4×10^5		nd	
Yeast propagation tank D	4.3×10^7		5.9×10^9		9.8×10^6		nd	
Fermenter	F5*	F9*	F5*	F9*	F5*	F9*	F5*	F9*
0 hours	1.8×10^4	7.1×10^2	8.2×10^6	6.2×10^4	3.1×10^3	8.8×10^1	nd	nd
6 hours	5.3×10^5	9.3×10^4	4.1×10^7	3.9×10^6	6.3×10^4	7.3×10^2	nd	nd
12 hours	9.4×10^5	5.3×10^5	6.3×10^7	5.8×10^7	2.7×10^4	7.7×10^4	nd	nd
18 hours	4.6×10^4	3.6×10^5	7.2×10^7	6.7×10^7	3.5×10^3	5.2×10^4	nd	nd
24h hours	4.4×10^4	8.0×10^4	7.8×10^7	7.0×10^7	5.9×10^3	3.9×10^4	nd	nd
36 hours	5.6×10^6	8.4×10^4	7.5×10^7	7.3×10^7	5.0×10^3	1.2×10^4	nd	nd
Dunder	nd		nd		nd		nd	
Mud	1.5×10^1		nd		nd		nd	
Caustic tank	nd		nd		nd		nd	
Acid tank	nd		nd		nd		nd	
Sanitizer tank	nd		nd		nd		nd	

*F5 & F9 refer to Fermenter 5 and Fermenter 9. These were the two observed for this study. F5 is an example of an old fermenter (smaller in volume 41000L) and F9 is a newer fermenter (larger in volume 79000L).

A one-way ANOVA (Table 3) showed significant differences in total bacteria counts between stages (F = 18.24, $p < 0.0001$). Post-hoc Tukey's test revealed that counts in the fermenters at 6 hours (mean = 5.3×10^5 CFU/mL), 12 hours (mean = 9.4×10^5 CFU/mL) and 36 hours (mean = 5.6×10^6 CFU/mL) were significantly higher than in earlier stages like the molasses wells and supply tank ($p < 0.05$). Lactic acid bacteria count also differed significantly between stages according to ANOVA (F = 26.11, $p < 0.0001$). Tukey's test indicated that abundances in the fermenters at 12 hours (mean = 2.7×10^4 CFU/mL), 18 hours (mean = 3.5×10^3 CFU/mL), and 24 hours (mean = 5.9×10^3 CFU/mL) were significantly greater than those of earlier tanks and propagations ($p < 0.01$). For wild yeasts, ANOVA showed an overall significant difference between different stages (F = 9.38, $p = 0.002$). Tukey's test revealed that counts in the front (mean = 8.2×10^3 CFU/mL), middle (mean = 7.8×10^3 CFU/mL) and far (mean = 9.6×10^2 CFU/mL) molasses wells were significantly higher than the supply tank (mean = 1.8×10^3 CFU/mL) and fermenters (not detected) ($p < 0.05$).

Microbial diversity analysis

The Shannon diversity index decreased throughout the rum fermentation process, starting from 1.49 in the middle molasses well and reaching to 0.68 at 36 hours (Table 4). Diversity also decreased as fermentation progressed, with the yeast propagation tanks exhibiting the highest diversity (reflecting a more balanced coexistence of bacterial and yeast taxa during early biomass build-up) and the later fermenter stages showing the lowest diversity.

Table 4 – Shannon diversity index values for microbial communities at each rum production stage.

Sample	Shannon Diversity Index
Front molasses well	1.10
Middle molasses well	1.49
Far molasses well	1.04
Molasses supply tank	1.03
Yeast propagation tank A	0.63
Yeast propagation tank B	1.18
Yeast propagation tank C	1.23
Yeast propagation tank D	1.13
Fermenter (F5) 0 hours	1.25
Fermenter (F5) 6 hours	1.22
Fermenter (F5) 12 hours	1.14
Fermenter (F5) 18 hours	1.10
Fermenter (F5) 24 hours	1.05
Fermenter (F5) 36 hours	0.68

The Bray-Curtis dissimilarity matrix compared microbial community composition between rum production stages (Table 5). The Bray-Curtis dissimilarity values reveal marked differences in microbial composition across rum production stages. Samples from distinct process points generally showed higher dissimilarity than those within the same stage.

