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Formation of 3-Methylbutanal and 3-Methylbutan-1-ol Recognized as Malty during Fermentation in Swiss Raclette-Type Cheese, Reconstituted Milk, and de Man, Rogosa, and Sharpe Broth

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ABSTRACT: This work aimed to determine the formation over time of 3-methylbutanal and 3-methylbutan-1-ol recognized as malty during the manufacture of Raclette-type cheese and the fermention of reconstituted skim milk, and filter-sterilized MRS broth. Using dynamic headspace-vacuum transfer in trap extraction followed by gas chromatography coupled with mass spectrometry−olfactometry (DHS-VTT-GC-MS-O) as a screening method for the malty compounds, five compounds (2-methylpropanal, 2- and 3-methylbutanal, and 2- and 3-methylbutan-1-ol) were identified as potential compounds causing the malty aroma in starter culture development and Raclette-type cheeses. Focus on compounds having a predominant sensorial effect (3-methylbutanal and 3-methylbutan-1-ol), spikings of leucine, ^{13}C-labeled leucine, α-ketoisocaproic acid, and α-ketoglutaric acid provided a better understanding of their formation pathway. This study highlighted the discrepancies in the formation of 3-methylbutanal and 3-methylbutan-1-ol between the growth media; namely, despite the presence of free leucine available in MRS and the addition of an excess, no increase of the target compounds was observed. The concentration of these compounds in MRS increased only when α-ketoglutaric acid or α-ketoisocaproic acid was added, and a preference for the pathway to α-hydroxyisocaproic acid instead of 3-methylbutanal was shown. In addition, a formation of 3-methylbutanal when the bacteria were not yet active was observed when spiking α-ketoisocaproic acid, which potentially indicates that this part of the metabolism could take place extracellularly. These results could potentially unveil other, not-yet-identified reactants, directly influencing the production of compounds responsible for the malty aroma in Raclette cheese.

KEYWORDS: *3-methylbutanal, 3-methylbutan-1-ol, malt aroma, Raclette, cheese, DHS-VTT, GC−MS, olfactometry*

INTRODUCTION

Malty aroma has been studied considerably in several food matrices, such as beers, spirits, and dairy products. This aroma has been mainly associated with branched-chain aldehydes, 2-methylpropanal, 2-methybutanal, and 3-methylbutanal, derived from degradation of valine, isoleucine, and leucine, respectively.[1,2] The formation of the branched chain aldehydes starts intracellularly first from the transamination of the former branched chain amino acids (AAs), which then leads to the formation of the corresponding intermediary α-ketoacids.[3−6] From then, different pathways can lead to the coupling to Coenzyme A, to the formation of their corresponding α-hydroxyacids, or to the direct or indirect decarboxylation of the α-ketoacids to the aldehydes.[1,3,5,7]

During the development of a new starter culture for Swiss Raclette-type cheese (a semi-hard cheese often ripened over at least three months and manufactured using pasteurized, thermized, or raw milk), such a malty undesirable off-flavor was detected, which had not yet been described in this type of cheese. This was first perceived through sensorial analysis and later analytically. This off-flavor was found to be unpleasant when the cheeses were tasted at room temperature or melted, which is a common way of tasting cheese throughout Switzerland. Since this off-flavor could be attributed to the use of a starter culture, the focus on the source of formation was put on these cultures. Starter cultures are usually not single-strain cultures but rather consist of a mix of one or more strain(s) of several species.

In certain cheese types, these compounds have been reported as having a desirable aroma, such as in cheddar[8,9] and Proosdij-type[10,11] cheeses, as it enhances chocolate and nutty notes. In other cheese types, like Manchego[12] or Egyptian Ras[13] cheese, this aroma is not desired due to its perception as unclean and burnt. The malty off-flavor has also been reported in raw milk for which the malty defect was still present after pasteurization.[14]

It is known that those compounds can be produced by lactic acid bacteria such as *Lactococcus lactis* (*L. lactis*) ssp., *Streptococcus salivarius* (*S. salivarius*) subsp. *thermophilus*, and *Lactobacillus delbrueckii* (*L. delbrueckii*) subsp. *lactis*. Those species are part of the raw milk flora (in raw milk cheese) and

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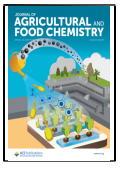

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in starter cultures. The activity of their corresponding enzymes then leads to the formation of those compounds. The development of starter cultures is not only important for the acidification properties of cheese but also for the production of flavor and texture in cheese.[11,15] As mentioned above, such starter cultures are usually a mixture of species and/or strains. This involves the selection of appropriate strains as well as the understanding of the interactions between the species/strains. In Swiss Raclette cheeses, mixtures of *L. lactis* subsp. *lactis*, *S. salivarius* subsp. *thermophilus*, *L. lactis* subsp. *cremoris*, and *L. lactis* subsp. *lactis* biovar *diacetylactis* are used as starter culture.

