LWT - Food Science and Technology 223 (2025) 117754

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Butter aroma compounds in plant-based milk alternatives through fermentation: screening of potential starter cultures and enhanced production in oat milk with *Lactococcus cremoris*

Sebastián M. Tapia [a], Ana Moreno-Ruiz [a], Andrea Tres [a], Nerea Moreno-Yerro [a], Patricia Arrubla [a], Miguel Gastón-Lorente [a], María Luz Mohedano [b], Raquel Virto [a], Dante Fratebianchi [a,*]

[a] National Centre for Food Technology and Safety (CNTA), San Adrian, 31570, Spain
[b] Centro de Investigaciones Biológicas Margarita Salas (CIB-CSIC), Madrid, 28040, Spain

ARTICLE INFO

Keywords:
Plant-based milk alternatives
Lactic acid bacteria
Diacetyl
Acetoin

ABSTRACT

Acetoin and diacetyl are aromatic compounds that confer creamy and butter aroma to products such as yogurt and cheese. As such, they can also impart desired sensory properties to plant-based products such as dairy analogues. These compounds are produced by lactic acid bacteria, but few strains can produce amounts high enough to be considered relevant strains for its use as fermentation starters. Therefore, in this study we aimed to identify a great acetoin and diacetyl bacteria producer using different reported methods. We identified the *Lactococcus cremoris* strain CNTA 939 as a great producer of these compounds and optimized its production in synthetic medium and in plant-based milk alternatives. In the latter, it was detected that the availability of free amino acids is crucial for allowing good fermentation performance by strain CNTA 939, and it was demonstrated that relevant acetoin and diacetyl titers (115 and 8 mg/L, respectively) can be achieved without adding external supplements.

1. Introduction

Diary analogues are plant-based products which are meant to resemble the texture, appearance, flavour and nutritional profile of dairy products such as milk, yogurt and cheese (Jeske et al., 2018; Kamath et al., 2022; Tangyu et al., 2019). Although global milk production is projected to grow at 1.5 % per year to 1039 Mt by 2032, the per capita consumption of dairy products is expected to decline in Europe, Oceania and North America partly due to the increasing consumption of plant-based products (OECD & FAO, 2023). Among them, consumption of plant-based milk alternatives (PBM) has increased over the last decade, drove by environmental sustainability awareness, health concerns such as cow's milk allergy and lactose intolerance, ethical disputes regarding the use of animals and the consumers lifestyle changes towards vegetarian and vegan foods (Jeske et al., 2018; Pua et al., 2022; Tangyu et al., 2019). The European and USA markets were worth $1.5 billion in 2015 and $1.8 billion in 2016, respectively (Jeske et al., 2018; Tangyu et al., 2019), and some reports foresee that PBM industry will witness a compound annual growth rate between 11.7 and 15 % up to a global market size of $47.55 to $123.1 billion by 2030 (Meticulous Research, 2023; Strategic Market Research, 2022). PBM are water extracts of plant material including legumes, nuts, seeds, cereals, and pseudo cereals. Although some raw plant material such as legumes and seeds show a protein content comparable to cow milk, the produced PBMs are often nutritionally unbalanced in terms of amino acid and vitamin content, and their organoleptic properties are not desired since they exhibit unpleasant green and beany flavours derived from aromatic compounds such as hexanal and hexanol found in the plant matrix (Tangyu et al., 2019). To improve its organoleptic properties, exogenous flavouring additives are added, yet they are perceived as artificial by the consumers. Therefore, leading companies have aimed to exclude those additives since clean label products are preferred by the consumers (Asioli et al., 2017). Fermentation can be a suitable approach to improve flavours in PBMs since microorganisms such as lactic acid bacteria (LAB) can produce volatile aroma compounds that impart attractive flavour profiles to the fermented foods (Peyer et al., 2016). Among them,

* Corresponding author.
E-mail address: dfratebianchi@cnta.es (D. Fratebianchi).

https://doi.org/10.1016/j.lwt.2025.117754
Received 18 January 2025; Received in revised form 2 April 2025; Accepted 5 April 2025
Available online 9 April 2025

diacetyl and acetoin, which are desired since they confer creamy and buttery flavours to the fermented dairy products such as yogurt, cheese and butter (Liu et al., 2021a; Liu et al., 2020; Xiao & Lu, 2014), positively contribute to the flavour of fermented PBMs (Peyer et al., 2016; Tangyu et al., 2019). Both acetoin and diacetyl are intermediate metabolites of the C4 compound pathway. Briefly, two molecules of pyruvate, which are produced by glycolysis, are condensed into α-acetolactate (α-AL) by the α-AL synthase. Then, diacetyl is formed from oxidative decarboxylation of α-AL, whereas acetoin is formed either by the enzymatic reduction of diacetyl or after enzymatic decarboxylation of α-AL carried out by the acetoin/diacetyl reductase and α-AL decarboxylase, respectively. Finally, acetoin can be reversibly converted into 2,3-butanediol (Hugenholtz et al., 2000). There are some LAB strains capable of metabolizing citrate such as the well-studied *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, which show typically high diacetyl and acetoin production (García-Quintáns et al., 2008; Hugenholtz & Starrenburg, 1992). Citrate is taken into the cell by the citrate permease (CitP) and converted into oxaloacetate by the citrate lyase complex (CitDEF), which then is transformed into pyruvate by the oxaloacetate decarboxylase (CitM) (García-Quintáns et al., 2008; Smid & Kleerebezem, 2014). Many LAB species have been associated with African and Asian traditional non-alcoholic fermented PBMs (Chileshe et al., 2020; Nout, 2009) and several have been granted the GRAS (Generally Recognized as Safe) (Liu et al., 2021b; Peyer et al., 2016) and/or QPS (Qualified Presumption of Safety) (Koutsoumanis et al., 2024) status. Therefore, isolating and selecting high acetoin- and diacetyl-producing strains would offer a great opportunity to be used as starter cultures to develop robust and reproducible fermented PBMs processes (Boeck et al., 2022; Youssef et al., 2020) and obtain dairy analogues with the valuable sensory attributes. However, since the ability to produce higher amounts of both diacetyl and acetoin is strain-dependent (Franciosi et al., 2009; Hugenholtz & Starrenburg, 1992; Passerini et al., 2013), many methods have been employed to identify them, such as the Voges-Proskauer (Speckman & Collins, 1982) and Kempler & McKay (Kempler & McKay, 1980) methods which detect the production of acetoin and citrate-fermenting strains, respectively. In addition, PCR has been used to identify *L. lactis* subsp. *lactis* biovar. *diacetylactis* through the detection of the genes encoding the CitP, CitDEF and CitM proteins and specific subspecies markers (Beimfohr et al., 1997; Passerini et al., 2013). However, the quantification of both compounds by chromatographic methods is the most effective approach to select the best producing strains.

Therefore, this work had two goals. On one hand, we aimed to identify LAB strains capable of producing high acetoin and diacetyl levels by screening isolates from different sources employing the reported methods and to compare them to determine whether they could be reliable for strain selection. On the other hand, we carried out fermentations with the best wild type strain in oat milk as a representative PBM to overproduce both acetoin and diacetyl and, hence, naturally improve the organoleptic profile of plant-based fermented products.

2. Material and methods

2.1. Bacterial strains and growth conditions

23 LAB strains belonging to the *Lactococcus* (7) and *Leuconostoc* (6) genera as well as 10 lactobacilli from diverse origins were used in this study and are listed in Table 1. Liquid precultures were carried out as follows: lactobacilli and *Leuconostoc* strains were grown overnight in MRS (Merck, Darmstadt, Germany) at 37 °C and 30 °C, respectively, whereas *Lactococcus* strains were cultivated overnight in M17 (Oxoid, Basingstoke, UK) at 30 °C. The genetically modified strains IL1403 [pFL3] and IL1403[pFL4] were provided by CIB-CSIC, Spain; their genotype is described in Table 2.

Table 1
Lactic acid bacteria strains used in the present study.