A heatmap (Figure 3) was generated to visualize the microbial profile across different sampling locations and media types within the rum production plant. Microbial levels were notably low in the wort cooler. In contrast, especially in the latter half of the sampling period, exhibited t higher microbial counts, particularly in the MRS media, relative to the wort cooler.

Table 5 – Bray-Curtis dissimilarity matrix comparing microbial community composition.

Sample Pair	Bray-Curtis Dissimilarity
Front vs Middle	0.41
Front vs Far	0.35
Middle vs Far	0.38
Supply vs Fermenter 0 hour	0.53
Fermenter 0 vs 12 hour	0.62
Fermenter 12 vs 24 hour	0.59
Fermenter 24 vs 36 hour	0.74
Supply vs Fermenter (F5) 6 hour	0.51
Supply vs Fermenter (F5) 12 hour	0.57
Supply vs Fermenter (F5) 18 hour	0.55
Supply vs Fermenter (F5) 24 hour	0.53
Supply vs Fermenter 36 hour	0.59
Fermenter (F5) 0 vs 18 hour	0.57
Fermenter (F5) 0 vs 24 hour	0.54
Fermenter (F5) 0 vs 36 hour	0.63
Fermenter (F5) 6 vs 12 hour	0.59
Fermenter (F5) 6 vs 18 hour	0.56
Fermenter (F5) 6 vs 24 hour	0.52
Fermenter (F5) 6 vs 36 hour	0.61
Fermenter (F5) 12 vs 18 hour	0.55
Fermenter (F5) 12 vs 36 hour	0.62
Fermenter (F5) 18 vs 24 hour	0.51
Fermenter (F5) 18 vs 36 hour	0.59
Fermenter (F5) 24 vs 36 hour	0.63

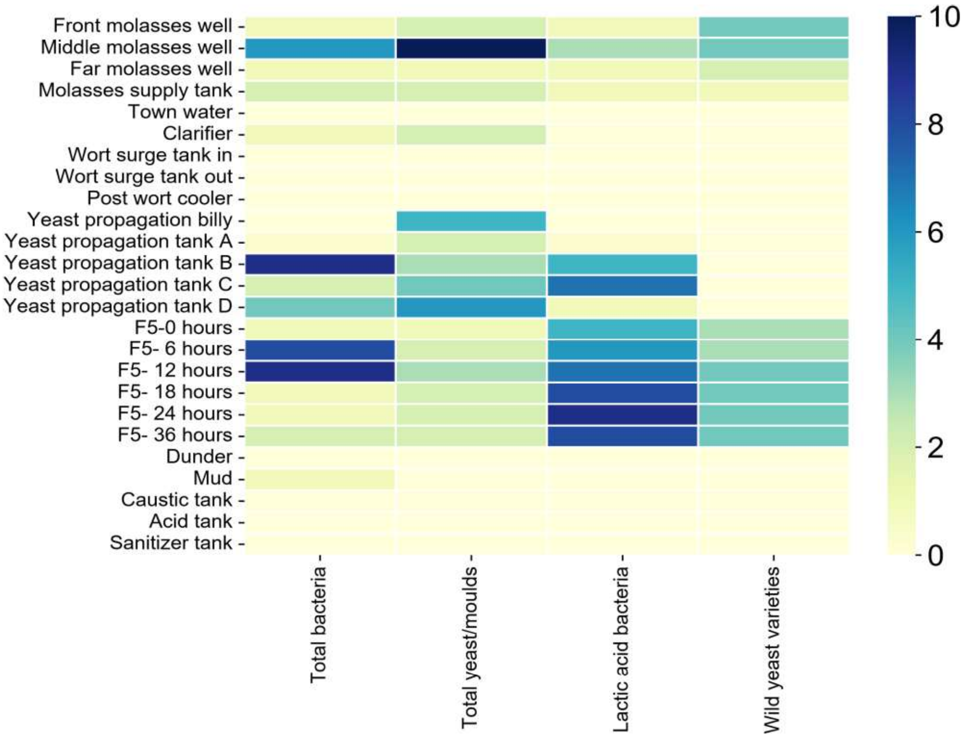

Fig. 2. Heatmap illustrating the microbial counts at various stages/sampling points. The colour scale ranges from yellow