The understanding of the corresponding metabolic pathways and conditions for those reactions is important for the selection of the starter cultures. The pathways for the production of 2- and 3-methylbutanal and their alcohol derivatives are known and described in the literature.[3−5,7,16] The substrate for the formation and the end product is the same for all species. However, the pathway taken by *Lactococcus* spp. is different from other species, such as *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *lactis*. There was nonetheless recently a report indicating that *L. lactis* subsp. *lactis* can also use the same pathway as *S. salivarius* subsp. *thermophilus*.[17] Production or inhibition of these compounds could be altered through alteration of the redox potential[18,19] or adding dissolved oxygen.[20] However, there are not many other reports about the control of these reactions available.

The first goal of this paper was to study the formation over time of compounds responsible for the malty aroma with a focus on those with the highest sensory impact (identified by dynamic headspace vacuum transfer in trap gas chromatography coupled with mass spectrometry and olfactometry [DHS-VTT-GC-MS-O][21]) in model Raclette cheeses, reconstituted skim milk (RSM), and filter-sterilized MRS (de Man, Rogosa, and Sharpe)[22] broth during fermentation using selected cultures. The second goal was to understand the influences of selected reactants belonging to the formation pathway of the chosen compounds. The results of this study will improve the production of Swiss Raclette or other cheeses for the purpose of developing a screening tool for the selection of cultures for specific aroma formation.

MATERIALS AND METHODS

Reagents. Chemicals were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland), Merck KGaA (Darmstadt, Germany), Chemrise (Moskow, Russia), VWR International SA (Nyon, Switzerland), and Biolife (Switzerland): 2-methylpropanal (Aldrich 240788), 2-methylbutanal (Aldrich W269107), 3-methylbutanal (Aldrich W269212), 2-methylbutan-1-ol (Aldrich 133051), 3-methyl-1-butanol (Aldrich W205702), 1-pentanol (Aldrich 76928), α-hydroxyisocaproic acid (Aldrich 219819), α-ketoisocaproic acid (Aldrich 68255), α-ketoglutaric acid (Merck K31946394), L-leucine (Merck K38661860), formic acid (Merck 100263), $^{13}C_6$-L-leucine (Aldrich 605239-100MG), 4-piperidinecarboxylic acid (Merck 814034), thiodiglycol (Aldrich 166782), 3-methylbutanal-d_6 (Chemrise), sulfosalicylic acid (VWR 20678.187), and MRS Broth with Tween 80 (Biolife 4017294).

Preparation of Starter Cultures and Bacterial Strains. *Single, Non-freeze-Dried Bacterial Strains.* *L. lactis* subsp. *cremoris* FAM-1233, and *L. lactis* subsp. *cremoris* FAM-17841 were kept as frozen stock in skim milk at −40 °C. Prior to use, a frozen ampoule was thawed and reactivated in reconstituted skim milk (RSM) at 30 °C for 16 h. This culture was then used to inoculate the experimental cultures, as described below.

Freeze-Dried Starter Cultures. All strains used to prepare starter culture mixtures were freeze-dried at Agroscope's facilities using the standard production protocol for *L. lactis* species (confidential). The single strains were then combined into the final mixture according to the recipe for the product. This mixture was made for a volume of 30 L of milk. To achieve the concentration in a low volume, the whole amount was suspended in 30 mL of reconstituted skim milk, and after 30 min at room temperature, this suspension was diluted 1000 times in the volume used for the experiment.

Preparation of Microbial Cultures in Reconstituted Skim Milk and Filter-Sterilized MRS Broth for Identification and Kinetic Studies of Malty Compounds. RSM was prepared from organic skim milk powder (Hochdorf Nutritec, Hochdorf, Switzerland) and water. Organic skim milk powder was added (9.5% w/w) to water with 0.01% w/w Glanapon 1594 (Bussetti & Co GesmbH, Marchtrenk, Austria), an antifoaming agent. The mixture was then stirred for a few minutes and heat-treated at 112 °C for 45 min. After this step, the RSM was stored at room temperature (RT = 25 °C) until further use.