Strain	Species	Source
CECT 539	*Lactococcus lactis*	Unknown
CNTA 159	*Lactococcus lactis*	Artichoke by-product
CNTA 162	*Lactococcus lactis*	Bean by-product
CNTA 939	*Lactococcus cremoris*	Altered cream
CNTA 1191	*Lactococcus lactis*	Sheep cheese
CNTA 1192	*Lactococcus lactis*	Sheep cheese
CNTA 1562	*Lactococcus lactis*	Sheep milk
CNTA 1201	*Companilactobacillus crustorum*	Cheese
CNTA 684	*Lactiplantibacillus plantarum*	Fermented olives
CNTA 1223	*Lactiplantibacillus plantarum*	Fermented lentils
CNTA 1232	*Lactiplantibacillus plantarum*	Cheese
CNTA 1238	*Lactiplantibacillus plantarum*	Sheep cheese
CNTA 1241	*Lactiplantibacillus plantarum*	Cheese
CNTA 1251	*Lactiplantibacillus plantarum*	Sheep cheese
CNTA 752	*Lacticaseibacillus rhamnosus*	Cream caramel
CNTA 756	*Lacticaseibacillus rhamnosus*	Vanilla cream caramel
CNTA 759	*Lacticaseibacillus rhamnosus*	Greek yogurt
CNTA 163	*Leuconostoc mesenteroides*	Garlic by-product
CNTA 165	*Leuconostoc mesenteroides*	Asparagus by-product
CNTA 170	*Leuconostoc mesenteroides*	Pea by-product
CNTA 184	*Leuconostoc mesenteroides*	Tomato by-product
CNTA 205	*Leuconostoc mesenteroides*	Sourdough
CNTA 607	*Leuconostoc mesenteroides*	Home-made chorizo

2.2. Biochemical assays

As a first screening to select potential good bacteria producers of acetoin and diacetyl, the strains were submitted to the Kempler & McKay (KMK) (Kempler & McKay, 1980) and Voges-Proskauer (VP) (Speckman & Collins, 1982) biochemical tests. Briefly, the LAB strains were grown overnight as was mentioned above, then streaked in KMK agar plates (5 g/L glucose; 2.5 g/L tryptone; 10 g/L skim milk powder; 15 g/L bacteriological agar; 10 g/L potassium ferricyanide; 0.25 g/L sodium citrate; 0.25 g/L iron citrate) and incubated during 48 h at their corresponding temperatures. Plates showing prussian blue colonies were considered as citrate-positive strains (Kempler & McKay, 1980). For the VP test, overnight precultures were inoculated into semi-synthetic medium (SSM; 5 g/L glucose; 10 g/L yeast extract; 9 g/L monopotassium phosphate; 7.5 g/L dipotassium phosphate; 0.2 g/L magnesium sulphate heptahydrate; 0.05 g/L manganese sulphate hydrate) at their corresponding temperatures for 24 h. Then 1 mL of supernatant was mixed with 0.5 mL of 1-naphthol solution (1 % w/v dissolved in ethanol) and 0.5 mL of 16 % w/v potassium hydroxide solution and incubated at 30 °C for 1 h. Supernatants that turned red were considered as positive phenotype regarding acetoin production (Passerini et al., 2013; Speckman & Collins, 1982).

2.3. Identification by molecular markers

The *Lactococcus* strains were grown overnight as indicated above and the DNA was extracted from the bacterial pellets using GenElute™ Bacterial Genomic DNA Kit (Merck, Darmstadt, Germany). Identification of the *Lactococcus lactis* subspecies was performed by PCR using the pair primers Lhis5F/Lhis6R and LLhis3F/LLhis4R (Beimfohr et al., 1997). The reaction products result in 1149 and 934 base pair amplicons in case the strain belongs to the subspecies *cremoris* and *lactis/diacetylactis*, respectively, whereas the latter pair primers give 343 base pair and no amplification to the subspecies *lactis* and *diacetylactis*, respectively. To detect the presence of the plasmidic *citP* gene encoding the citrate transporter and the chromosomal *citM-citI-citCDEFXG* operon encoding the citrate lyase complex and the oxalate decarboxylase responsible for conversion of citrate into pyruvate, which are specific to the subspecies *diacetylactis*, the pair primers citM_F/citG_R (7209 bp amplicon) and cit1P/cit2P (1327 bp amplicon) (Passerini et al., 2013) were used. The PCR reactions to identify the subspecies and to detect the *citP* gene were carried out using the IMMOLASE™ DNA polymerase

Table 2
Genetically modified lactic acid bacteria strains used in the present study.

Strain	Species	Characteristic and genotype	Reference
IL1403 [pFL3]	*Lactococcus lactis* subsp. *lactis* bv. *diacetylactis*	Carries the plasmid pFL3 that harbours the *tetL* gene conferring tetracycline resistance and the *citP* gene encoding the Cit P transporter from the plasmid pCIT264 under the control of the *Streptococcus pneumoniae polA* promoter	(Bourel et al., 1996; Magni et al., 1994)
IL1403 [pFL4]	*Lactococcus lactis* subsp. *lactis* bv. *diacetylactis*	Carries the plasmid pFL3 plasmid that harbours the *tetL* gene and the *citP* gene with its native promoter from the plasmid pCIT264.	Magni et al. (1994)

(Meridian bioscience®, London, UK) following the provider instructions. Meanwhile, the PCR to detect the *citM-citI-citCDEFXG* operon was performed using the Phusion™ High–Fidelity DNA Polymerase (Thermo Fisher Scientific, MA, USA) following the provider instructions. DNA from IL1403[pFL4] strain was used as positive control in both *citP* and *citM-citI-citCDEFXG* PCR reactions. Primer sequences are listed in Table 3.

2.4. Chemical analyses

Sugars in the samples were extracted with water (heating to 60–70 °C for 5 min, followed by agitation at 800 rpm, centrifugation and filtration of the resulting supernatant) and analyzed by reversed-phase liquid chromatography using a Waters HPLC® system (Waters Corporation, Milford, MA, USA) equipped with a Luna® Omega 3 μm Sugar column 100 Å, (LC Column 250 × 4.6 mm, Phenomenex, Torrance, CA, USA) and refractive index detector (2414 Refractive Index Detector Waters). Organic acids were analyzed by reversed-phase liquid chromatography using a Waters HPLC® system (Waters Corporation) equipped with an Aminex® HPX-87H Column (300 × 7.8 mm, BIO-RAD, Hercules, CA, USA) and determined by PDA detector (2998 PDA Detector Waters) at 210 nm. Acetoin and diacetyl (2,3-butanedione) were determined by gas chromatography using a gas chromatograph 7890A (Agilent Technologies, Palo Alto, CA, USA) equipped with a capillary column DB-WAX (30 m × 0.25 mm, 0.25 μm) with flame ionization detector (GC-FID). The oven temperature program was as follows: the initial temperature (50 °C) was held for 4.0 min, then increased to 140 °C at 15 °C/min, held for 1.0 min, and finally ramped to 200 °C at 12 °C/min and held for 2.0 min. The injector and detector temperatures were 250 °C and 300 °C, respectively. One microliter was injected into the column, in split mode, with a split ratio of 50:1. In all analytical methods external calibration was performed and duplicate samples were analyzed.

2.5. Flask fermentation conditions for strains screening

Flask experiments were performed to quantify the ability of the selected strains to produce both acetoin and diacetyl. Overnight precultures were inoculated in SSM-G (SSM with 30 g/L glucose instead of 5 g/L) and SSM-GC (SSM-G with 8.8 g/L of sodium citrate dihydrate) media (Passerini et al., 2013) at initial concentration values of 1×10^6 cells/mL and incubated at 30 °C for 24 h. Fermentation products were quantified by the methods described above.

Table 3
Primer sequences used in this study.

