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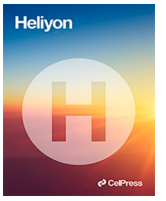

Research article

The effect of selected Non-*Saccharomyces* yeasts and cold-contact fermentation on the production of low-alcohol marula fruit beer

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A R T I C L E I N F O

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Pichia fermentans
Pichia kluyveri

A B S T R A C T

The last decade has seen increased consumer demand for zero and low-alcohol beverages. Cold-contact fermentation (CCF) in combination with non-*Saccharomyces* can be an effective method for producing low-alcohol fruit beverages with desirable qualities. Thus, the aim of this study was to develop a CCF process to produce low-alcohol marula fruit beer using selected non-*Saccharomyces* yeasts. The effect of temperature (°C), and time (h) on alcohol (% v/v), pH, total titratable acidity (LAE/mL) and specific gravity (SG) was evaluated using response surface methodology. Sterile marula fruit juice was inoculated with *Metschnikowia pulcherrima*, *Pichia fermentans*, or *Pichia kluyveri* respectively. Higher final SG values were observed for temperatures between 8 °C and 15 °C. Above 15 °C, the SG decreased with an increase in temperature and time. Fermentation at temperatures below 10 °C produced zero to low-alcohol marula fruit beer (0.00–0.20 % v/v) with an attenuation rate above 80 %. This was confirmed by the significance of quadratic models for SG ($p \leq 0.01$), and alcohol ($p = 0.00$) for the three selected yeasts. Overall, *P. kluyveri* produced the lowest alcohol levels, followed by *M. pulcherrima* and *P. fermentans*, respectively. The study confirmed that cold-contact fermentation with non-*Saccharomyces* yeasts can be an effective biological method to produce low-alcohol marula fruit beer in line with the emerging consumer demand for low-alcohol beverages.

1. Introduction

Low-alcohol beverages (LACB) (≤3.5 % v/v) have been in existence for hundreds of years [1]. Their re-emergence as a principal alternative to high-alcohol beverages (HACB) has been a consequence of increasing health-oriented lifestyle trends, religious prohibitions, changing consumer preferences, and stricter legislation [2,3]. Specifically, the promotion of teetotalism and related drinking trends which avert health risk factors associated with the consumption of HACB have enabled this adoption [4,5]. Recurrent health risks associated with the consumption of HACB include premature death, allergenic induction, liver cirrhosis, pancreatitis, gastrointestinal diseases, stroke, hepatitis, mental health disorders, elevated blood pressure, cancer, obesity, elevated blood pressure, and peptic ulcers [6–8]. To this end, the World Health Organization has set a global target to reduce the harmful use of alcohol by the year

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2025 [9].

Physical and biological processes are currently the main strategies used to produce LACB [10,11]. The physical method involves the gentle removal of alcohol from a fermented standard beer matrix while avoiding the loss of flavour compounds and sensory attributes, such as palate fullness, body, and freshness [12,13]. This method may require the use of expensive and specialised equipment [14]. Alcohol is typically completely or partially removed by thermal treatments, and/or membrane separation processes [11,15]. In contrast to physical methods, biological processes produce LACB by limiting the formation of ethanol during beer fermentation, reducing wort fermentability through modified mashing regimes, using special strains of fermenting yeasts, which are incapable of fermenting maltose, and continuous culture of free and immobilised yeast, or cold-contact process [1,8,16]. Attempts to use brewing yeast mutants deficient in tricarboxylic acid cycle enzymes have been made [10].

Some strains of *Saccharomyces cerevisiae* have been genetically modified to be alcohol dehydrogenase-negative and thus do not produce alcohol at significant concentrations [17]. *S. cerevisiae* mutant strains can produce high levels of sugar alcohols and glycerol, and low levels of ethanol [18,19]. Comparably, strains of *Saccharomycodes ludwigii* are capable of fermenting glucose, sucrose, and fructose, but not maltose (the most abundant sugar of the wort) and maltotriose. This results in a beer with low alcohol and high lactic acid [20,21]. *S. cerevisiae* mutants that lack fumarase and 2-ketoglutarate dehydrogenase activity are considered the most acceptable for obtaining LACB [14]. Other suggested species include *Scheffersomyces shehatae*, *S. pastorianus*, *Wickerhamomyces anomalus*, *Lactobacillus*, *Kluyveromyces bulgaricus*, *K. fragilis*, and *Pichia kluyveri* [10,16]. The role of non-fermentative yeasts isolated from marula fruits is yet to be elucidated in marula fruit beer fermentation [22]. Similarly, cold-contact fermentation (CCF) or cold-contact process (CCP) is a well-established approach used for the reduction of alcohol levels [23].

Cold-contact fermentation (CCF) can effectively restrict yeast metabolic activity and the formation of ethanol by using low-to-near 0 °C temperatures [24]. The fermentation temperature is essential since it affects yeast reproduction rates [25]. The benefit of low-temperature fermentation is the production of beer with improved physicochemical properties (i.e., specific gravity, alcohol, pH, total titratable acidity) [26]. A study by Kucharczyk and Tuszyński [25] demonstrated the benefits of low-temperature fermentation, especially decreased formation of undesirable components and volatile compounds such as fusel alcohols, acetaldehyde, and vicinal diketones (e.g., diacetyl). For example, undesirable compounds such as sulphur have been eliminated with carbon dioxide by stripping wort at a low temperature and under pressure [23]. Thus, compared to other alcohol reduction methods, the combination of special yeasts (i.e., non-*Saccharomyces*) with CCF is substantially advantageous [24,27]. Given these benefits, the aim of this study was to develop a CCF process to produce low-alcohol marula fruit beer using *Metschnikowia pulcherrima*, *P. fermentans*, and *P. kluyveri*.

2. Methodology

2.1. Marula fruit processing

Fresh marula fruits were obtained from an independent farmer at Magona village, Limpopo, South Africa (23.0206° S, 30.8886° E). The fruits were transported under cool conditions (<20 °C) to the laboratory for processing. The fruits were washed with running tap water and allowed to dry at 25 °C for 1.5 h. Thereafter, the skins were carefully turned inside-out to separate them from the flesh and the skinless fruits were then transferred to a juicer (JMM70, Kenwood South Africa, Johannesburg, South Africa) to extract the juice. Fresh marula juice was sterilised and stored frozen at −20 until needed.