To determine which compounds are responsible for the malty off-aroma, three chosen mixtures of strains (RdV 18, RdV 22, and RdV 27) were used in the starter culture development (Supporting Information, Table S1) and one *L. lactis* subsp. *cremoris* strain (FAM-1233) from the Agroscope culture collection was grown in RSM, as described below, for the purpose of simulating Swiss Raclette cheese. The bacterial cultures in RSM were inoculated at a 1‰ (v/v) ratio and incubated at 30 °C for 18 h.

For kinetics and spiking studies, fermentations were done in RSM and in a model medium, filter-sterilized de Man, Rogosa, and Sharpe (MRS) broth.[22] For these studies, *L. lactis* subsp. *cremoris* FAM-17841 from the Agroscope culture collection was used. Figure 1 describes the execution of kinetic and spiking studies. Bacteria were cultivated at 30 °C in both RSM and filter-sterilized MRS broth. For kinetic studies (Figure 1a), after collection of the aliquot, the number of viable cells was determined on MRS agar plates after anaerobic incubation at 30 °C for a minimum of 24 h. For spiking studies, α-ketoisocaproic acid, α-ketoglutaric acid, L-leucine, and ^{13}C-L-leucine were added to the media in concentrations as specified in Figure 1b,c. All samples were placed right after their collection point in the freezer at −40 °C until further analysis.

Mini Raclette Cheesemaking. Mini Raclette cheeses were manufactured for the identification of compounds responsible for the malty flavor according to the recipe of Swiss-type Raclette cheese. Starter cultures were added at a concentration of 5×10^6 cfu mL^{-1} (colony forming unit per milliliter) in milk.

The molds used to collect the strained cheese curd were made from perforated square polyethylene terephthalate (PETG), 4.6 cm in length. A weight of 750 g was applied to each mini cheese for 1 h at room temperature (RT = 25 °C); then, the weight was removed. The mini cheeses were then left at RT for 24 h. In the case of the kinetic experiment, 1 g of fresh cheese was collected every hour for the first 7 h and one sample at 24 h of maturation. All samples were frozen after collection in order to stop the maturation at an exact time. After 24 h, the pH of the mini cheeses was measured (achieved pH: 5.08−5.18), and depending on the number of samples

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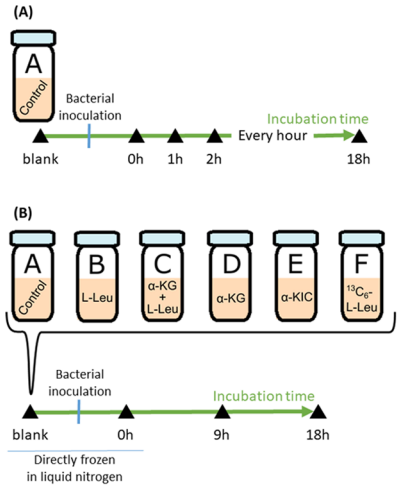

(C)

	Spiking models [mM]					
Compound	A	B	C	D	E	F
α-KIC					0.5	
L-leucine		1	1			
α-KG			4	4		
$^{13}C_6$-l-leucine						2.42

Figure 1. (a) Scheme describing the kinetic studies. (b) Scheme describing the spiking studies. (c) Concentrations in [mM] of reactants spiked in the reaction media (MRS and RSM). (triangle) Sample collection time point.

required, the cheeses were then cut into pieces and placed in individual vacuum bags. Ripening was done in a temperature-controlled cellar at 10−11 °C and samples were collected after one week, five weeks, and two months. After ripening, samples were stored at −40 °C until analysis.

Gas Chromatography−Mass Spectrometry (GC−MS). *Sample Preparation for Extraction.* For olfactometry analyses by DHS-VTT and qualitative analyses by HS-SPME, 4 mL of reconstituted skim milk/MRS-cultures or 4 g of mini cheese were added to 20 mL headspace crimp glass vials for DHS-VTT analyses and screw glass vials for HS-SPME analyses. For HS-SPME analyses, 30% w/v sodium chloride was added to displace headspace equilibrium.[23]

Dynamic Headspace Vacuum Transfer in-Trap (DHS-VTT). For the quantitative analyses of kinetic and spiking studies, 2 mL samples with 50 μL of an internal standard solution composed of 8 mg kg^{-1} 3-MB-d_6 and 8 mg kg^{-1} 1-pentanol in PEG_{200} (poly(ethylene glycol)200) to normalize the results were added to 20 mL crimp glass vials. Calibration curves were established by the method of standard additions in RSM and filter-sterilized MRS broth matrices (Supporting Information, Table S2). Samples that were outside of the linear range of the calibration curve were diluted in order to fit in the range. Each sample was measured in duplicate. A clean piece of a Topper8 10 × 10 cm (Systagenix, North Yorkshire, United Kingdom) gauze swab was inserted to prevent boiling and foaming of the sample under vacuum.