Primer	Sequence (5' → 3')	Reference
Lhis5F	CTTCGTTATGATTTTACA	Beimfohr et al. (1997)
Lhis6R	AATATCAACAATTCCATG	Beimfohr et al. (1997)
LLhis3F	AAAGAATTTTCAGAGAAA	Beimfohr et al. (1997)
LLhis4R	ATTTAGAATTGGTTCAAC	Beimfohr et al. (1997)
cit1P	ATGATGAATCACCCG	Passerini et al. (2013)
cit2P	ACTTCATGAATATGAC	Passerini et al. (2013)
citM_F	ATGAATGCAGCCAAGTTAG	Passerini et al. (2013)
citG_R	TGATGTGAACCGTTAGTTAC	Passerini et al. (2013)

2.6. Acetoin and diacetyl production optimization assays

To optimize the production of acetoin and diacetyl, the selected strains CNTA 159 and CNTA 939 were inoculated in flasks containing 50 mL of M17 liquid medium supplemented with 30 g/L of glucose (M17-G). After 24 h fermentation in a laboratory shaker set at 30 °C and 150 rpm, the pH of the culture was increased up to 6.8–7.0 using 1 M NaOH and was incubated for another 24 h. Samples were taken to determine the production of acetoin and diacetyl after 24 and 48 h of fermentation. Then, bioreactor assays were performed to test the effect of pH on the production of these compounds. Fermentations were carried out with the CNTA 939 strain in 500 mL Applikon MiniBio bioreactors (Getinge Applikon, Delft, Netherlands) at two different conditions. Briefly, overnight CNTA 939 precultures were used to inoculate 400 mL of M17-G and the pH was maintained at 6.5 (M17-G-N) or 4.8 (M17-G-A). The temperature was kept at 30 °C with a stirring speed of 300 rpm for 48 h. Dissolved oxygen was maintained over 20 % along the process. Samples were taken after 24 and 48 h of fermentation to quantify sugar consumption and the production of organic acids and C4 compounds. Moreover, as proof of concept to validate the relation between citrate metabolism and acetoin and diacetyl production, strains IL1403[pFL3] and IL1403[pFL4] were inoculated in flasks containing 50 mL of M17 medium supplemented with 30 g/L of glucose and 8.8 g/L of sodium citrate dihydrate (M17-GC) plus 1 μg/mL of tetracycline and were incubated at 30 °C for 24 h. As control, the strains were grown in M17-G plus 1 μg/mL of tetracycline medium as well. Samples were taken at the beginning and at the end of fermentation to quantify acetoin, diacetyl and organic acids.

2.7. Oat milk fermentation

Fermentations of oat milk were carried out in 500 mL Applikon MiniBio bioreactors using the CNTA 939 strain as fermentation starter. Oat syrup containing high amounts of sugars and protein was kindly provided by the Spanish food company AMC GLOBAL. For each bioreactor assay, oat milk was prepared dissolving oat syrup in distilled water to a final concentration of 130 g/L and then it was sterilized. To optimize acetoin and diacetyl production in oat milk different key nutrients were added (Table 4). Amino acids were supplemented adding 1.92 g/L yeast synthetic drop-out medium supplements without uracil. Vitamins, minerals and trace elements were supplemented as concentrated stocks to obtain the final amount according to Jensen & Hammer (1993). 100X vitamin stock was prepared as follows: 9.77 mg/L biotin, 98.44 mg/L

Table 4
Combination of the different stock nutrients tested in oat milk to optimized acetoin and diacetyl production. OM: oat milk; AA: Amino acids; V: Vitamins; M: Mineral and trace elements; YE: Yeast extract and F: Flavourzyme® treatment.

Condition	AA	V	M	YE	F
OM					
OM_AA	X				
OM_V		X			
OM_AA_M	X		X		
OM_AA_V	X	X			
OM_AA_V_M	X	X	X		
OM_YE				X	
OM_F					X

nicotinic acid, 97.85 mg/L riboflavin, 205.64 mg/L pyridoxine-HCL, 101.18 mg/L thiamine-HCl. 1000X folic acid stock was prepared dissolving folic acid in 0.1 M NaOH at a concentration of 1015.22 mg/L. 100X salt stock solution was composed of: 292.2 g/L sodium chloride, 28.6 g/L monopotassium phosphate, 4.95 g/L magnesium chloride, 0.28 g/L iron sulphate heptahydrate and 4.88 g/L potassium sulphate. 10000X micronutrients stock was prepared as follows: 554.95 mg/L calcium chloride, 24.97 mg/L copper sulphate pentahydrate, 7.26 mg/L sodium molybdate dihydrate, 247.32 mg/L boric acid, 38.95 mg/L cobalt chloride, 158.33 mg/L manganese chloride tetrahydrate and 28.76 mg/L zinc sulphate heptahydrate. Oat milk supplemented with 5 g/L of yeast extract (Springer® 0351, BioSpringer, Maisons-Alfort, France) was used as control. To enrich its amount in assimilable nitrogen, oat milk was treated with the peptidase mix Flavourzyme® (Novozymes, Copenhagen, Demark) as follows: 0.2 % w/v of enzyme was added to oat milk and was incubated at 50 °C for 4 h. Then, the treated milk was sterilized. All fermentations were carried out for 48 h in 500 mL Applikon MiniBio bioreactors with 400 mL working volume. An overnight preculture of CNTA 939 strain in M17 medium was inoculated at 1 % v/v. The temperature was controlled and maintained at 30 °C. A constant air flux was set at 0.1 vvm and initial stirring speed was set at 300 rpm. Dissolved oxygen was maintained at 20 % and controlled by stirring cascade up to 600 rpm. pH was maintained at 6.5 using a 2 M KOH solution. Samples were taken at 24 and 48 h to quantify sugar consumption and the production of organic acids, acetoin and diacetyl.

2.8. Statistical analyses

All One-way ANOVA, Tukey's multiple comparisons and *t*-test analyses were performed using GraphPad Prism software version 10.3.0 for Windows 10 64-bits (www.graphpad.com).

3. Results and discussion

3.1. Selection of best acetoin- and diacetyl-producing strains and comparison of the screening methods

23 LAB strains were submitted to the VP and KMK assays. The results (Table 5) indicated that three lactococci, five lactobacilli and one *Leuconostoc* showed positive VP phenotype, meaning that were able to produce and accumulate acetoin. All *Lactococcus* strains except one showed positive KMK phenotype suggesting they are citrate-fermenting bacteria. On the other hand, three lactobacilli and only one *Leuconostoc* showed positive KMK phenotype. The molecular characterization (Table 6) showed that six *Lactococcus* strains belonged to the subspecies *lactis* and only one to the subspecies *cremoris*. However, the biovariety *diacetylactis* was not identified. Unexpectedly, the presence of both *citP* and *citM-citI-citCDEFXG* genes was not detected in any *Lactococcus* strain, not even in those that showed positive KMK phenotype and belonged to the subspecies *lactis*. The control strain IL1403[pFL4] was the only one that harboured all citrate transport and catabolic genes. Thus, there is no evident relation between KMK results and the molecular characterization. Based on the previous results, seven strains showing different positive VP and KMK phenotypes were selected (*Lactococcus* strains CNTA 539, CNTA 159, CNTA 162 and CNTA 939; and lactobacilli strains CNTA 1232, CNTA 1238 and CNTA 1241). The above-mentioned strains were cultured in SSM-G and SSM-GC to test whether they can consume citrate and overproduce acetoin and diacetyl or not, and to select the best producing strains. Among *Lactococcus*, the strains CNTA 159 and CNTA 939 produced 60.9 mg/L and 111.9 mg/L of acetoin, respectively, after 24 h in the SSM-G condition. Moreover, the CNTA 159 strain was able to produce 8.47 mg/L of diacetyl in this condition (Fig. 1A). However, none of the *Lactococcus* strains was able to consume citrate, including those that showed positive KMK phenotypes, and hence, no increase was observed in the production of both acetoin and diacetyl in the SSM-GC condition compared to SSM-G. Under the SSM-GC condition, strain CNTA 939 produced 119.4 mg/L of acetoin, whereas no acetoin production was detected for strain CNTA 159 (Fig. 1A). Recent studies used positive KMK and VP phenotypes and the 939 bp amplicon detection as criteria to declare many *L. lactis* strains as subspecies *lactis* bv. *diacetylactis* (Fusieger et al., 2020) yet our results indicated that this approach is not suitable because there is not a clear relation between a KMK positive phenotype and citrate consumption. Therefore, the KMK biochemical test is not reliable to detect citrate-fermenting LAB. Based on our results and those obtained by Passerini et al. (2013), the best method to detect a citrate-fermenting *Lactococcus* strain, and in consequence, a potential good acetoin and diacetyl producer is the detection of citrate catabolic genes by PCR. Detection of the α-AL synthase, acetoin/diacetyl reductase and α-AL decarboxylase genes implied in C4 compounds production from pyruvate could complement the above-mentioned approach to identify promising acetoin/diacetyl-producing strains. As for the lactobacilli strains tested, different results were obtained since they consumed little amounts of citric acid (Fig. 1B) and they were only able to produce

Table 5
Biochemical phenotype showed by the 23 tested LAB strains. VP: (++) Strong red signal in the medium; (+) Weak red signal in the medium; (−) No colour change in the medium. KMK: (+) Prussian blue colonies; (−) White colonies. NG: No growth.