Most alcoholic fermentations (rum, whisky, wine, etc.) deliberately occur in the presence of persistent and varied microbiome beyond the main yeast introduced into the fermentation [21], such as lactic acid bacteria and wild yeast varieties, to enhance the flavour profile and sensory characteristics of the final product. However, these varied microbiomes can significantly impact the main fermentation efficiency, potentially leading to stuck fermentation or plant shutdown for cleaning before restarting the fermentation. This highlights the critical need to understand and manage these varied microbiomes to ensure smooth and uninterrupted operation of the rum production processes [13].

The presence of a diverse microbial population in rum manufacturing underscores the importance of implementing robust control measures to ensure the desired fermentation outcomes. Managing microbial biomes is crucial to ensure smooth and uninterrupted rum production processes. The diverse microbial population in rum manufacturing emphasizes the importance of robust control measures, including stringent sanitation protocols, microbial population monitoring, and optimized fermentation conditions. These measures aim to harness the beneficial aspects of the microbial community while minimizing potential challenges.

Microbial Community Dynamics in Molasses Feedstock

The results reveal an intriguing microbial landscape associated with molasses during rum production. In the molasses storage stages (front, middle, and far molasses wells), a diverse array of bacteria was observed, as indicated by the relatively high total bacterial counts. This aligns with the expected chronic microbial contamination linked to molasses production and storage, where a multitude of bacteria can coexist. The high bacterial counts observed in the molasses wells corresponds with studies showing complex, multi-species communities in sugarcane molasses and other feedstocks [22-24].

Previous research has demonstrated that a range of osmotolerant, acidophilic, and ethanol-tolerant bacteria can survive in sugarcane molasses, tolerating the high sugar content, low water activity, and relatively low pH [1, 14, 25]. This aligns with the current study, where a diverse bacterial population adapted to these selective pressures were observed proliferating in the molasses feedstock wells. Despite conditions typically considered restrictive to microbial growth, specialized species possess adaptations enabling them to persist in sugarcane molasses, as noted by both Green [1] and the present research. The ability of these bacteria to withstand the challenges of sugarcane molasses contributes to the development of complex microbial communities revealed in this rum production environment.

While *Saccharomyces* yeast performs the core sugar conversion during rum fermentation, the structure of the feedstock microbiome as seen in this study will have important implications on process efficiency and product outcomes. As noted by previous studies [21, 26], bacterial metabolites such as organic acids, bacteriocins, and surfactants can inhibit yeast growth and fermentation productivity. Overproduction of such compounds by uncontrolled feedstock bacteria may hamper yields [22, 26]. However, bacterial enzymes may produce key flavour compounds absent in sterile fermentations [1, 3]. Thus, complete microbial exclusion could reduce product complexity. Therefore, further community analysis should assess taxa-function dynamics in feedstocks.

The diverse bacterial biome in molasses seem to provide a reservoir of functional traits that carryover into fermentation. As found in other studies, molasses-derived *Lactobacillus* strains can persist and enhance fermentation through micronutrient provisioning [24]. Rum feedstocks also likely contain cellulolytic bacteria that increase substrate availability as reported in other ethanol systems [27].

Lactic acid bacteria selection during fermentation

The increasing relative abundance of lactic acid bacteria (LAB) observed over the course of rum fermentation agrees with previous metagenomic surveys that have shown *Lactobacillus* to predominate in various alcoholic fermentation systems [22, 28]. Through DNA-based community profiling approaches, studies have revealed LAB, and particularly *Lactobacillus* species, become highly dominant members of the microbial consortia present during latter stages of industrial-scale fermentations.