Headspace Solid Phase Micro Extraction (HS-SPME). For α-keto acid semi-quantitative analysis, 200 μL of the sample, 50 μL of 10% w/w HCl, and 50 μL of MeOH were added to 20 mL glass vials with screw caps. The vials were stirred in an MPS2 autosampler agitator at 90 °C for 45 min at 500 rpm to esterify the acids. After esterification, the pH was adjusted to pH 2.5 with 215 μL of 0.5 M NaOH to avoid damaging the SPME fiber. Calibration curves were established by the method of standard additions of α-ketoisocaproic acid (α-KIC) and α-hydroxyacid (α-HIC) in RSM and filter-sterilized MRS broth matrices (Supporting Information, Table S2). A calibration curve for α-ketoglutaric acid (α-KG) could not be established. Each sample was measured in duplicate. SPME was chosen as the extraction method in this case to analyze the esterified acids as the extraction capacity is inferior to DHS-VTT, giving a lower background noise for such analysis.

Extraction Parameters. *DHS-VTT.* Volatiles were extracted by dynamic headspace vacuum transfer in trap extraction (DHS-VTT)[21] using a Tenax TA/Carbosieve III ITEX (in tube extraction) trap (BGB Analytik AG, Böckten, Switzerland) under reduced pressure using a vacuum pump Buchi V-300 (Büchi, Flawil, Switzerland). The trap was conditioned according to the supplier's temperature recommendations (300 °C for 1 h) under a nitrogen stream of 100 mL min^{-1}. For extraction, the syringe temperature was fixed at 100 °C and the ITEX trap at 35 °C. For kinetic studies, samples were incubated for 15 min at 45 °C and volatiles were then extracted for 10 min at 500 Pa. For the identification of malty compounds, volatiles were extracted for 45 min at 60 °C and 500 Pa without agitation. The sorbent and syringe were dried under a nitrogen stream for 20 min at 220 mL min^{-1}. Trapped volatiles were desorbed from the sorbent for 2 min with a nitrogen flow of 100 mL min^{-1} at 240 °C in a programmed temperature vaporizer (PTV) injector of type CIS4 (Gerstel AG, Sursee, Switzerland) in the vent mode at 50 mL min^{-1} and 0 kPa for 30 s.

The injector was equipped with a glass liner filled with Tenax TA and cooled to 10 °C using liquid nitrogen. The injector was then heated at a rate of 12 °C s^{-1} to 240 °C. The purge flow to the split vent was set at 100 mL min^{-1} after 2 min. After injection, the trap was reconditioned according to the supplier's temperature recommendation (300 °C) for 15 min under a nitrogen flow of 100 mL min^{-1}.

HS-SPME. Samples were extracted using a 2 cm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm Stableflex fiber (Supelco, Bellefonte, PA, U.S.A.), which was conditioned according to the supplier's recommendations (270 °C for 30 min). The mixed coating fiber chosen here allows the analysis of a wide range of compounds due to the bipolar phase[24−27] and a 2 cm fiber increases the amount of volatiles extracted compared to a 1 cm fiber. Previous reports using this fiber for a similar type of matrix gave good results.[26,28−30] For the qualitative analyses of fermented skim milk, MRS cultures, and mini cheeses, the headspace was extracted for 60 min at 60 °C with an agitation rate of 500 rpm without preheating. For the analysis of esterified α-keto acids, the headspace was extracted for 30 min at 90 °C with an agitation rate of 250 rpm. Trapped volatiles were desorbed for 2 min at 250 °C in the injector, which was in the splitless mode for 2 min, and then the split valve was opened after 3 min (split flow: 50 mL min^{-1}).

GC−MS Parameters. The analyses were completed using an MPS2 autosampler (Gerstel AG) on an Agilent 7890B GC system coupled to an Agilent 5977A mass selective detector (MSD) (Agilent Technologies, Basel, Switzerland). Volatile compounds were separated on an OPTIMA FFAPplus-fused silica capillary column (polyethylene glycol nitroterephthalate, cross-linked, 60 m × 0.32 mm × 1.0 μm film; MACHEREY-NAGEL, Düren, Germany) with helium as the carrier gas at a constant flow of 2.5 mL min^{-1} (29.873 cm s^{-1}).