2.2. Experimental design

Preliminary experiments (data not shown) were conducted to determine appropriate ranges for input factors (i.e., temperature (°C), and time (h)) and their effect on the responses (i.e., alcohol (% v/v), pH, total titratable acidity (TTA) (LAE/mL) and specific gravity (SG)). This data was used for the experimental design in Design-Expert (13.0.5.0, Stat-Ease Inc., Minneapolis, USA) (Table 1**)**. Central Composite Design (CCD) was the selected response surface design type. A total of 13 experimental combinations were generated (Table 2).

2.3. Yeast culture and inoculum preparation

M. pulcherrima (Y0842), *P. fermentans* (B4LY2), and *P. kluyveri* (MY2) were provided by the Agricultural Research Council (ARC) Infruitec-Nietvoorbij culture collection (Stellenbosch, South Africa). The inoculums were prepared by picking yeast cells from 48 h yeast and mould agar (Oxoid Limited, UK) using sterile pipette tips and inoculating them into 5 mL yeast extract agar (Sigma-Aldrich, USA) broth in 10 mL test tubes. The tubes were then incubated for 24 h at 28 °C.

Table 1
Process parameters selected for optimization: fermentation temperature and time.

Parameters	Code	High level (+1)	Medium level (0)	Low level (−1)
Temperature (°C)	X_1	20	15	10
Time (h)	X_2	336	252	168

h – hour.

Table 2
Experimental design for low-alcohol marula fruit beer production.

Run	Temperature (°C)	Time (h)
1	15	252
2	10	168
3	15	133.21
4	15	252
5	8	252
6	20	168
7	22.07	252
8	10	336
9	20	336
10	15	252
11	15	252
12	15	370.79
13	15	252

h – hour.

2.4. Marula fruit cold-contact fermentation

Sterile 70 mL marula fruit juice (13 °P) was aliquoted into 100 mL Erlenmeyer flasks and then inoculated separately with the prepared *M. pulcherrima* (Y0842), *P. fermentans* (B4LY2), or *P. kluyveri* (MY2) cultures at a concentration of 2.3×10^7 cells/mL. The inoculated flasks were incubated according to the fermentation conditions of each experimental run (Table 2). All experiments were conducted in triplicate.

2.5. Determination of physiochemical properties

2.5.1. Alcohol (% v/v) and specific gravity (SG)

The alcohol content and SG of the marula fruit beer were determined using an alcohol and extract meter (Alex 500, Anton Paar, Austria). The instrument was first calibrated by measuring readings for distilled water. Thereafter, a standard sample of 3 % ethanol was measured.

2.5.2. pH

A portable pH meter (HI8424, Hanna Instruments (Pty) Ltd., Johannesburg, South Africa) was first calibrated with standard buffers of pH 4.00 and 7.00. Thereafter, the pH of the fermented product was determined.

2.5.3. Total titratable acidity (TTA) (LAE/mL)

The approved method of the American Association of Cereal Chemists (AACC) 02–31 [28] was used to determine the TTA whereby 10 g of the sample was dissolved in 100 mL distilled water. The solution was mixed well and 0.5 mL of 1 % phenolphthalein indicator was added. Finally, standardised 0.1 N sodium hydroxide was used to titrate the prepared solution until a faint pink colour was observed. The TTA in Lactic Acid Equivalent/millimetre (LAE/mL) was measured using the formula: total titratable acidity = volume (mL) required/20.

2.6. Statistical analysis

All experiments and analyses were conducted in triplicate and expressed as mean ± standard deviation (SD). Means were separated using Duncan's Multiple Range Test (DMRT) and significant differences were accepted at $p < 0.05$. Analysis of variance (ANOVA) was performed to determine the significance of the generated models. Design-Expert (13.0.5.0, Stat-Ease Inc., Minneapolis, USA) was used to determine the responses (Y) of the second-order polynomial equations, the coefficient of determination (R^2), the 'predicted R-squared' and 'adjusted R-squared', the coefficient of variance (CV), and the 'probability F' value.

3. Results and discussion

3.1. The effect of input factors on physicochemical properties

3.1.1. The effect of temperature and time on specific gravity (SG)

The effect of temperature (°C), and time (h) on the physiochemical properties of marula fruit juice during fermentation was evaluated using response surface methodology. The physicochemical properties of the marula fruit juice used were as follows: pH = 4.83; TTA (LAE/mL) = 0.37; SG = 1.06. The lowest SG for all yeasts (*M. pulcherrima*, *P. fermentans*, and *P. kluyveri*) was 1.01 at 15.00–22.07 °C after 252–370.79 h (Table 3). Conversely, the highest SG (1.04) in marula fruit juice fermented with *P. fermentans* was observed at 10.00 °C after 168 and 336 h. Overall, high SG values were observed for temperatures between 8.00 °C and 15.00 °C. Above 15.00 °C, the SG decreased with an increase in temperature and time. The high SG that was observed with *P. fermentans* can be

Table 3
The effect of temperature (°C) and time (h) on the SG, TTA (LAE/mL), pH, and alcohol content (% v/v) of marula fruit beer fermented with *Metschnikowia pulcherrima*, *Pichia fermentans* and *P. kluyveri*.