The high LAB abundances reported in this study, especially during active rum fermentation in the pH 4-6 range, can potentially contribute meaningful sensory attributes to the final product [5]. As noted, LAB physiology is adapted to the rum fermentation conditions of ethanol and acid stress. Lactic acid production lowers pH levels, which LAB tolerate far better than most competing microbes. Additionally, LAB metabolism can lead to the generation of characteristic flavour-active compounds like acetoin and diacetyl, imparting buttery sensory notes valued in spirits [24]. Specific *L. plantarum* isolates from rum have been shown to produce flavour-impacting esters and fusel alcohols through detailed strain characterization.

However, LAB overpopulation if left unchecked has the potential to negatively impact rum fermentations. Excessive LAB levels, such as above reported thresholds of 108 CFU/mL, are associated with impaired outcomes like increased volatile acidity formation or substrate nutrient sequestration inhibiting yeast [29, 30]. These scenarios compromise ethanol yields and consistency. The absence of LAB counts exceeding 108 CFU/mL in this study suggests the rum production process implementing pH controls and environmental parameters reflective of LAB preferences avoids such inhibited functionality [31]. The quantification of LAB succession dynamics will guide tactical methods for balancing flavour contributions against fermentation performance risks.

Further analysis of the data indicates that LAB consistently constitute a significant portion of the total bacterial count, suggesting their crucial role in the fermentation dynamics. At the initial stages of the process, such as in the molasses wells and supply tank, LAB comprise a relatively small percentage of the total bacterial population, ranging from 0.1 % to 0.3 %. This indicates the presence of other bacterial species alongside LAB, which may be attributed to the chronic microbial contamination expected in fuel ethanol fermentations [22]. However, as the fermentation progresses and the process moves into yeast propagation tanks, a significant increase in the percentage of LAB is observed. In yeast propagation tank B, for instance, LAB represent an impressive 55.3 % of the total bacterial population, indicating their dominant presence in this stage. Similarly, yeast propagation tanks C and D show significant proportions of LAB, accounting for approximately 8.3 % and 22.8 % of the total bacterial population, respectively.

The most notable finding is the prevalence of LAB in the fermenters, F5 and F9. Lactic Acid Bacteria (LAB) consistently exhibit high percentages compared to other bacterial species throughout the fermentation duration. At the start (0 hours), LAB already account for 45.6 % and 10.7 % of the total bacterial population in F5 and F9 fermenters, respectively. As the fermentation progresses, the dominance of LAB becomes more pronounced, with percentages reaching approximately 92.8 % and 94.4 % in F5 and F9 fermenters at 36 hours, respectively. These trends agree with the work of previous scholars [13], who observed LAB levels up to 87 % of total bacteria in a dry grind ethanol facility. Lactic acid bacteria (LAB) levels reached 10^5-10^8 CFU/mL in their fermenters.

The lower abundance of lactic acid bacteria (LAB) in the molasses compared to the later stages of rum production can be attributed to several factors, with the dilution of molasses with town water being a significant contributing factor. During the initial stages, such as the front, middle, and far molasses wells, the molasses is in its concentrated form, with a higher sugar content and lower water activity. These conditions may limit the growth of LAB, which have an optimum activity range that favours a slightly higher water activity and lower sugar concentration as discussed by other authors [1, 14, 25]. As a result, other bacterial species may dominate the microbial population in the concentrated molasses due to their ability to adapt and thrive in these conditions [1]. When the molasses is subsequently diluted with town water in later processes, it alters the composition of the microbial population. The dilution increases the water activity and reduces the sugar concentration, bringing the environment within the optimum range for LAB activity [32]. Lactic acid bacteria are known to thrive in environments with a moderate sugar concentration and slightly higher water activity, allowing them to efficiently convert sugars into lactic acid [32]. Consequently, the dilution with town water provides a more favourable environment for the growth and proliferation of LAB, leading to an increase in their abundance.

Overall, the data supports the notion that lactic acid bacteria are the predominant bacterial species in rum fermentations. Their increasing proportions and dominance throughout the fermentation stages underscore their importance in the overall process and warrant further investigation into their metabolic activities and interactions within the fermentation environment.