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For olfactometry analyses and identification of malty compounds by injection of their pure standards, the oven temperature program was as follows: 7 min at 40 °C then heated to 220 °C at a rate of 10 °C min^{-1} with a final hold time of 10 min to make a total run time of 35 min. For esterified α-keto acid analyses, the oven temperature was programmed as follows: 5 min at 80 °C then heated to 220 °C at 5 °C min^{-1} with a final holding time of 8 min to make a total run time of 41 min. For all other analyses, the oven temperature program was as follows: 10 min at 40 °C then heated to 220 °C at a rate of 5 °C min^{-1} with a final hold time of 24 min to make a total run time of 65 min.

For the identification of malty compounds, pure compounds and a sample were analyzed as well on a HP-5MS UI ((5%-phenyl)-methylpolysiloxane, 30 m × 0.25 mm × 0.25 μm film, Agilent) with helium as a carrier gas at a constant flow of 1.2 mL min^{-1} (26.091 cm s^{-1}). The oven temperature program was as follows: 10 min at 40 °C then heated to 240 °C at a rate of 5 °C min^{-1} with a final hold time of 10 min to make a total run time of 60 min.

The MS settings were as follows: transfer line at 250 °C, source temperature at 230 °C, and the analytes monitored in the SCAN mode were between 30 and 250 amu without solvent delay. The autosampler was controlled with a Cycle Composer V.1.5.4 (CTC Analytics, Zwingen, Switzerland) and a PTV injector with Maestro1 software V.1.4.8.14/3.5 (Gerstel AG). Extracted ion counts (EICs) were integrated using MassHunter Quantitative Analysis software version B.08.00 (Agilent Technologies). The NIST/EPA/NIH mass spectral library (NIST14) version 2.2 (National Institute of Standards and Technology (NIST), Gaithersburg, MD, U.S.A.) was used for peak identification.

Olfactometry Parameters. Odor-active compounds were analyzed using a two-way odor detection port (ODP2; Gerstel AG) connected to a 7890B Agilent gas chromatograph (2W-GC-O) coupled with a 5977A MSD (Agilent Technologies) and equipped with an MPS2 autosampler and MassHunter software version B.08.00 (Agilent Technologies). The olfactometric ports were designed for two panelists to detect volatile compounds simultaneously. The dimensions and capillaries after GC separation were adapted to obtain an optimal and timely synchronized signal between the physical detectors and the two sniffers:[31] a column to the MS of a length of 80 cm × 0.1 mm and two columns to the olfactory ports of a length of 3 m × 0.53 mm, all of which were deactivated, fused silica columns (Agilent Technologies). The sample was split into the following proportions: MSD:ODP:ODP 0.12:0.88:0.88, corresponding to a flow rate of 0.3 mL min^{-1}:1.1 mL min^{-1}:1.1 mL min^{-1}. The transfer line for each ODP was heated at 220 °C to prevent the condensation of volatiles in the capillaries.

Olfactory analyses were performed by a trained panel of 10 sniffers. Individuals participating in the olfactometric analyses were trained as described by Fuchsmann et al.[32] The focus here was placed on the aromas related to malty notes. Comments were registered and processed at the end of each session with AcquiSniff software version 6.5.9 (INRA, Clermont-Ferrand, France). Aromagrams were computed, recorded, and processed with AcquiSniff software, as described in the literature.[33]

Cultures and strains selected for olfactometry analyses were based on a rapid orthonasal olfactory screening performed by three trained panelists. The static olfactory screening took place in the following format: the three panelists smelled the different cultures and denoted each one in four categories (neutral, light malty, malty, and strong malty). RdV 18 and 22 were chosen as non-malty references, and RdV 27 and FAM-1233 were chosen as malty ones.

Analyses of Total L-Leucine and L-Glutamic Acid by HPLC-UV. *Hydrolyzing and Derivatizing the Samples.* RSM and MRS samples in spiking studies were analyzed for their total amino acid content. Sample preparation was based on a procedure described by Waters.[34] Briefly, hydrolysate samples were obtained after 24 h of acid hydrolysis (HCl 6 M) at 110 °C. Then, 100 μL of the hydrolysate was evaporated to dryness and reconstituted with 20 mM HCl, which contained α-aminobutyric acid (AAbA) as the internal standard. To convert the amino acids into highly stable derivatives, 20 μL of the reconstituted sample was added to 60 μL of borate buffer and 20 μL of the derivatization reagent (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate). The mixture was mixed immediately using a vortex and heated in a block to 55 °C for 10 min.