Code	Inputs		Responses											
	Temperature (°C)	Time (h)	SG			TTA (LAE/mL)			pH			Alcohol (% v/v)		
	X_1	X_2	Y_1			Y_2			Y_3			Y_4		
Exp.[a] Run			*M. pulcherrima*	*P. fermentans*	*P. kluyveri*	*M. pulcherrima*	*P. fermentans*	*P. kluyveri*	*M. pulcherrima*	*P. fermentans*	*P. kluyveri*	*M. pulcherrima*	*P. fermentans*	*P. kluyveri*
1	15.00	252.00	1.01 ± 0.04	1.01 ± 0.03	1.01 ± 0.12	0.42 ± 0.21	0.42 ± 0.06	0.42 ± 0.29	3.82 ± 0.02	3.88 ± 0.04	3.78 ± 0.01	1.35 ± 0.01	1.75 ± 0.01	1.19 ± 0.01
2	10.00	168.00	1.03 ± 0.13	1.04 ± 0.15	1.02 ± 0.02	0.92 ± 0.17	0.92 ± 0.26	1.02 ± 0.25	3.85 ± 0.07	3.87 ± 0.03	3.92 ± 0.08	0.13 ± 0.02	0.00 ± 0	0.06 ± 0
3	15.00	133.21	1.03 ± 0.02	1.03 ± 0.10	1.03 ± 0.02	0.87 ± 0.15	0.86 ± 0.17	0.83 ± 0.31	4.59 ± 0.05	4.75 ± 0.02	4.72 ± 0.01	0.68 ± 0	0.21 ± 0	0.24 ± 0.01
4	15.00	252.00	1.01 ± 0.04	1.01 ± 0.03	1.01 ± 0.12	0.42 ± 0.21	0.42 ± 0.06	0.42 ± 0.29	3.82 ± 0.02	3.88 ± 0.04	3.78 ± 0.01	1.35 ± 0.01	1.75 ± 0.01	1.19 ± 0.01
5	8.00	252.00	1.03 ± 0	1.03 ± 0.01	1.03 ± 0.01	0.54 ± 0.17	0.52 ± 0.26	0.52 ± 0.23	3.95 ± 0.02	3.87 ± 0.01	3.84 ± 0.04	0.00 ± 0	0.00 ± 0	0.01 ± 0.01
6	20.00	168.00	1.01 ± 0.05	1.01 ± 0.01	1.02 ± 0.02	0.55 ± 0.12	0.61 ± 0.15	0.53 ± 0.17	3.66 ± 0.04	3.76 ± 0.01	3.77 ± 0.02	0.73 ± 0.01	0.65 ± 0.01	0.44 ± 0.02
7	22.07	252.00	1.01 ± 0.01	1.01 ± 0.01	1.01 ± 0.01	0.59 ± 0.21	0.58 ± 0.38	0.57 ± 0.20	3.73 ± 0.03	3.77 ± 0.02	3.77 ± 0.02	0.91 ± 0.02	0.67 ± 0.01	0.29 ± 0.02
8	10.00	336.00	1.02 ± 0.39	1.04 ± 0.15	1.02 ± 0.17	0.98 ± 0.26	0.98 ± 0.10	1.02 ± 0.26	3.95 ± 0.02	3.96 ± 0.07	3.92 ± 0.08	0.20 ± 0	0.35 ± 0.01	0.06 ± 0.02
9	20.00	336.00	1.01 ± 0.01	1.01 ± 0.01	1.01 ± 0.01	0.51 ± 0.10	0.61 ± 0.12	0.51 ± 0.25	3.74 ± 0.01	3.78 ± 0.01	3.75 ± 0.01	1.54 ± 0.02	1.31 ± 0.02	1.23 ± 0.01
10	15.00	252.00	1.01 ± 0.04	1.01 ± 0.03	1.01 ± 0.12	0.42 ± 0.21	0.42 ± 0.06	0.42 ± 0.29	3.82 ± 0.02	3.88 ± 0.04	3.78 ± 0.01	1.35 ± 0.01	1.75 ± 0.01	1.19 ± 0.01
11	15.00	252.00	1.01 ± 0.04	1.01 ± 0.03	1.01 ± 0.12	0.42 ± 0.21	0.42 ± 0.06	0.42 ± 0.29	3.82 ± 0.02	3.88 ± 0.04	3.78 ± 0.01	1.35 ± 0.01	1.75 ± 0.01	1.19 ± 0.01
12	15.00	370.79	1.01 ± 0.01	1.01 ± 0.01	1.01 ± 0.01	0.43 ± 0.25	0.46 ± 0.21	0.51 ± 0.10	3.84 ± 0.03	3.93 ± 0.03	3.82 ± 0.03	1.51 ± 0.01	1.47 ± 0.01	1.09 ± 0.01
13	15.00	252.00	1.01 ± 0.04	1.01 ± 0.03	1.01 ± 0.12	0.42 ± 0.21	0.42 ± 0.06	0.42 ± 0.29	3.82 ± 0.02	3.88 ± 0.04	3.78 ± 0.01	1.35 ± 0.01	1.75 ± 0.01	1.19 ± 0.01

[a] Exp. – experimental; h – hour; SG – specific gravity; TTA – total titratable acidity.

attributed to its inability to metabolize sucrose, a characteristic linked to the absence of β-fructosidase, which hydrolyses sucrose into glucose and fructose [29]. *P. kluyveri* is reportedly unable to ferment maltose and fructose, a characteristic associated with the lack of maltase and fructokinase [30,31]. As a result, *P. kluyveri* must be combined with *S. cerevisiae* to avoid sluggish fermentation and achieve the desired quality parameters [32]. Since marula fruit juice does not contain maltose, but mainly glucose, fructose, and sucrose [33,34], it can be suggested that *P. kluyveri* relied on the fermentation of glucose and sucrose. Conversely, *M. pulcherrima* has been shown to assimilate mainly glucose, fructose, sucrose, and cellobiose but not arabinose [35]. However, the fermentation of the carbon sources is limited given the yeast's dependence on oxidative metabolism [36]. Lastly, this study observed a high attenuation rate (83.3 %) for all three yeasts. In a study by Methner et al. [37], an average attenuation rate of above 80 % by *Saccharomycopsis fibuligera* at 20 °C, optimally producing a low-alcohol beer at around 0.8 % v/v.

3.1.2. The effect of temperature and time on total titratable acidity (TTA)

The highest TTA was observed for fermentations conducted at 10.00 °C for 336 h, followed by fermentations conducted at 10.00 °C for 168 h for all three yeasts (Table 3). Conversely, the lowest TTA (0.42) was observed with all yeasts when fermentations were conducted at 15 °C for 252 h. These results were within the range of TTA values of 0.31–1.10 %, found in other marula fruit beer production studies [38,39]. Generally, the concentration of TTA is dependent on the synthesis of organic acids such as acetic acid, citric acid, lactic acid, malic acid, propionic acid, and succinic acid present in the juice marula fruit and produced during alcoholic fermentation [40,41]. According to Liszkowska and Berlowska [42], the synthesis of organic acid is regulated by yeast metabolic activities, which are dependent and driven by a decrease in temperature, which is in agreement with the findings in this study, as shown by fermentations conducted at 10.00 °C for 168 and 336 h (Table 3) As a result, changes in TTA may be a better indication of the success rate of the fermentation process than changes in pH [43]. Low TTA and high pH are often associated with fermentations that are highly susceptible to bacterial spoilage [44,45].