Impact of wild yeasts on rum quality

While wild yeasts were present at lower levels in the molasses, they can still impact flavour by producing significantly higher/fusel alcohols and esters, contributing fruity and floral notes typical of rum. Key flavour compounds such as the methyl ketones 2-pentanone and 2-heptanone which impart sweet, fruity, and slightly buttery aromas are produced by *Pichia* species [1, 5, 33], contributing to the distinct rum profile. *Hanseniaspora spp.* are known for producing high levels of ethyl acetate, contributing fruity nuances [1, 5, 33]. Further culture-based analysis of isolates, combined with untargeted metabolomic profiling, is warranted to definitively correlate the identified microbes to rum volatile chemistry.

Further down, the clarification process involves heating the molasses mixture to 70°C for approximately one hour to facilitate the sedimentation of solids. This step is not primarily intended to target the elimination of wild yeast populations but rather to facilitate the removal of unwanted particulate matter. However, the temperature and duration used in this step may have some impact on reducing the overall yeast population, including wild yeast [34]. A temperature of 70°C is generally considered high enough to kill most yeast strains, including many wild yeast varieties [35-37]. Wild yeast strains are often less heat-tolerant compared to *Saccharomyces cerevisiae*, which is the primary yeast used in rum fermentation. As such, no wild yeasts are seen in this study beyond the clarifier [5].

The absence of wild yeasts in propagation tanks highlights effective control measures during rum fermentation. Various studies have shown that raw ingredients variably inoculate wild yeasts, causing unpredictable fermentations [38, 39]. While providing flavour complexity, uncontrolled wild yeasts also risk stuck fermentations and spoilage [38]. The BDC production process avoided such problems through stringent sanitation and inoculum selection. Limiting wild yeasts in propagations tanks concentrates desirable *Saccharomyces* strains for consistent fermentations.

By focusing on specific yeast strains that are known for their desirable fermentation characteristics, the production process can achieve greater consistency and quality in the final rum product. It is important to note that although wild yeast varieties were not detected in the propagation tanks, it does not guarantee their absence throughout the entire fermentation process. Careful monitoring of the fermentation stage is necessary to ensure the continued absence of wild yeast contamination and to promptly address any potential issues that may arise.

Microbial community shift

Bacterial diversity decreased and *Lactobacillus* dominance increased during rum fermentation, indicating strong ecological filtering of the microbiome. These community shifts agree with previous findings that acidic pH, alcohol toxicity, and nutrient limitation select for acidophilic and alcohol-tolerant microbes [1, 14]. In the BDC fermentations, the average pH dropped from 5.4 to 3.8, likely inhibiting less acid-tolerant bacteria compared to *Lactobacillus*. Ethanol concentrations exceeding 6% probably eliminated bacteria unable to tolerate high levels of alcohol [40]. Meanwhile, *Saccharomyces cerevisiae* populations aligned with its tolerance of up to 10% ethanol , thereby outcompeting other microbes [41].

At the start of fermentation, the population of *S. cerevisiae* is typically low since the inoculation of yeast into the fermenter has just occurred, and the yeast population consisted of the initially added yeast cells. It is worth noting that the initial population of *Saccharomyces cerevisiae* was slightly higher in the smaller tank (F5) compared to the larger tank (F9). This difference in the initial population can be attributed to the use of the same inoculum volume for both tanks. Since the smaller tank has a lower volume, the same inoculum volume would result in a higher population density. As fermentation progressed, the yeast populations in both tanks exhibited a significant increase. At 6 hours, there was a substantial growth in *Saccharomyces cerevisiae* populations in both F5 and F9. This indicates successful yeast adaptation and proliferation in the fermentation medium. The exponential growth agrees with models of *S. cerevisiae* consuming sugars and producing ethanol under favourable conditions [9]. Yeast cells utilize the available fermentable sugars, such as glucose and fructose, through the process of glycolysis, leading to the production of ethanol and carbon dioxide through fermentation. The favourable fermentation conditions, including sufficient nutrients, promote yeast growth, resulting in a significant increase in the *S. cerevisiae* population [5, 9, 12, 23]. The exponential growth phase continued until 12 hours, with further increases in yeast counts in both tanks. This growth phase aligns with the expected behaviour of *Saccharomyces cerevisiae* during the early stages of fermentation, as the yeast cells consume the available sugars and produce ethanol and other by-products [5, 23]. This phase is characterized by a high metabolic activity of *S. cerevisiae* and a rapid increase in the yeast population.