HPLC-UV Parameters. L-Leucine and L-glutamic acid analyses were conducted using a Vanquish HPLC system (Thermo Fisher Scientific, Reinach, Switzerland) equipped with an ultraviolet (UV) detector. Chromatographic separation was carried out on an AccQ-Tag Ultra analytical column (2.1 × 100 mm, 1.7 μm) (Waters, Baden, Switzerland) using the mobile phase and gradient described by Waters.[34] Each analysis was measured in duplicate. The flow rate was held at 0.7 mL min^{-1}. A volume of 0.5 μL of the sample was injected onto the column operating at 55 °C. UV chromatograms were recorded at 260 nm. Calibration standards were prepared after appropriate dilution of working solutions. For the working solution preparation, an amino acid standard solution (2.5 μmoL mL^{-1} in HCl 0.1 N) was used.

Analyses of $^{13}C_6$-L-Leucine by HPLC-MS/MS. *Extraction of Free Amino Acids.* An extraction solution with HCl 0.1 M and 2% (v/v) thiodiglycol in Milli-Q water was prepared. Then, 500 mg of sample was added to 25 mL of the extraction solution in a 50 mL glass bottle. The mixture was mixed for 60 min and left to decant. Next, 10 mL of the upper liquid was sampled in another 25 mL Schott glass bottle. Five milliliters of 6% (w/v) sulfosalicylic acid in Milli-Q water and left to agitate for another 5 min. The solution was filtered in a disposable plastic test tube using a syringe filter of 0.20 μm porosity and analyzed by HPLC-MS/MS.

Sample Preparation for HPLC-MS Analyses. Fifty microliters of the extracted sample, 15 μL of 13 μM AAbA internal standard solution, and 0.935 mL of 0.1% formic acid (FA) solution in H_2O-MeOH (8:2) were added to an HPLC vial of 1.5 mL for a total volume of 1 mL. Calibration curves of L-leucine and $^{13}C_6$-L-leucine were established for each sample using the method of standard addition in the extracted sample. Each sample was analyzed in duplicate.

HPLC-MS/MS Parameters. AAbA, $^{13}C_6$-L-leucine, and L-leucine were detected by an Agilent 6460 MS/MS coupled with a 1290 Infinity LC. A reversed-phase Agilent Poroshell 120 EC-C18 (2.1 × 100 mm, 2.7 μm) column was used for chromatographic separation. The amino acids were separated by a gradient solution. The mobile phase (A) consisted of water with 0.1% formic acid, and the mobile phase (B) was methanol with 0.1% FA. The gradient profile was as follows: 0−2.00 min, 10.0% B; 2.00−5.00 min, 10% B → 90.0% B; 5.00−7.00 min, 90.0% B; 7.00−8.00 min, 90.0% B → 10% B;

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Table 1a. Intensity of Extracted Ion Counts (EIC) of Identified Compounds Responsible for Malty Aroma in RSM Cultures and Mini Cheeses ($N_{replicates}$ = 5)[a]

	Extracted ion counts (mean intensity ± standard deviation)					
compound	RdV 18 (milk)	RdV 22 (milk)	RdV 27 (milk)	FAM-1233 (milk)	RdV 18 (cheese)	RdV 27 (cheese)
2-methylpropanal (*m/z* 72)	$1.49 \times 10^4 \pm 1.67 \times 10^3$	$1.83 \times 10^4 \pm 4.89 \times 10^3$	$5.56 \times 10^5 \pm 1.44 \times 10^5$	$4.85 \times 10^4 \pm 1.25 \times 10^4$	$3.03 \times 10^4 \pm 6.74 \times 10^3$	$6.68 \times 10^4 \pm 1.64 \times 10^4$
2-methylbutanal (*m/z* 86)	753 ± 55	896 ± 376	$9.31 \times 10^4 \pm 1.6 \times 10^4$	$2.31 \times 10^3 \pm 337$	$2.29 \times 10^3 \pm 1.73 \times 10^3$	$9.57 \times 10^3 \pm 2.99 \times 10^3$
3-methylbutanal (*m/z* 86)	678 ± 231	715 ± 314	$4.06 \times 10^5 \pm 1.16 \times 10^5$	$2.69 \times 10^4 \pm 7.13 \times 10^3$	$7.13 \times 10^3 \pm 2.88 \times 10^3$	$1.51 \times 10^5 \pm 6.70 \times 10^4$
2-methylbutan-1-ol (*m/z* 70)	$1.52 \times 10^5 \pm 8.22 \times 10^3$	$1.63 \times 10^5 \pm 4.91 \times 10^4$	$2.44 \times 10^7 \pm 1.64 \times 10^6$	$7.48 \times 10^6 \pm 2.85 \times 10^5$	$1.31 \times 10^6 \pm 1.15 \times 10^6$	$4.54 \times 10^7 \pm 2.74 \times 10^6$
3-methylbutan-1-ol (*m/z* 70)						

[a]RdV 18 and 22 were the non-malty references, and RdV 27 and FAM-1233 were the malty references.