3.1.3. The effect of temperature and time on pH

The lowest pH measurements for *M. pulcherrima*, *P. fermentans*, and *P. kluyveri* were 3.66, 3.76, and 3.77, respectively observed at 20 °C after 168 h (Table 3). In contrast, the highest pH (4.75) was observed with *P. fermentans* fermentations conducted at 15 °C for 133.21 h. The initial pH of the marula fruit juice affects the fermentation process, as the pH produces an acidic fermentation environment [46]. While the pH of marula fruit juice has been determined to range between 4.01 and 4.38 [34,47], marula fruit beers generally have a pH of approximately 2.80–3.88 [39,48]. Thus, a decrease in the average pH over time is correlated to the conversion of sugars to alcohol and other by-products and the subsequent acidification associated with an increase in the concentration of organic acids. The accumulation of these acids, especially acetic acid, lactic acid, and propionic acid showed significant pH reduction during

Table 4
Quadratic model analysis of variance (ANOVA) of low-alcohol marula fruit beer production using *Metschnikowia pulcherrima*, *Pichia fermentans*, and *P. kluyveri*.

Yeast	*M. pulcherrima*		*P. fermentans*		*P. kluyveri*	
Source	F-value	*p*-value	F-value	*p*-value	F-value	*p*-value
SG						
Model	22.24	0.00*	7.90	0.01*	9.14	0.01*
X_1 – Temperature	54.38	0.00*	24.73	0.00*	13.35	0.01*
X_2 – Time	23.46	0.00*	2.54	0.16	13.35	0.01*
X_1X_2	3.20	0.12	0.00	1.00	1.82	0.22
X_1^2	17.05	0.00*	6.90	0.03*	9.70	0.02*
X_2^2	17.05	0.00*	6.90	0.03*	9.70	0.02*
TTA						
Model	2.37	0.15	2.38	0.14	2.38	0.14
X_1 – Temperature	2.65	0.15	1.63	0.24	3.47	0.10
X_2 – Time	1.66	0.24	1.18	0.31	0.85	0.39
X_1X_2	0.11	0.76	0.03	0.87	0.00	0.96
X_1^2	2.77	0.14	3.07	0.12	2.41	0.16
X_2^2	5.58	0.05	7.04	0.03*	6.05	0.04*
pH						
Model	1.54	0.29	2.01	0.19	2.60	0.12
X_1 – Temperature	1.46	0.27	0.53	0.49	0.55	0.48
X_2 – Time	2.24	0.18	3.06	0.12	5.22	0.06
X_1X_2	0.00	0.96	0.03	0.87	0.00	0.96
X_1^2	0.37	0.56	1.24	0.30	0.26	0.63
X_2^2	3.26	0.11	4.46	0.07	6.54	0.04*
Alcohol						
Model	53.06	0.00*	66.34	0.00*	23.16	0.00*
X_1 – Temperature	96.26	0.00*	43.06	0.00*	17.10	0.00*
X_2 – Time	38.54	0.00*	51.01	0.00*	18.09	0.00*
X_1X_2	9.87	0.02*	1.24	0.30	5.69	0.05
X_1^2	115.86	0.00*	185.99	0.00*	66.04	0.00*
X_2^2	12.74	0.01*	77.75	0.00*	16.12	0.01*

* - significant at $p < 0.05$; SG – specific gravity; TTA – total titratable acidity.

marula fruit fermentation [40]. At a higher temperature and higher pH, certain non-*Saccharomyces* yeasts such as *Torulaspora delbrueckii* and *Starmerella bacillaris* show increased glycerol concentrations in the final products [49,50]. At lower temperatures and lower pH, the reverse effect was observed by Jolly et al. [36]. This observation confirmed our findings, specifically, for fermentations conducted at 8.00 °C for 252 h, 10.00 °C 336 h, and 15.00 °C 133.21 (Table 3). This may be attributed to yeast's production of glycerol to prevent dehydration by balancing the intracellular osmolarity with that of the acidic environment [51].

3.1.4. The effect of temperature and time on alcohol (% v/v) production

At the fermentation temperature between 15 °C and 20 °C, and fermentation time above 252 h, a comparatively higher alcohol content (1.09–1.75 % v/v) was observed for all three yeasts (Table 3). At 15 °C, alcohol increased between 133.21 h and 252 h and declined at 370.79 h. Zero alcohol content was observed for marula fruit beer fermented at 8.00 °C for 252 h. Overall, *P. kluyveri* produced the lowest alcohol levels, followed by *M. pulcherrima* and *P. fermentans,* respectively. In other marula fruit beer studies, the alcohol content ranged between 0.5 and 5 % v/v [48,52]. In wine, *P. kluyveri* was shown to produce 0.36 g of ethanol per gram of sugar, while *P. fermentans* produced 0.04 g of ethanol per gram of sugar [30]. In a study by Simões et al. [29], the alcohol content in low-alcohol beer after 240 h was 0.52 and 0.17 % v/v at 14 °C for *P. fermentans*, and *P. kluyveri*, respectively. This finding was consistent with our findings of 0.65 and 0.44 % v/v after 168 h, and 0.21 and 0.24 % v/v after 133.21 h for *P. fermentans*, and *P. kluyveri*, respectively (Table 3). Another study was able to show that *P. fermentans* was intolerant to high temperatures and could not produce alcohol at temperatures above 37 °C [31]. Elsewhere, *P. kluyveri* was able to show an increase in alcohol production at increasing temperatures during the fermentation of agave [53]. The anamorphic form of *M. pulcherrima* (*Candida pulcherrima*) has an oxidative metabolic function that allows it to limit the rate of sugar metabolism and alcohol production at low temperatures [36]. Correspondingly, alcohol production by *C. pulcherrima* increased from 3.9 % v/v at 15 °C to 4.4 % v/v at 20 °C but declined to 3.7 % v/v at 28 °C during the production of Chenin Blanc wine [54]. Overall, all three yeast strains produced low alcohol levels, when compared to the maximum alcohol content of 2 % required for low-alcohol fruit beers [55].