Between 18 and 24 hours of fermentation, yeast populations reached a stable level, indicating the onset of the stationary phase as described in other studies [42, 43]. This stability suggests that the yeast cells had consumed a significant portion of the fermentable sugars and had also begun to face environmental limitations, such as nutrient depletion and accumulation of ethanol, which can suppress further yeast growth [1, 14]. Interestingly, populations in F5 and F9 converged by 24 hours, emphasizing successful yeast adaptation despite initial disparities. This convergence likely resulted from inoculum standardization and microenvironment homogenization rather than fermenter size effects [44].

The lack of detectable microorganisms in the town water and dunder is encouraging, as it prevents potential contamination that could adversely affect fermentation or produce off-flavours. The absence of microbes in the dunder used in rum production is significant as it enables precise pH control and nutrient allocation towards selected yeasts, promoting consistent outcomes [45].

No microbes were detected in the caustic, acid, or sanitizer tanks, which can be entirely contributed to the inhospitable chemical environments. However, dilute solutions of caustic and or acid could allow microbial survival [46]. One previous study examined the antimicrobial efficacy of diluted alkaline and acidic tank cleaning solutions commonly used in food processing facilities. They found that many bacteria, including *Salmonella* and *Listeria* species, could survive and even proliferate in dilute hydroxide and nitric acid solutions [47]. Proper storage tank design and solution strength maintenance are crucial for preserving antimicrobial properties. While current conditions at the BDC appear to prevent growth, continued monitoring and quality control are essential to ensure microbial integrity of cleaning agents.

Verifying and troubleshooting Clean-in-Place protocols.

The detection of bacteria in supposedly sterile propagation tanks indicates potential recontamination or inadequate sanitization despite clean-in-place (CIP) protocols. CIP alone cannot guarantee sterility without verification testing, as survivors may persist in protected niches [46, 47]. Dead legs, flawed gaskets, or micro-cracks could harbor cells by limiting sanitizer contact [48]. Airborne contamination likely also occurs post-CIP before sealing tanks. Lapses during yeast transfer may introduce bacteria.

While a multifaceted approach to validation of the CIP system is proposed for BDC, implementing comprehensive sterility testing has numerous challenges. Swabbing every tank surface may be unfeasible (time consuming and cost of analytical consumables) and could still miss certain micro-niches. The detection limits during CIP validation used as thresholds influence results, and varies by study [49, 50]. Monitoring CIP parameters can be labour-intensive and subject to sensor errors [46, 47, 51]. Significant effort would be required to overhaul tank designs and protocols.

These collective issues underscore the need for practical alternatives such as microbial quantification of rinse waters to indicate sanitization efficacy without interior surface sampling. Pairing periodic tank sterilizations with molecular monitoring addressing limits of culture-reliant methods could offer complementary validation of disinfection completeness. Ongoing efforts to optimize protocols through industrially relevant validation are thus warranted to ensure microbial decontamination efficiency.

Ecological implications of microbial populations

Significant ((F=12.4, $p<0.01$) community shifts occurred across the process. Theis again confirms the successional changes, which found *lactobacilli* proliferation early in rum fermentation followed by *Saccharomyces* dominance [1]. The assembly patterns suggest rum microbiota are shaped by ecological filtering, just like the environmental selection pressures reported in other ethanolic fermentation systems [52].