Strain	Species	VP	KMK
CECT 539	*Lactococcus lactis*	+	–
CNTA 159	*Lactococcus lactis*	–	+
CNTA 162	*Lactococcus lactis*	+	+
CNTA 939	*Lactococcus cremoris*	++	+
CNTA 1191	*Lactococcus lactis*	–	+
CNTA 1192	*Lactococcus lactis*	–	+
CNTA 1562	*Lactococcus lactis*	–	+
CNTA 1201	*Companilactobacillus crustorum*	–	NG
CNTA 684	*Lactiplantibacillus plantarum*	+	–
CNTA 1223	*Lactiplantibacillus plantarum*	+	–
CNTA 1232	*Lactiplantibacillus plantarum*	+	+
CNTA 1238	*Lactiplantibacillus plantarum*	+	+
CNTA 1241	*Lactiplantibacillus plantarum*	+	+
CNTA 1251	*Lactiplantibacillus plantarum*	–	–
CNTA 752	*Lacticaseibacillus rhamnosus*	+	–
CNTA 756	*Lacticaseibacillus rhamnosus*	–	–
CNTA 759	*Lacticaseibacillus rhamnosus*	–	–
CNTA 163	*Leuconostoc mesenteroides*	–	–
CNTA 165	*Leuconostoc mesenteroides*	–	–
CNTA 170	*Leuconostoc mesenteroides*	–	+
CNTA 184	*Leuconostoc mesenteroides*	–	–
CNTA 205	*Leuconostoc mesenteroides*	–	–
CNTA 607	*Leuconostoc mesenteroides*	–	–

Table 6
Molecular characterization of the *Lactococcus* strains. IL1403[pFL4] DNA was used as positive control for *citP* and *citM-citI-citCDEFXG* PCR reactions. NP: Not performed.

Strain	Lhis5F/ Lhis6R	LLhis3F/ LLhis4R	subspecie	*citP*	*citM-citI-citCDEFXG*
CECT 539	934	343	*lactis*	–	–
CNTA 159	934	343	*lactis*	–	–
CNTA 162	934	343	*lactis*	–	–
CNTA 939	1149	ND	*cremoris*	-	–
CNTA 1191	934	343	*lactis*	–	–
CNTA 1192	934	343	*lactis*	–	–
CNTA 1562	1149	343	*lactis*	–	–
IL1403 [pFL4]	NP	NP	*lactis* bv. *diacetylactis*	+	+

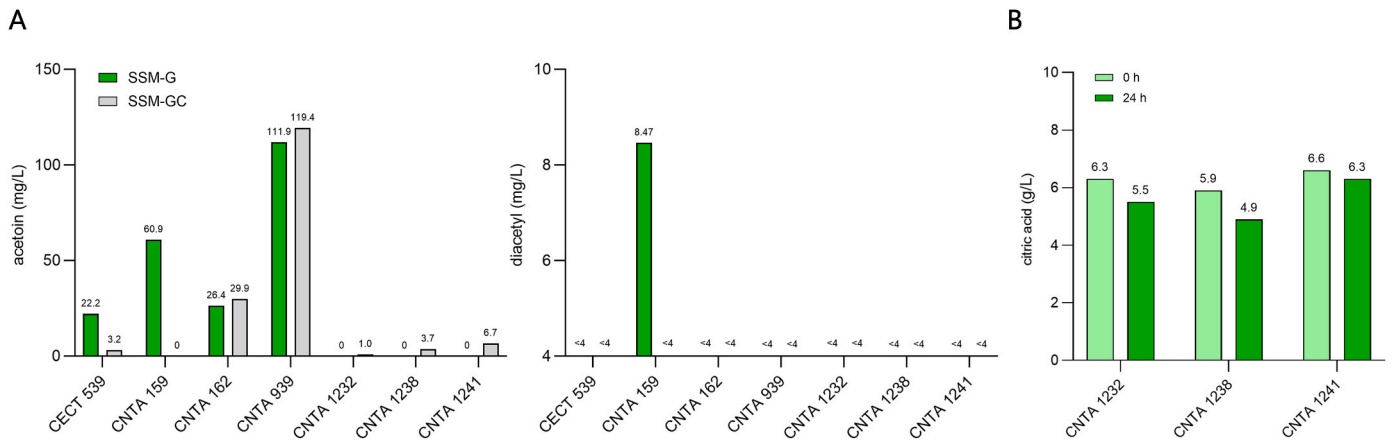

Fig. 1. (A) Production of acetoin and diacetyl after 24 h of fermentation by the tested LAB strains and (B) Initial and residual citric acid concentration after 24 h of fermentation with three *L. plantarum* strains.

acetoin in the condition SSM-GC, yet in much lower concentration compared with the amount produced by the *Lactococcus* strains CNTA 159 and CNTA 939 (Fig. 1A). The latter strain showed a strong positive VP phenotype but the remaining strains that were VP positive showed a weak red colour signal concordant with the lower acetoin production. Therefore, the VP method is reliable to detect a good acetoin producer only when a strong signal is observed. The results indicated that strains CNTA 159 and CNTA 939 were the best acetoin or diacetyl producers and were selected to optimize the production of both compounds.

3.2. Optimization of acetoin and diacetyl production

After 24 h of fermentation in M17-G medium, strain CNTA 939 produced 508 mg/L of acetoin and 19 mg/L of diacetyl (Fig. 2A), whereas strain CNTA 159 produced only 36 mg/L of acetoin and diacetyl production was below the quantification limit (<4 mg/L) in two of three replicas (Supplementary Table 2). To maintain metabolic activity and promote sugar consumption, the pH was manually adjusted from 4.3–4.5 to 6.8–7.0 and fermentation was extended for an additional 24 h. During this time, strain CNTA 939 increased acetoin and diacetyl production to 651 mg/L and 25 mg/L, respectively. However, the strain CNTA 159 was unable to increase the production of acetoin and diacetyl (Fig. 2A). The fact that strain CNTA 939 produced more acetoin and diacetyl in M17-G medium with manual pH adjustment compared with SSM-G medium with no pH adjustment (Fig. 2A vs. 1A) indicates that the production of these compounds can be increased when metabolic