3.2. Multi-response of process factors

Fisher's F-value was used to examine the best fit for each model (alcohol, pH, TTA, and SG) (Table 4). The significance of each model, and model terms (X_1, X_2, X_1X_2, X_1^2 and X_2^2, where X_1 = temperature and X_2 = time) was determined by Probability > F (*p*-value) (Table 4). Thus, for *p*-value <0.05, the model was significant. Likewise, significant model terms influenced the response. The reverse was true for both the model and model terms. The model for SG was significant for *M. pulcherrima* (*p*-value = 0.00), *P. fermentans* (*p*-value = 0.01), and *P. kluyveri* (*p*-value = 0.01). The model terms X_1, X_1^2 and X_2^2 were significant for all three yeasts. The model term X_2 was only significant for *M. pulcherrima* (*p*-value = 0.00) and *P. kluyveri* (*p*-value = 0.01). Only the model term X_2^2 (*p*-value = 0.04) was significant for TTA. The TTA model term X_2^2 for *P. fermentans* (0.03) and *P. kluyveri* (0.04) was significant. The model for pH was not significant for all three yeasts. The model for alcohol was significant for *M. pulcherrima* (*p*-value = 0.00), *P. fermentans* (*p*-value = 0.00), and *P. kluyveri* (*p*-value = 0.00). Equally, the alcohol model terms X_1, X_2, X_1^2 and X_2^2 were significant for all three yeasts. The alcohol model term X_1X_2 was only significant for *M. pulcherrima* (*p*-value = 0.02).

Table 5
Fit statistics of low-alcohol marula fruit beer production models using *Metschnikowia pulcherrima*, *Pichia fermentans*, and *P. kluyveri.*

	M. pulcherrima	P. fermentans	P. kluyveri
SG			
CV	0.28	0.62	0.36
R^2	0.94	0.85	0.87
Adjusted R^2	0.90	0.74	0.77
Predicted R^2	0.58	−0.07	0.06
Adequate Precision	13.10	7.32	7.61
TTA			
CV	29.02	27.87	30.48
R^2	0.63	0.63	0.63
Adjusted R^2	0.36	0.36	0.37
Predicted R^2	−1.64	−1.64	−1.63
Adequate Precision	3.58	3.76	3.65
pH			
CV	5.34	5.40	5.16
R^2	0.52	0.59	0.65
Adjusted R^2	0.18	0.30	0.40
Predicted R^2	−2.39	−1.92	−1.49
Adequate Precision	4.68	5.42	5.65
Alcohol			
CV	12.20	13.35	23.02
R^2	0.97	0.98	0.94
Adjusted R^2	0.96	0.96	0.90
Predicted R^2	0.82	0.85	0.59
Adequate Precision	21.89	20.05	12.11

CV – coefficient of variation; SG – specific gravity; TTA – total titratable acidity.

Generally, *M. pulcherrima*, *P. fermentans*, and *P. kluyveri* require 14–25 °C to properly metabolize carbon and nitrogen sources to produce by-products over time [56,57]. As shown by a decrease in the initial SG value (1.06) in the marula fruit juice, temperature and time affected the final SG values of the fermented product (Table 3). A suitable temperature range (15.00–22.07 °C) and time (252–370.79 h) positively influenced specific gravity. The metabolism of carbon sources into alcohol and other by-products are dependent on the viable cells) [58]. In contrast, temperature and time did not influence the TTA and pH of production models for all yeasts. During the fermentation process, TTA and pH have an inversely proportional relationship [59]. However, this relationship could not be ascertained as shown by the insignificant models (Table 4). This is attributed to the marula fruit's high initial TTA, and lower pH [41,46]. As a result, only insignificant changes can be observed with increasing or decreasing temperature and/or time (Table 3). Temperature and time influenced the production of alcohol by *M. pulcherrima*, *P. fermentans*, and *P. kluyveri* as shown by the significant values of the model and model terms (Table 4). This was an expected result since alcohol production depends on the yeast's temperature tolerances to effectively break down carbon sources over time.

The Coefficient of Variation (CV), R-squared (R^2), Adjusted R^2, Predicted R^2 and Adequate Precision described the model fit statistics (Table 5). R^2 measured the amount of variation around the mean explained by the model. The Predicted R^2 was in reasonable agreement with the Adjusted R^2 when the difference was less than 0.2. A difference greater than 0.2 indicated a problem with the data or the model. The Predicted R^2 values in the SG model for *M. pulcherrima* and *P. kluyveri* were not as close to the Adjusted R^2 values (i.e., the difference is more than 0.2). In this case, a different order polynomial could improve the model. Adequate Precision measured the signal-to-noise ratio. Adequate Precision was only below 4 for the TTA model for all three yeasts (Table 5). The Predicted R^2 values in the TTA model for *M. pulcherrima*, *P. fermentans*, and *P. kluyveri* were negative. Similarly, the Predicted R^2 values in the pH model for *M. pulcherrima*, *P. fermentans*, and *P. kluyveri* were −2.39, −1.92, and −1.49, respectively. The negative Predicted R^2 values suggest that the overall mean is a better predictor of the response than the current model. Alternatively, a higher-order model such as cubic could show better prediction. The agreement between the Predicted R^2 and the Adjusted R^2 was observed for alcohol production by *M. pulcherrima* and *P. fermentans*. A ratio greater than 4 was a desirable, adequate signal. Thus, the model could be used to navigate the design space.