Table 1b. Intensity of Olfactory Signal ($N_{replicates}$ = 10) and Literature Odor Detection Threshold of Identified Compounds Responsible for Malty Aroma in RSM Cultures and Mini Cheeses[c]

	Olfactory signal (mean intensity x mean detection)						odor detection threshold (lit.)[35]		
compound	RdV 18 (milk)	RdV 22 (milk)	RdV 27 (milk)	FAM-1233 (milk)	RdV 18 (cheese)	RdV 27 (cheese)	in air (mg m^{-3})	in water (mg kg^{-1})	in oil (mg kg^{-1})
2-methylpropanal (*m/z* 72)	0	0	0.2	0.1	0	0.25	0.001	0.00049	0.043[a]
2-methylbutanal (*m/z* 86)	0.05	0	1.95	1.9	0.18	3.18	0.01	0.0015	0.14[a]
3-methylbutanal (*m/z* 86)							0.00035	0.0005	0.013[a]
2-methylbutan-1-ol (*m/z* 70)	0	0	1.6	2.4	0.025	3.83	0.14	1.2	0.48[b]
3-methylbutan-1-ol (*m/z* 70)							0.0061	0.22	0.1[b]

[a]Vegetable oil. [b]Deodorized oil. [c]RdV 18 and 22 were the non-malty references, and RdV 27 and FAM-1233 were the malty references.

Table 2. Combinations of Strains Used for the Influence of Strains on the Production of 3-Methylbutanal and 3-Methylbutan-1-ol

strain number	*L. lactis* subsp. *lactis* FAM-17906	*L. lactis* subsp. *lactis* FAM-17940	*L. lactis* subsp. *cremoris* FAM-23219	*L. lactis* subsp. *lactis biovar diacetylactis* FAM-21959	*S. thermophilus* FAM-20898	*S. thermophilus* FAM-20910
mix 1	X	X				
mix 2	X	X	X			
mix 3					X	X
mix 4			X		X	X
mix 5	X	X			X	X
mix 6	X	X	X	X	X	X

and 8.00−10.00 min, 10% B. A volume of 2 μL was injected and eluted at a flow rate of 0.250 mL min^{-1} with the column temperature at 30 °C.

The source temperature was set at 300 °C with a nitrogen gas flow of 5 L min^{-1} and the nebulizer at 25 psi. Data were collected by single reaction monitoring (SRM) in the positive mode. The MS/MS parameters were optimized for each individual analyte by injecting a standard solution of the target compounds with a CE of 10 V and a dwell time of 200 ms for all compounds. The fragmentor voltage was set at 90 V for $^{13}C_6$-L-leucine and L-leucine and at 80 V for AAbA. The parent ions and product ions were respectively monitored for $^{13}C_6$-L-leucine (*m/z* 138, *m/z* 91.1), L-leucine (*m/z* 132, *m/z* 86.1), and AAbA (*m/z* 104, *m/z* 58.2).

RESULTS

GC−MS−O Screening for Malty Compounds. Five molecules were identified as malty (Tables 1a and 1b): 2-methylpropanal (2-MP), 2-methylbutanal (2-MB), 3-methylbutanal (3-MB), 2-methylbutan-1-ol (2-MBol), and 3-methylbutan-1-ol (3-MBol). Aromagrams of the olfactory class malty can be found in the Supporting Information (Figure S1). 2-MB and 3-MB could not be separated by olfactometry due to slight coelution. MS results of these two structural isomers could, however, separate them. Quantitated results of kinetic analyses determined that 3-MB was more abundant than 2-MB in these cultures (data not shown). Odor thresholds from the literature[35] show that 2-MP, 3-MB, and 3-MBol have the lowest detection threshold in air (Tables 1a and 1b). Matheis et al.[36] were recently able to separate 2-MBol and 3-MBol using two-dimensional high-resolution GC−MS. However using our method, these two compounds could not be separated on the selected column, though the high ratio of *m/z* 55 over *m/z* 57 gave an indication that mostly 3-MBol was present (Supporting Information, Table S3). Moreover, the odor threshold of 3-MBol is lower in air, water, and oil than 2-MBol (Table 1b). Taking into account their literature

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odor threshold, detection frequencies, and odor intensities by GC−O, the focus was set on 3-MB and 3-MBol.