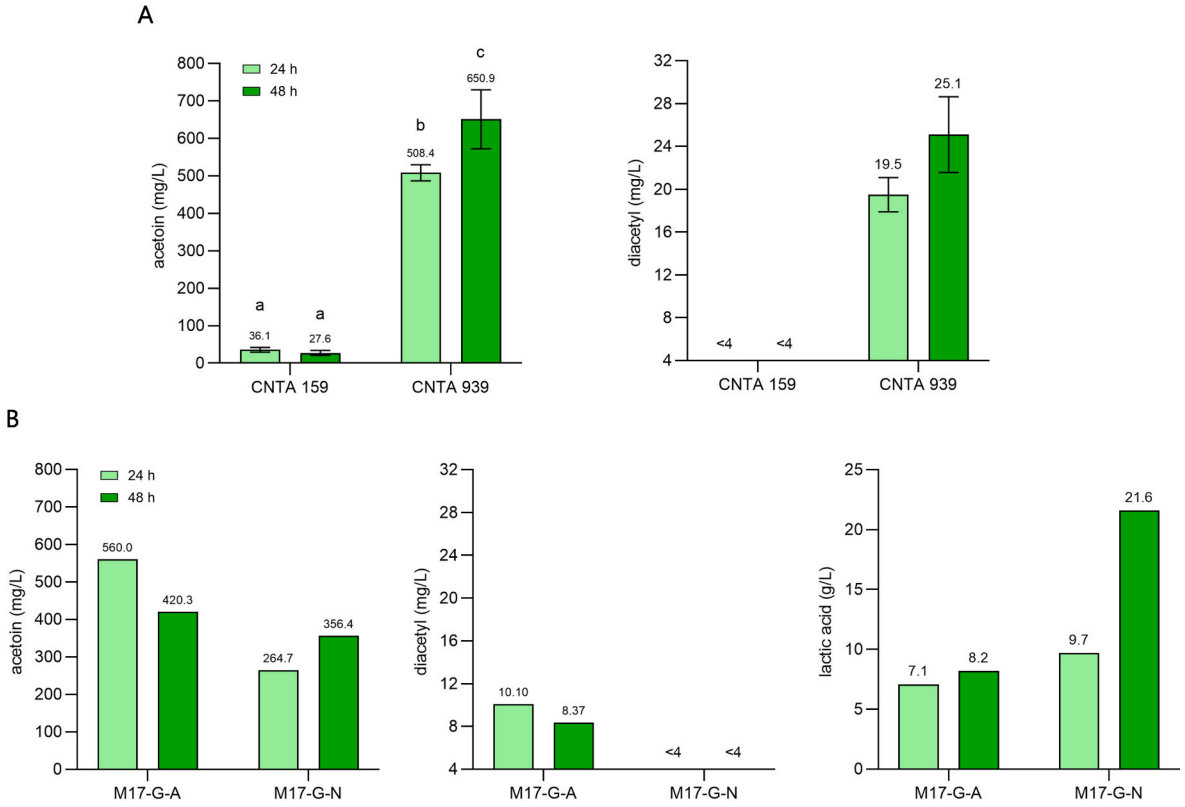

Fig. 2. (A) Production of acetoin and diacetyl by the strains CNTA 159 and CNTA 939 after 24 and 48 h of fermentation in M17-G medium. Experiments were carried out by triplicates. Statistical differences were obtained through an ANOVA analysis. (B) Production of acetoin, diacetyl and lactic acid by strain CNTA 939 at neutral (M17-G-N) and acidic pH (M17-G-A) after 24 and 48 h of fermentation.

activity is kept at high levels throughout fermentation, which could be achieved if sugars are consumed under appropriate environmental and process conditions. Moreover, the production of these compounds is strain-dependent and strain CNTA 939 showed the best capability to produce both acetoin and diacetyl from glucose. It has been reported that the metabolic flux towards the production of C4 compounds in *L. lactis* is increased at acidic pH (García-Quintáns et al., 2008). Thus, fermentations were carried out in bioreactor at constant neutral (M17-G-N) and acidic (M17-G-A) pH to control and increase the production of both acetoin and diacetyl. After 24 h of fermentation 560 mg/L of acetoin and 10.1 mg/L of diacetyl were produced in the M17-G-A condition. Meanwhile, at neutral pH only 265 mg/L of acetoin were produced and diacetyl was below of detection limit (Fig. 2B). After 48 h, the acetoin and diacetyl concentrations decreased to 420 mg/L and 8.37 mg/L, respectively, in the M17-G-A condition, whereas in the M17-G-N condition acetoin production slightly increased to 356 mg/L and diacetyl remained below the detection limit (Fig. 2B). Based on lactic acid production in both conditions, acidic pH not only favoured a higher acetoin and diacetyl production but also improved the respective yields relative to sugar consumption (Fig. 2B & Supplementary Table 3). Thus, we demonstrated that production and yield of C4 compounds can be increased by setting acidic pH conditions throughout fermentation, which is in line with previous observations (García-Quintáns et al., 2008). However, there are other factors that might play a relevant role in modulating acetoin/diacetyl production such as the dissolved oxygen, and hence, the redox balance of the cells due to the C4 compound 2, 3-butanediol pathway by which the equilibrium between acetoin and 2,3-butanediol can be shifted depending not only on the activity of the butanediol dehydrogenase enzyme but also the NADH/NAD^+ ratio (Suttikul et al., 2023). Another factor that may impact on acetoin/diacetyl production is the size of inoculum (Liu et al., 2021b). Thus, such factors should be addressed to better control the production of these compounds.

Since we have not identified a wild citrate fermenting *Lactococcus lactis*. sp *lactis* bv. *diacetylactis* in this study, we wanted to demonstrate as proof of concept the potential amounts of acetoin and diacetyl that can be produced with this particular specie. Therefore, we carried out fermentations with the strains IL1403[pFL3] and IL1403[pFL4] (Table 2) which harbour the *citP* gene under the control of *S. pneumoniae polA* promoter and its native promoter, respectively. After 24 h, although the IL1403[pFL3] produced 61.8 mg/L of acetoin and no diacetyl in the M17-G condition, the same strain was able to produce 1423 mg/L of acetoin and 13.7 mg/L of diacetyl in the M17-GC condition and all citrate was consumed (Fig. 3). Conversely, even though in the M17-G condition the strain IL1403[pFL4] produced 51 mg/L of acetoin and diacetyl was below the limit of detection, acetoin production in the M17-GC condition increased only slightly up to 131 mg/L and an insignificant citrate consumption was observed (Fig. 3). Citrate metabolism in *L. lactis* subsp. *lactis* bv. *diacetylactis* is induced at acidic pH (below 5) (Magni et al., 1999). However, the pH value was over 5 after 24 h of fermentation in the condition M17-GC (Supplementary Table 4). Thus, since the strain IL1403[pFL4] carries the citrate permease under the control of its inducible P1 promoter at low pH the levels of the citrate permease activity were low at the above-mentioned conditions, and hence, insignificant citrate consumption and low increment of both acetoin and diacetyl production were observed compared to IL1403[pFL3]. The results indicated that a *L. lactis* subsp. *lactis* bv. *diacetylactis* strain showing great capacity to consume citrate is an ideal candidate to obtain both acetoin and diacetyl at high amounts.

3.3. Oat milk supports limited fermentative capacity to L. cremoris CNTA 939

Since the food industry remains heavily inclined towards the use of microorganisms deemed natural, CNTA 939 strain (best wild-type acetoin and diacetyl producer in synthetic medium) was selected over IL1403[pFL3] and IL1403[pFL4] strains as fermentation starter to overproduce these compounds in a PBM matrix such as oat milk. When oat milk was fermented with CNTA 939, a slight glucose consumption of 4.7 g/L was observed after 48 h, and 0.15 g/L and 3.57 g/L of acetic and lactic acid were produced, respectively (Fig. 4B–OM). However, a little amount of acetoin was produced and diacetyl was below the quantification limit (Fig. 4A–OM). Interestingly, sugar consumption increased to 16.1 g/L when 5 g/L yeast extract was supplemented to oat milk (Fig. 4B, OM_YE) and, in consequence, 1.74 g/L and 14.09 g/L of acetic and lactic acid were produced (Fig. 4A, OM_YE). In addition, production of acetoin increased to 368 mg/L and 297 mg/L after 24 and 48 h of fermentation, respectively. These results indicate that oat milk by itself does not support all nutrients that are required by the strain CNTA 939 to ferment optimally and overproduce acetoin and diacetyl.