Coded (or pseudo) prediction equations for *M. pulcherrima* (equations, (1), (2), (3), (4)), *P. fermentans* (equations, (5), (6), (7), (8)), and *P. kluyveri* (equations, (9), (10), (11), (12)) were used to identify the relative significance of the factors by comparing the factor coefficients. The coefficient estimate represented the expected change in response Y (where $Y_1 = SG$, $Y_2 = TTA$, $Y_3 = pH$, $Y_4 = Alcohol$) per unit change in X (where X_1 = Temperature, X_2 = Time) when all remaining factors were held constant. Variance Inflation Factor (VIF) which measured the variance around the coefficient estimate was 1 for orthogonal factors. VIFs greater than 1 indicated multicollinearity. Thus, the higher the VIF the more critical the correlation of factors. The positive sign in front of the terms indicated a synergetic effect, whereas the negative sign indicated an antagonistic effect. The coded prediction equations characterizing the influence of different considered variables on the response are shown below:

Metschnikowia pulcherrima

$$Y_1 = 1.01 - 0.0073X_1 - 0.0048X_2 + 0.0025X_1X_2 + 0.0044X_1^2 + 0.0044X_2^2 \quad (1)$$

$$Y\_(2) = 0.4185 - 0.0961X\_(1) - 0.0760X\_(2) - 0.0271X\_(1)\ X\_(2) + 0.1054X\_(1)^{\ 2} + 0.1496X\_(2)\hat{}(2)\ \_(\quad (2)$$

$$Y_3 = 3.82 - 0.0885X_1 - 0.1097X_2 - 0.0058X_1X_2 - 0.0475X_1^2 + 0.1417X_2^2 \quad (3)$$

$$Y_4 = 1.35 + 0.4048X_1 + 0.2561X_2 + 0.1833X_1X_2 - 0.4763X_1^2 - 0.1579X_2^2 \quad (4)$$

Pichia fermentans

$$Y_1 = 1.01 - 0.0110X_1 - 0.0035X_2 + 0.0000X_1X_2 + 0.0063X_1^2 + 0.0063X_2^2 \quad (5)$$

$$Y_2 = 0.4185 - 0.0737X_1 - 0.0629X_2 - 0.0137X_1X_2 + 0.1086X_1^2 + 0.1644X_2^2 \quad (6)$$

$$Y_3 = 3.88 - 0.0543X_1 - 0.1310X_2 - 0.0183X_1X_2 - 0.0894X_1^2 + 0.1698X_2^2 \quad (7)$$

$$Y_4 = 1.75 + 0.3195X_1 + 0.3477X_2 + 0.0767X_1X_2 - 0.7121X_1^2 - 0.4604X_2^2 \quad (8)$$

Pichia kluyveri

$$Y_1 = 1.01 - 0.0048X_1 - 0.0048X_2 - 0.0025X_1X_2 + 0.0044X_1^2 + 0.0044X_2^2 \quad (9)$$

$$Y_2 = 0.4185 - 0.1175X_1 - 0.0583X_2 - 0.0046X_1X_2 + 0.1051X_1^2 + 0.1664X_2^2 \quad (10)$$

$$Y_3 = 3.78 - 0.0524X_1 - 0.1616X_2 - 0.0050X_1X_2 - 0.0385X_1^2 + 0.1940X_2^2 \quad (11)$$

$$Y_4 = 1.19 + 0.2431X_1 + 0.2500X_2 + 0.1983X_1X_2 - 0.5123X_1^2 - 0.2531X_2^2 \quad (12)$$

The behaviour of the process was further explained by 3D surface and 2D contour graphs. The terms with significant effects are shown in each model graph (Figs. 1–3, Supplementary S1 – S3). The graphs for SG and TTA took an inverted-dome shape, with higher

values observed at increasing temperatures and time (Fig. 1A – B, 2A – B, 3A – B). At a constant temperature of 10 °C, the SG was the highest as shown by a dark red shading between 168 and 210 h for all three yeasts (Figs. 1A, 2A and 3A; Supplementary S1A, S2A, S3A). Lower SG values were observed for higher temperatures (15–22.07 °C) and longer fermentation times (252–336 h) as shown by a dark blue shading (Figs. 1A, 2A and 3A; Supplementary S1A, S2A, S3A). At a constant temperature of 15 °C, the TTA was lower between 168 and 252 h for all three yeasts as shown by a dark blue shading (Figs. 1B, 2B and 3B; Supplementary S1B, S2B, S3B). The surface graphs for pH took a planar shape, not clearly showing a smooth transition towards lower or high points (Figs. 1C, 2C and 3C; Supplementary S1C, S2C, S3C). This further illustrates the insignificance of the quadratic pH model and model terms for all three yeasts (Table 4). The surface graphs for alcohol production were domed-shaped, with lower alcohol production points (shaded light blue) observed at lower temperatures and a relatively shorter time (Figs. 1D, 2D and 3D; Supplementary S1D, S2D, S3D). The graphs further illustrate the reverse effect of temperature and time on alcohol production, with a higher temperature and time associated with higher temperatures (15–20 °C), and time longer fermentation times (252–3736 h) (shaded red). The lowest alcohol (0.00–0.20 % v/v) formation was observed for a fermentation temperature below 10 °C. At this temperature, alcohol formation increased over time (168–336 h) as shown by the transition from a light blue shade to a light yellow shade (Fig. 1D, Supplementary S1D).

Fig. 1**(A–B)**. 3D surface graphs showing the effect of temperature and time on specific gravity (A) and total titratable acidity (B) in marula fruit beer fermented by *Metschnikowia pulcherrima*.

Fig. 2 **(C–D)**. 3D surface graphs showing the effect of temperature and time on pH (C) and alcohol (D) in marula fruit beer fermented by *Pichia fermentans*.

Fig. 3 **(C–D)**. 3D surface graphs showing the effect of temperature and time on pH (C) and alcohol (D) in marula fruit beer fermented by Pichia kluyveri.