The increase in total bacteria likely reflects proliferation of amylolytic lactobacilli utilizing sugars from molasses [1, 3, 5, 7, 14]. The changes in wild yeasts indicate replacement by *Saccharomyces spp.* as fermentation selects for specialist bacteria. While ANOVA revealed significant differences exist between stages, post-hoc Tukey's tests were essential to pinpoint which specific pairs of means varied. These comparisons revealed total bacteria and *lactobacilli* increased primarily during active fermentation, while wild yeasts declined after molasses wells.

Overall, the quantitative statistical analyses validate the hypothesized compositional changes discussed in this paper. They provide numerical evidence that community assembly in rum fermentation follows a predictable ecological succession pattern. Extending sampling resolution and combining ANOVA with high-throughput sequencing could further elucidate rum microbial dynamics.

The marked decrease in Shannon diversity and increase in Bray-Curtis dissimilarity over fermentation aligns with ecological theory, where strong selective pressures reduce community biodiversity. The Shannon index declined from 1.49 in the middle molasses well to 0.68 at 36 hours of fermentation. This reveals decreasing community richness and evenness, aligning with niche theory where strong selective pressures reduce biodiversity. The quantitative evidence verifies the compositional shifts reflecting microbial community succession in rum. Environmental filtering likely eliminates less tolerant species, allowing adapted taxa like lactobacilli and *Saccharomyces* to dominate in synchronisation with changing conditions. Characterizing ecological dynamics through diversity metrics can thus elucidate assembly patterns and guide targeted interventions to manage community structure.

The Bray-Curtis dissimilarity matrix revealed clear compositional differences between microbial communities across rum production stages. Samples from distinct process points generally showed higher dissimilarity than those within the same stage, indicating succession of microbial populations during processing. For instance, the front and middle molasses wells had a Bray-Curtis value of 0.41, whereas the dissimilarity between the front well and initial fermenter was 0.53. This quantitatively demonstrates community shifts between production steps, likely reflecting environmental filtering [14, 53]. The decreased Shannon index and increased Bray-Curtis dissimilarity over rum fermentation aligns with successional patterns reported in other fermented beverages. Studies on wine, beer, and cider also demonstrate declining alpha diversity and increasing beta diversity over fermentative progression as environmental conditions select for specialists [54, 55].

Dissimilarity progressively increased between later fermentation timepoints, reaching values of 0.63 between 0 hour and 36 hours. Even adjacent samples like 12 hours and 18 hours showed dissimilarity of 0.55. These patterns align with ecological theory, where strong selective pressures reduce biodiversity over time by eliminating less adapted organisms [56]. Specific taxa thrive under newly arising conditions, driving community divergence.

The high dissimilarity between initial supply/fermenter samples and late fermentation stages further indicates the extent of ecological turnover. This successional change in community assembly follows hypothesized metabolic transitions during rum production [37]. Ultimately, the Bray-Curtis analysis provided numerical evidence verifying predictable community shifts reflecting environmental selection pressures and microbial adaptation during the rum fermentation process.

Conclusion

The culture-based analysis revealed predictable successional patterns, with lactic acid bacteria proliferating in early fermentation, succeeded by Saccharomyces dominance. The detection of wild yeasts in initial ingredients provides valuable insight into potential inconsistencies in fermentation performance between batches, as fluctuations in wild yeast populations may influence the metabolic profile, flavour development, and overall fermentation kinetics.

The observed shifts in microbial composition across fermentation stages address key knowledge gaps around rum microbiome variability and succession that motivated the study. Quantifying population shifts fills gaps in understanding how processing steps shape microbial assembly. The sporadic presence of spoilage microbes explains batch-to-batch differences reported in industry. Characterizing this ecological variability is the first step toward targeted interventions to control microbiota.

The culture-based survey established a crucial foundation for higher-resolution community characterization using genomic techniques. These results will inform starter culture designs and microbiological control strategies to enhance process efficiency and product quality. Elucidating rum microbiome dynamics and functionality is essential for the evolution of this vital industry. Moving forward, selectively promoting beneficial *lactobacilli* and yeast strains while inhibiting potential spoilage microbes will be vital. The development of tailored starter cultures and microbial management regimes will be informed by these results.

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