3.4. Optimization of the production of acetoin and diacetyl in fermented oat milk

Yeast extract provides a variety of nutrients (Tao et al., 2023) and to identify those that, when absent, limit the fermentation capacity of the strain CNTA 939, concentrated stocks of different nutrients including amino acids, vitamins and minerals were prepared. When amino acids were added to oat milk, practically similar amounts of sugars were consumed after 48 h compared to oat milk supplemented with yeast extract (Fig. 4B, OM_AA) and acetic and lactic acid production was 0.19 g/L and 17.8 g/L, respectively. As for the C4 compounds, acetoin production titers after 24 and 48 h were 36.0 mg/L and 68.2 mg/L, respectively, and notably 7.46 mg/L of diacetyl were produced after 48

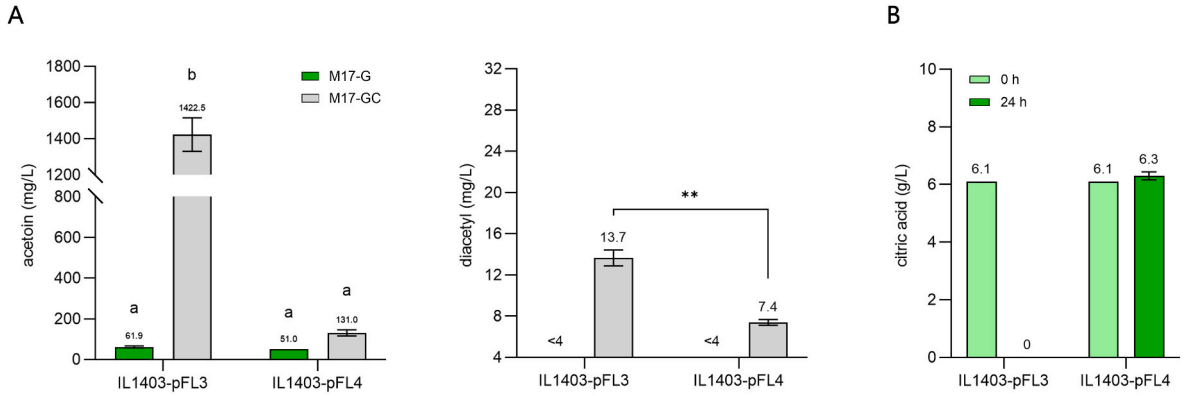

Fig. 3. (A) Production of aromatic compounds of the strains IL1403-pFL3 and IL1403-pFL4 after 24 of fermentation. Experiments were carried out by duplicate. (B) Residual citric acid concentration after 24 h of fermentation. Statistical differences were obtained through an ANOVA (acetoin and citric acid) and *t*-test analysis (diacetyl). p-values for *t*-test are indicated as follow: 0.1234 (ns), 0.0332(*), 0.0021(**), 0.0002(***) and <0.0001 (****).

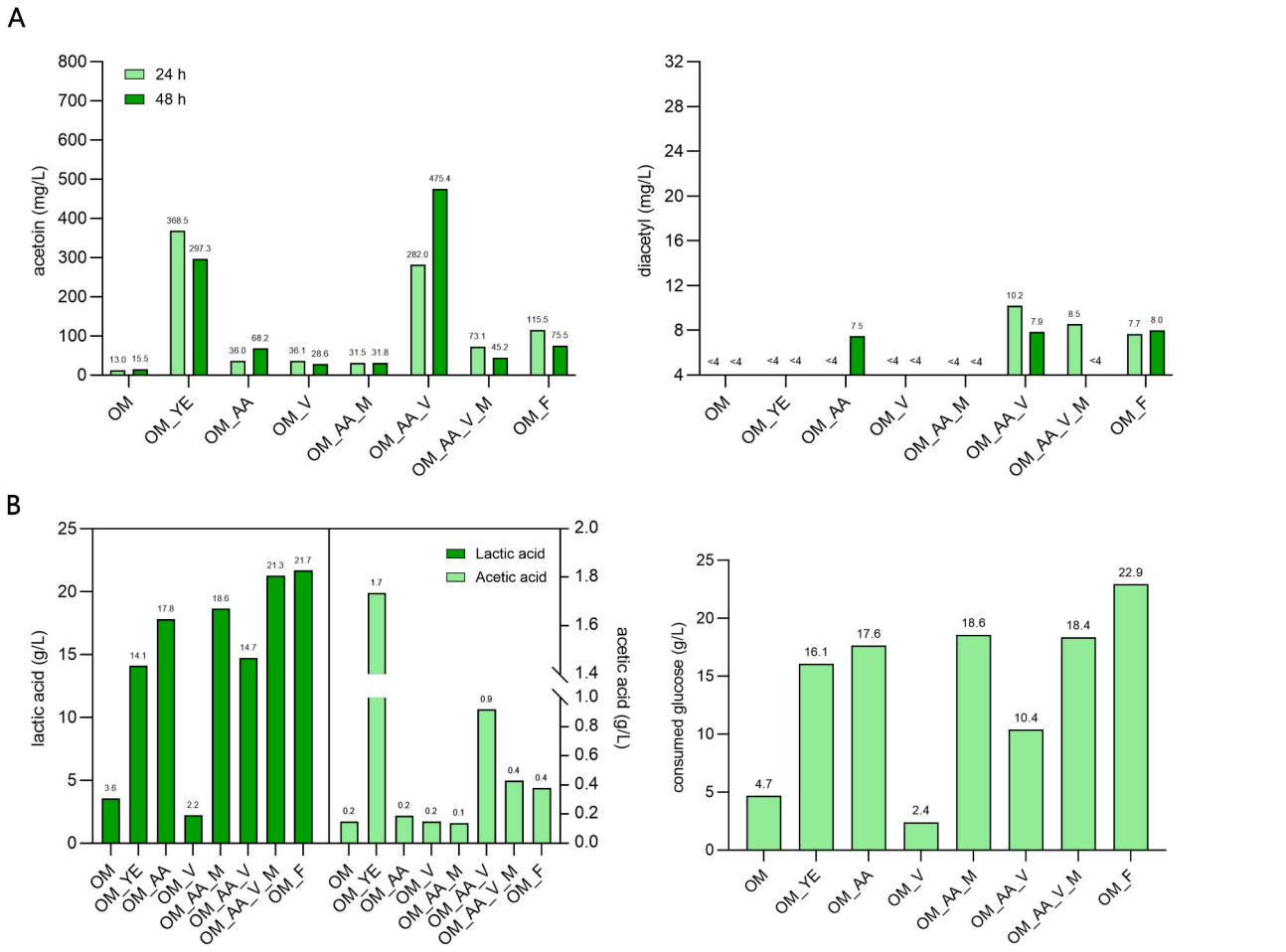

Fig. 4. Production of fermentative compounds in oat milk fermented with the strain CNTA 939. (A) Production of acetoin (left) and diacetyl (right) after 24 and 48 h of fermentation. (B) Production of lactic and acetic acid (left) and glucose consumed (right) after 48 h of fermentation. OM: oat milk; OM_YE: oat milk with yeast extract, OM_AA: Oat milk with amino acids; OM_V: Oat milk with vitamins; OM_AA_M: Oat milk with amino acids and minerals; OM_AA_V: Oat milk with amino acids and vitamins; OM_AA_V_M: Oat milk with amino acids, vitamins and minerals; OM_F: Oat milk treated with Flavourzyme®.