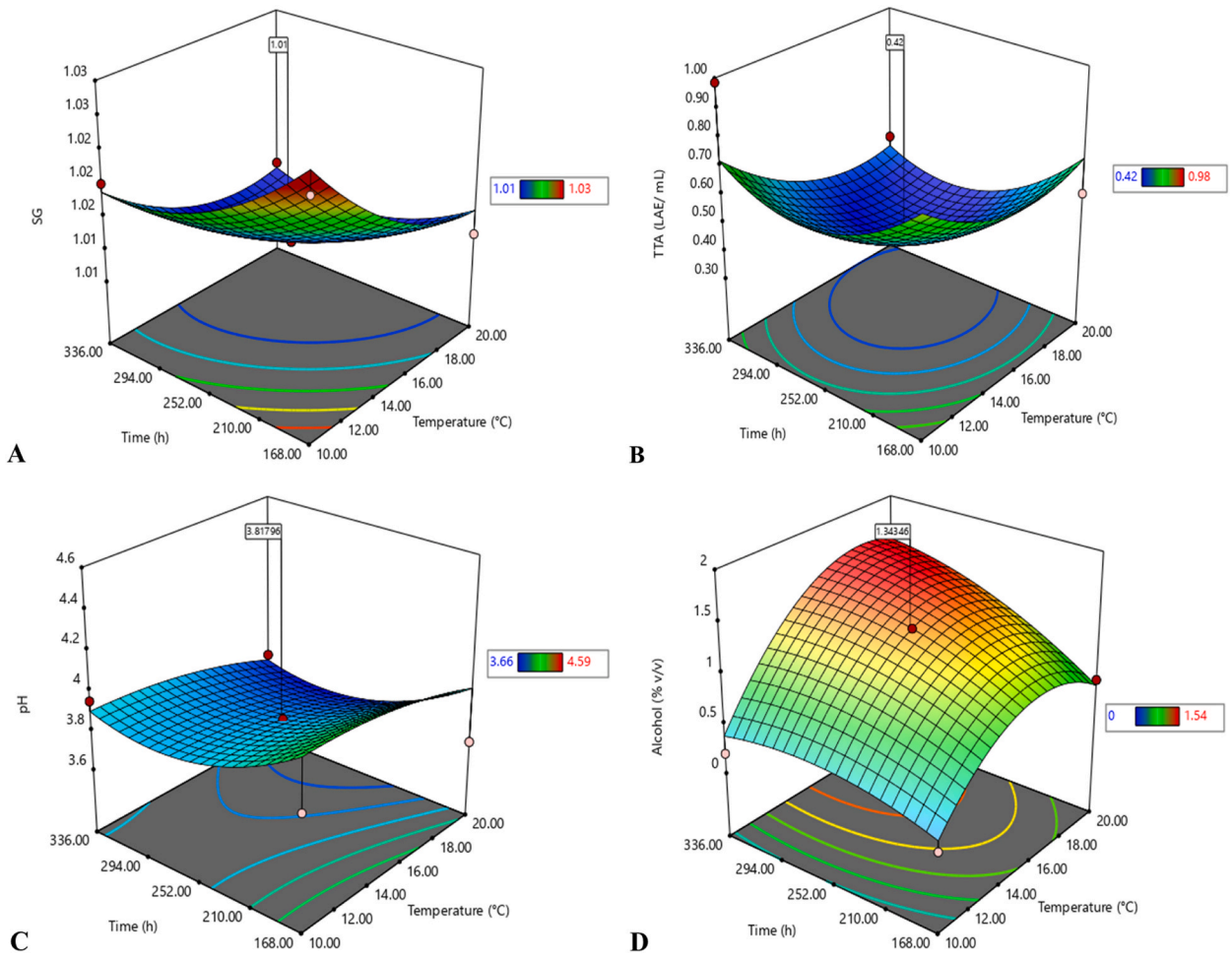

Fig. 1. (C–D). 3D surface graphs showing the effect of temperature and time on pH (C) and alcohol (D) in marula fruit beer fermented by *Metschnikowia pulcherrima*.

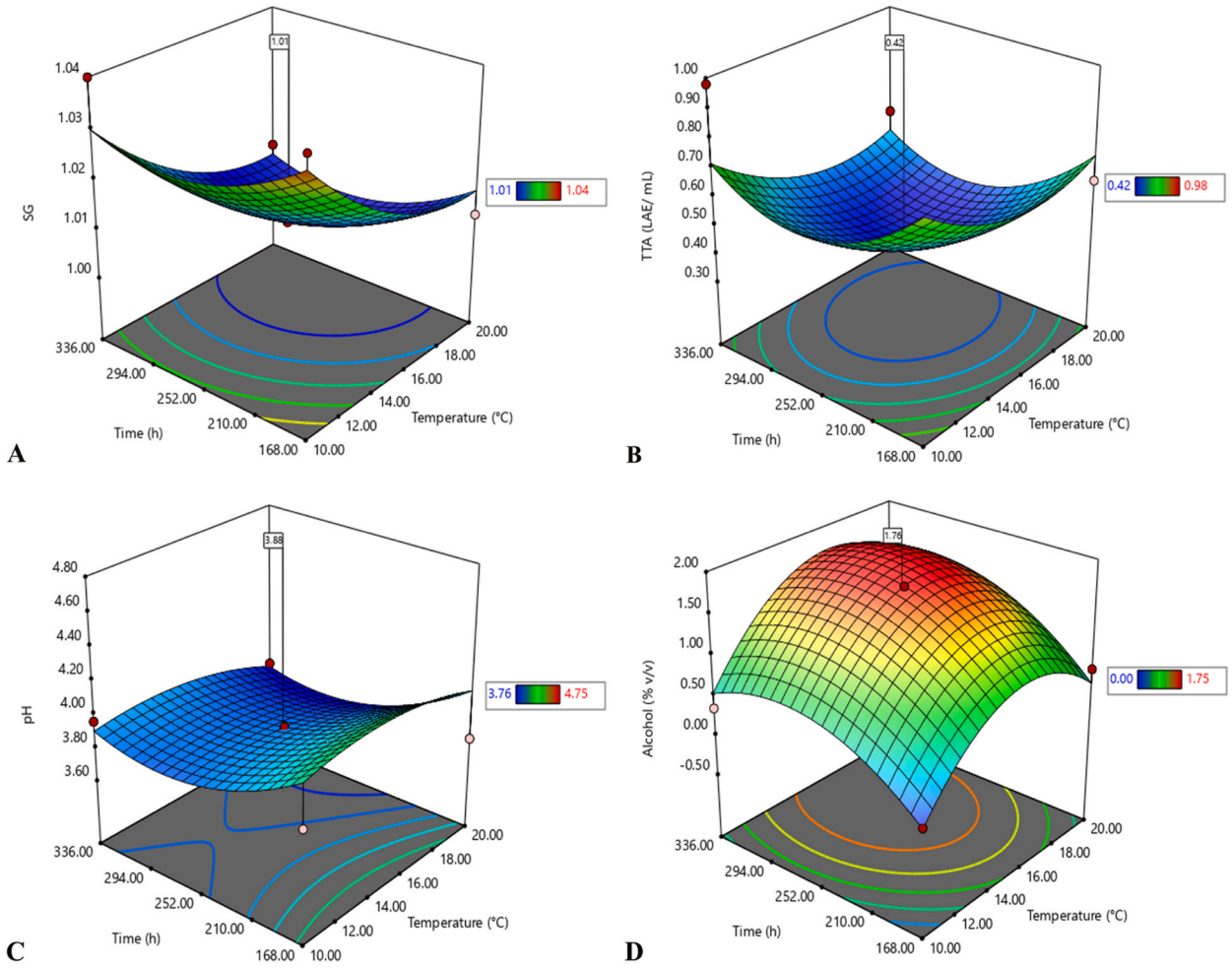

Fig. 2. (A–B). 3D surface graphs showing the effect of temperature and time on specific gravity (A) and total titratable acidity (B) in marula fruit beer fermented by *Pichia fermentans*.