h (Fig. 4A, OM_AA). On the other hand, the sole addition of vitamins did not impact on the consumption of sugars, yet a slight increment of acetoin was observed compared to oat milk without nutrients (Fig. 4A and B, OM_V). The best results were obtained when amino acids and vitamins were added together, since acetoin production increased up to 475.4 mg/L after 48 h, which means that production was 7 times higher compared to oat milk supplemented with only amino acids (Fig. 4A, OM_AA_V vs. OM_AA). Interestingly, the addition of minerals had an opposite effect on the production of both acetoin and diacetyl. When amino acids and minerals were added, the production of acetoin was reduced to 31.8 mg/L after 48 h and diacetyl was below the detection limit (Fig. 4A, OM_AA_M vs. OM_AA). The same pattern was observed when amino acids, vitamins and minerals were added together, since acetoin production was reduced to 45.2 mg/L and diacetyl was below the detection limit (Fig. 4A, OM_AA_V_M vs. OM_AA_V). Therefore, the results indicated that amino acids are key nutrients required by the strain CNTA 939 to ferment oat milk and that addition of vitamins favours both acetoin and diacetyl production when amino acids are present. As many LAB, *Lactococcus* strains are characterized by being auxotrophic for a variety of amino acids including leucine, isoleucine, valine, methionine, histidine and glutamic acid (Chopin, 1993; Jensen & Hammer, 1993). Considering the amino acid requirements identified in strain CNTA 939, we tested an alternative to external supplementation to provide oat milk with free amino acids. Oat milk proteins contain all the amino acids required by *L. lactis* (Mäkinen et al., 2017) yet they are not free and available to the starter. However, they can be released by proteolytic treatment (Bonke et al., 2020). Therefore, we treated oat milk with the commercial peptide mix Flavourzyme® and carried out the fermentation. Surprisingly, the strain CNTA 939 showed a strong fermentation capacity in which all fermentable sugars were consumed after 24 h (Fig. 4B & Supplementary Table 5) and production of acetic and lactic acid were 0.38 g/L and 21.67 g/L after 48 h, respectively (Fig. 4B, OM_F). Most importantly, acetoin and diacetyl production were similar to that obtained when external amino acids were added (Fig. 4A, OM_F vs. OM_AA), and surpassed their aroma threshold concentrations in water by 2-fold and approximately 1500-fold, respectively (50 and 0.005 mg/L) (Peyer et al., 2016). Therefore, we demonstrated that oat milk can be converted into a good fermentation substrate for *Lactococcus* strains such as CNTA 939 after proteolytic treatment and overproduce acetoin and diacetyl at amounts which can potentially improve the sensory properties of PBM.

4. Conclusion

This study tested various reported methodologies to select a LAB strain capable of producing significant amounts of the butter- and cream-like aromatic compounds acetoin and diacetyl. The objective was to identify citrate-fermenting LAB, ideally wild *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* strains, as it is reported to be one of the best producers of these compounds from citrate. We first tested the KMK method but found it unsuitable due to numerous false positives. In the case of *Lactococcus* strains, we found that molecular characterization by PCR to

detect the citrate catabolic gene *citP* and operon *citM-citI-citCDEFXG* is suitable for performing a first screening and then submit those to a fermentation assay in a citrate-containing medium to test acetoin/diacetyl production and citrate consumption. For the remaining LAB tested (*Leuconostoc* and lactobacilli), similar molecular methods should be developed by designing specific primers for them. We also demonstrated that the VP method is reliable to detect acetoin-producing LAB strains from simple sugars only when a strong signal is observed. Based on this method we could identify the strain *Lactococcus cremoris* CNTA 939 as a potential acetoin/diacetyl producer, which was then confirmed after quantification of the acetoin and diacetyl produced in synthetic medium. To optimize the production of these compounds we demonstrated the importance of ensuring good environmental conditions such as an acidic pH along the process, to allow the strain to ferment great amounts of sugar and increase both acetoin and diacetyl yields. Other factors such as dissolved oxygen and inoculum size must be addressed to further improve the production of these compounds. Even though the greatest amounts of both acetoin and diacetyl in synthetic medium were obtained using a recombinant *L. lactis* subsp. *lactis* bv. *diacetylactis* strain with strong capacity to metabolize citrate without the necessity to control pH, the strong preference for wild type strains for industrial purposes led us to choose the CNTA 939 strain to further explore the production of these compounds in PBM matrices such as oat milk. We observed that oat milk did not provide all the required nutrients, identified as mainly amino acids, to support a great fermentation capacity by this strain, and demonstrated that external supplementation can be avoided by carrying out a proteolytic treatment to the oat milk before its sterilization. The fermentation capability of the strain CNTA 939 substantially increased in the treated oat milk, where the production of acetoin and diacetyl readily surpassed their respective aroma thresholds in water. This approach could therefore be a suitable strategy to improve the sensory attributes of fermented PBM products without adding external flavour agents.

CRediT authorship contribution statement

Sebastián M. Tapia: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Ana Moreno-Ruiz:** Writing – original draft, Methodology. **Andrea Tres:** Methodology. **Nerea Moreno-Yerro:** Methodology. **Patricia Arrubla:** Methodology. **Miguel Gastón-Lorente:** Methodology. **María Luz Mohedano:** Writing – review & editing, Methodology. **Raquel Virto:** Writing – review & editing, Supervision, Funding acquisition. **Dante Fratebianchi:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization.

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.

Acknowledgements

We thank Paloma López for the critical reading of the manuscript.

The present work was conducted within the PC130-131 PBAFER project, funded by the Spanish Government of Navarre.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2025.117754.

Data availability

Data will be made available on request.

References

Asioli, D., Aschemann-Witzel, J., Caputo, V., Vecchio, R., Annunziata, A., Næs, T., & Varela, P. (2017). Making sense of the "clean label" trends: A review of consumer food choice behavior and discussion of industry implications. *Food Research International, 99*(July), 58–71. https://doi.org/10.1016/j.foodres.2017.07.022

Beimfohr, C., Ludwig, W., & Schleifer, K.-H. (1997). Rapid genotypic differentiation of *Lactococcus lactis* subspecies and biovar. *Systematic & Applied Microbiology, 20*(2), 216–221. https://doi.org/10.1016/S0723-2020(97)80068-9

Boeck, T., Ispiryan, L., Hoehnel, A., Sahin, A. W., Coffey, A., Zannini, E., & Arendt, E. K. (2022). Lentil-based yogurt alternatives fermented with multifunctional strains of lactic acid bacteria — techno-functional, microbiological, and sensory characteristics. *Foods, 11*(14), 2013. https://doi.org/10.3390/foods11142013

Bonke, A., Sieuwerts, S., & Petersen, I. L. (2020). Amino acid composition of novel plant drinks from oat, lentil and pea. *Foods, 9*(4), 1–13. https://doi.org/10.3390/foods9040429

Bourel, G., Bekal, S., Diviès, C., & Prévost, H. (1996). Citrate permease gene expression in *Lactococcus lactis* subsp. *lactis* strains IL1403 and MG1363. *FEMS Microbiology Letters, 145*(3), 367–370. https://doi.org/10.1111/j.1574-6968.1996.tb08602.x

Chileshe, J., van den Heuvel, J., Handema, R., Zwaan, B. J., Talsma, E. F., & Schoustra, S. (2020). Nutritional composition and microbial communities of two non-alcoholic traditional fermented beverages from Zambia: A study of *mabisi* and *munkoyo*. *Nutrients, 12*(6), 1–19. https://doi.org/10.3390/nu12061628

Chopin, A. (1993). Organization and regulation of genes for amino acid biosynthesis in lactic acid bacteria. *FEMS Microbiology Reviews, 12*(1–3), 21–37. https://doi.org/10.1016/0168-6445(93)90056-F

Franciosi, E., Settanni, L., Cavazza, A., & Poznanski, E. (2009). Biodiversity and technological potential of wild lactic acid bacteria from raw cows' milk. *International Dairy Journal, 19*(1), 3–11. https://doi.org/10.1016/j.idairyj.2008.07.008

Fusieger, A., Martins, M. C. F., de Freitas, R., Nero, L. A., & de Carvalho, A. F. (2020). Technological properties of *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* obtained from dairy and non-dairy niches. *Brazilian Journal of Microbiology, 51*(1), 313–321. https://doi.org/10.1007/s42770-019-00182-3

García-Quintáns, N., Repizo, G., Martín, M., Magni, C., & López, P. (2008). Activation of the diacetyl/acetoin pathway in *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* CRL264 by acidic growth. *Applied and Environmental Microbiology, 74*(7), 1988–1996. https://doi.org/10.1128/AEM.01851-07

Hugenholtz, J., Kleerebezem, M., Starrenburg, M., Delcour, J., De Vos, W., & Hols, P. (2000). *Lactococcus lactis* as a cell factory for high-level diacetyl production. *Applied and Environmental Microbiology, 66*(9), 4112–4114. https://doi.org/10.1128/AEM.66.9.4112-4114.2000