4. Conclusion

Low-alcohol marula fruit beer was produced by cold-contact fermentation using *M. pulcherrima*, *P. fermentans*, and *P. kluyveri*. Temperature and time influenced the specific gravity (SG) and level of alcohol produced. Above 15 °C, the SG values decreased with increased temperature and time. Fermentation at temperatures below 10 °C produced low-alcohol marula fruit beer (0.00–0.20 % v/v) with an attenuation rate above 80 %. The lowest alcohol content was observed for marula fruit beer fermented at 8 °C for 252 h. Overall, *P. kluyveri* showed the lowest production of alcohol, followed by *M. pulcherrima* and *P. fermentans*, respectively. Cold-contact fermentation by non-*Saccharomyces* was shown to be an effective biological method to produce low-alcohol marula fruit beer in line with the emerging demand for low-alcohol beverages. However, the sugar profile and flavour compounds of the final beer product produced by the selected yeast under the optimum fermentation conditions should be further investigated. The impact of cold-contact fermentation using non-*Saccharomyces* yeast on the sensory profile and quality of the product should be investigated before the production process can be upscaled.

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Consent for publication

All authors gave permission for the research article to be published.

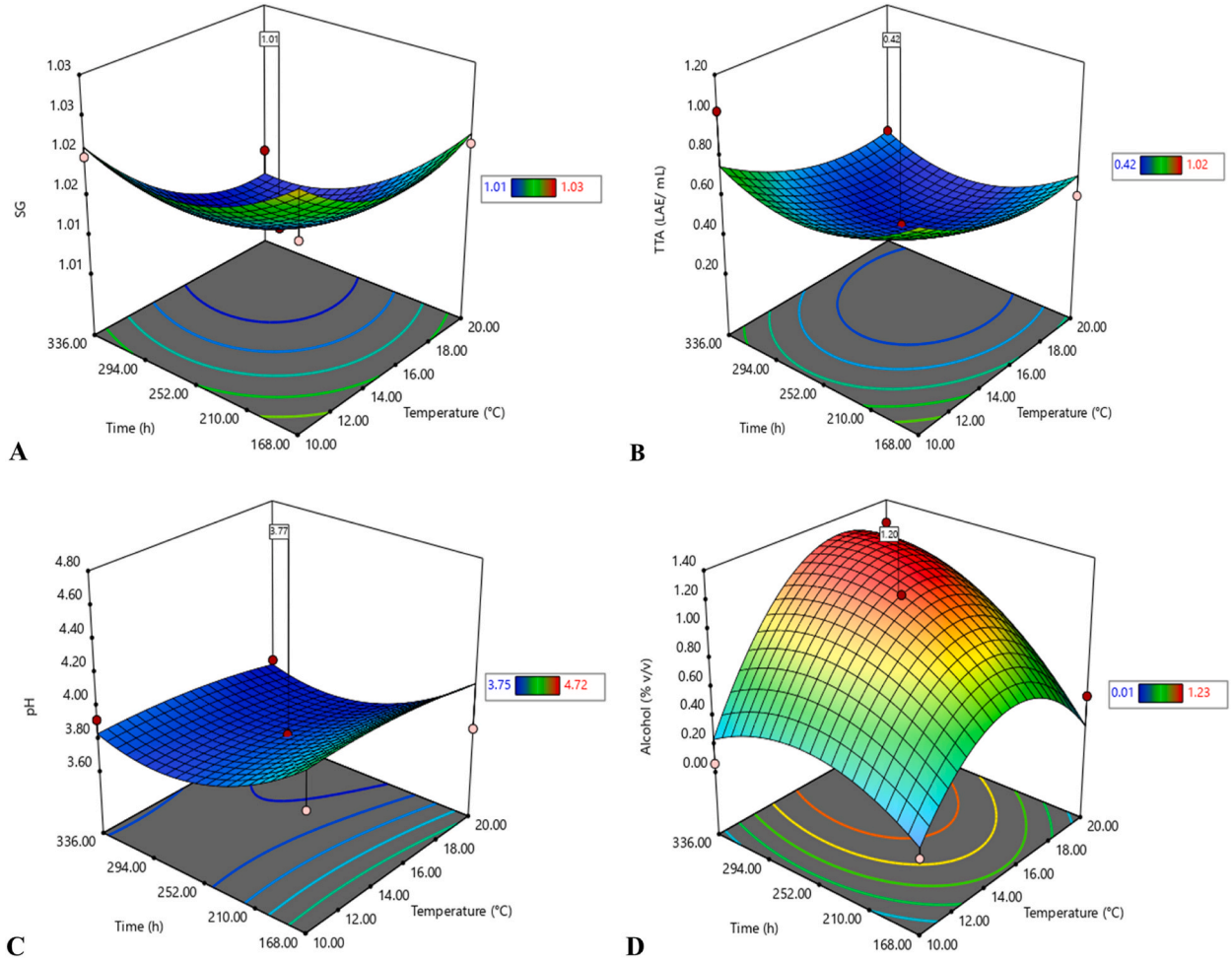

Fig. 3. (A–B). 3D surface graphs showing the effect of temperature and time on specific gravity (A) and total titratable acidity (B) in marula fruit beer fermented by *Pichia kluyveri*.

CRediT authorship contribution statement

Edwin Hlangwani: Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Heinrich W. du Plessis:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Bhekisisa C. Dlamini:** Writing – review & editing, Validation, Resources, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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