Hugenholtz, J., & Starrenburg, M. C. (1992). Diacetyl production by different strains of *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* and *Leuconostoc* spp. *Applied Microbiology and Biotechnology, 38*(1), 17–22. https://doi.org/10.1007/BF00169412

Jensen, P. R., & Hammer, K. (1993). Minimal requirements for exponential growth of *Lactococcus lactis*. *Applied and Environmental Microbiology, 59*(12), 4363–4366. https://doi.org/10.1128/aem.59.12.4363-4366.1993

Jeske, S., Zannini, E., & Arendt, E. K. (2018). Past, present and future: The strength of plant-based dairy substitutes based on gluten-free raw materials. *Food Research International, 110*, 42–51. https://doi.org/10.1016/j.foodres.2017.03.045

Kamath, R., Basak, S., & Gokhale, J. (2022). Recent trends in the development of healthy and functional cheese analogues-a review. *LWT, 155*, Article 112991. https://doi.org/10.1016/j.lwt.2021.112991

Kempler, G. M., & McKay, L. L. (1980). Improved medium for detection of citrate-fermenting *Streptococcus lactis* subsp. *diacetylactis*. *Applied and Environmental Microbiology, 39*(4), 926–927. https://doi.org/10.1128/aem.39.4.926-927.1980

Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., De Cesare, A., Gilbert, F., Lindqvist, R., Nauta, M., Nonno, R., Peixe, L., Ru, G., Simmons, M., Skandamis, P., Suffredini, P., Cocconcelli, P. S., Fernández Escámez, P. S., … EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards). (2024). Update of the list of qualified presumption of safety (QPS) recommended microbiological agents intentionally added to food or feed as notified to EFSA 20: Suitability of taxonomic units notified to EFSA until March 2024. *EFSA Journal, 22*(7), Article e8882. https://doi.org/10.2903/j.efsa.2024.8882

Liu, J.-M., Chen, L., Dorau, R., Lillevang, S. K., Jensen, P. R., & Solem, C. (2020). From waste to taste—efficient production of the butter aroma compound acetoin from low-value dairy side streams using a natural (nonengineered) *Lactococcus lactis* dairy isolate. *Journal of Agricultural and Food Chemistry, 68*(21), 5891–5899. https://doi.org/10.1021/acs.jafc.0c00882

Liu, J.-M., Chen, L., Jensen, P. R., & Solem, C. (2021a). Food grade microbial synthesis of the butter aroma compound butanedione using engineered and non-engineered *Lactococcus lactis*. *Metabolic Engineering, 67*(August Sep), 443–452. https://doi.org/10.1016/j.ymben.2021.08.006

Liu, J.-M., Fehér, C., Cao, M., Lu, F., & Jensen, P. R. (2021b). Editorial: Lactic acid bacteria: Microbial metabolism and expanding applications. *Frontiers in Bioengineering and Biotechnology, 9*(November), Article 794164. https://doi.org/10.3389/fbioe.2021.794164, 1–4.

Magni, C., de Mendoza, D., Konings, W. N., & Lolkema, J. S. (1999). Mechanism of citrate metabolism in *Lactococcus lactis*: Resistance against lactate toxicity at low pH. *Journal of Bacteriology, 181*(5), 1451–1457. https://doi.org/10.1128/JB.181.5.1451-1457.1999

Magni, C., Felipe, F. L., Sesma, F., López, P., & Mendoza, D. (1994). Citrate transport in *Lactococcus lactis* biovar *diacetylactis*: Expression of the plasmid-borne citrate permease P. *FEMS Microbiology Letters, 118*(1–2), 75–82. https://doi.org/10.1111/j.1574-6968.1994.tb06806.x

Mäkinen, O. E., Sozer, N., Ercili-Cura, D., & Poutanen, K. (2017). Protein from oat. In *Sustainable protein sources* (pp. 105–119). Elsevier. https://doi.org/10.1016/B978-0-12-802778-3.00006-8.

Meticulous Research. (2023). Plant-based milk market by type (almond milk, soy milk, coconut milk, oat milk, rice milk), formulation (unflavored, flavored), distribution channel (B2B, B2C [modern groceries, convenience store, specialty store, online retail]) - global forecast to 2030. https://www.meticulousresearch.com/product/plant-based-milk-market-5304. (Accessed 2 December 2024).

Nout, M. J. R. (2009). Rich nutrition from the poorest - cereal fermentations in Africa and Asia. *Food Microbiology, 26*(7), 685–692. https://doi.org/10.1016/j.fm.2009.07.002

OECD, & FAO. (2023). Dairy and dairy products market situation. In *OECD-FAO Agricultural Outlook 2023-2032* (pp. 202–213). https://www.fao.org/documents/card/en/c/cc6361en.

Passerini, D., Laroute, V., Coddeville, M., Le Bourgeois, P., Loubière, P., Ritzenthaler, P., Cocaign-Bousquet, M., & Daveran-Mingot, M. L. (2013). New insights into *Lactococcus lactis* diacetyl- and acetoin-producing strains isolated from diverse origins. *International Journal of Food Microbiology, 160*(3), 329–336. https://doi.org/10.1016/j.ijfoodmicro.2012.10.023

Peyer, L. C., Zannini, E., & Arendt, E. K. (2016). Lactic acid bacteria as sensory biomodulators for fermented cereal-based beverages. *Trends in Food Science & Technology, 54*, 17–25. https://doi.org/10.1016/j.tifs.2016.05.009

Pua, A., Tang, V. C. Y., Goh, R. M. V., Sun, J., Lassabliere, B., & Liu, S. Q. (2022). Ingredients, processing, and fermentation: Addressing the organoleptic boundaries of plant-based dairy analogues. *Foods, 11*(6), 875. https://doi.org/10.3390/foods11060875

Smid, E. J., & Kleerebezem, M. (2014). Production of aroma compounds in lactic fermentations. *Annual Review of Food Science and Technology, 5*(1), 313–326. https://doi.org/10.1146/annurev-food-030713-092339

Speckman, R. A., & Collins, E. B. (1982). Specificity of the westerfeld adaptation of the Voges-Proskauer test. *Applied and Environmental Microbiology, 44*(1), 40–43. https://doi.org/10.1128/aem.44.1.40-43.1982

Strategic Market Research. (2022). Plant-based milk market by product (pea milk, rice milk, almond milk, wheat milk, hemp milk, coconut milk, soy milk, others), by formulation (plain, flavoured), by end use (food & beverage industry, retail/household, foodservice industry), by application. https://www.strategicmarketresearch.com/market-report/plant-based-milk-market. (Accessed 16 December 2024).

Suttikul, S., Charalampopoulos, D., & Chatzifragkou, A. (2023). Biotechnological production of optically pure 2,3-butanediol by *Bacillus subtilis* based on dissolved oxygen control strategy. *Fermentation, 9*(1). https://doi.org/10.3390/fermentation9010015

Tangyu, M., Muller, J., Bolten, C. J., & Wittmann, C. (2019). Fermentation of plant-based milk alternatives for improved flavour and nutritional value. *Applied Microbiology and Biotechnology, 103*(23–24), 9263–9275. https://doi.org/10.1007/s00253-019-10175-9

Tao, Z., Yuan, H., Liu, M., Liu, Q., Zhang, S., Liu, H., Jiang, Y., Huang, D., & Wang, T. (2023). Yeast extract: Characteristics, production, applications and future perspectives. *Journal of Microbiology and Biotechnology, 33*(1), 151–166. https://doi.org/10.4014/jmb.2207.07057

Xiao, Z., & Lu, J. R. (2014). Generation of acetoin and its derivatives in foods. *Journal of Agricultural and Food Chemistry, 62*(28), 6487–6497. https://doi.org/10.1021/jf5013902

Youssef, C. El, Bonnarme, P., Fraud, S., Péron, A. C., Helinck, S., & Landaud, S. (2020). Sensory improvement of a pea protein-based product using microbial co-cultures of lactic acid bacteria and yeasts. *Foods, 9*(3). https://doi.org/10.3390/foods9030349