Influence of Individual Strains on the Formation of 3-Methylbutanal and 3-Methylbutan-1-ol. Individual strains of RdV 27 were incubated in RSM as well as some combinations of these strains, as detailed in Table 2. Figure 2 shows the peak areas of 3-MB (m/z 86) and 3-MBol (m/z 70) of the different strains and mixtures analyzed by HS-SPME-GC−MS. Different concentrations of 3-MB and 3-MBol could be observed between the fermentation of strain combinations as well as for individual strains.

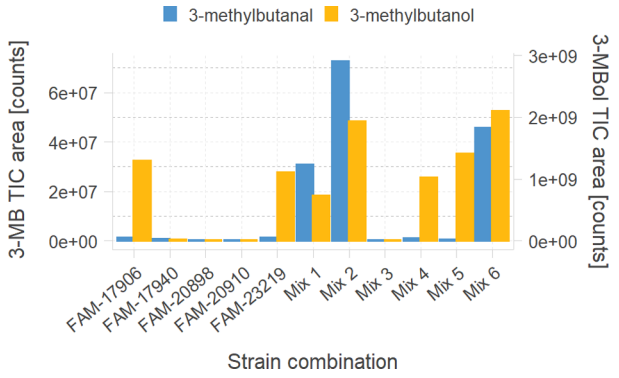

Figure 2. 3-Methylbutanal (m/z 86) and 3-methylbutan-1-ol (m/z 70) amounts in EIC (extracted ion count) produced by various single or combined strains after 18 h of incubation at 30 °C in reconstituted skim milk ($N_{replicates}$ = 1).

Kinetic Studies in Mini Raclette Cheeses, RSM, and Filter-Sterilized MRS Broth. The kinetics of 3-MB and 3-MBol were tracked in mini Raclette cheeses, RSM, and filter-sterilized MRS broth to understand their metabolic formation in cheese. Kinetic profiles of 3-MB and 3-MBol analyzed by HS-SPME-GC−MS in mini cheeses with RdV 27 as a starter culture are illustrated in Figure 3. Notably, 3-MB increased over time from the beginning of ripening, reached a maximum after 7 h of pressing, and then declined until the end of the two-month ripening period. In contrast, 3-MBol production started a few hours after the increase of 3-MB and reached a maximum after one week of ripening in the cellar. The kinetics in mini Raclette cheeses was repeated with the same strains but with a different *L. lactis* subsp. *lactis* biovar *diacetylactis* and quantitated (Supporting Information, Figure S2). Results showed similar kinetics with final concentrations after two months of ripening in the cellar of 10.77 μM for 3-MB and 32.1 μM for 3-MBol.

Kinetic profiles of 3-MB and 3-MBol produced by *L. lactis* subsp. *cremoris* FAM-17841 in RSM and MRS are illustrated in Figure 4. Figure 4b shows that the kinetic profile of RSM is comparable to that of mini cheeses. The results showed an explicit increase in 3-MB in the first 8 h of culture incubation followed by 3-MBol steadily increasing between 2 and 18 h.

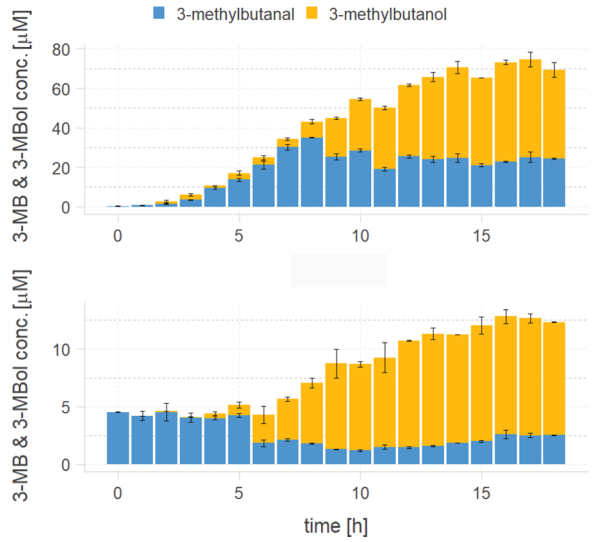

Figure 4. Kinetic profile of 3-methylbutanal and 3-methylbutan-1-ol in [μM] produced by *L. lactis* subsp. *cremoris* FAM-17841 cultivated in reconstituted skim milk (top) and filter-sterilized MRS broth (bottom) at 30 °C between 0 and 18 h. Samples were analyzed and quantitated by DHS-VTT-GC−MS ($N_{replicates}$ = 2).

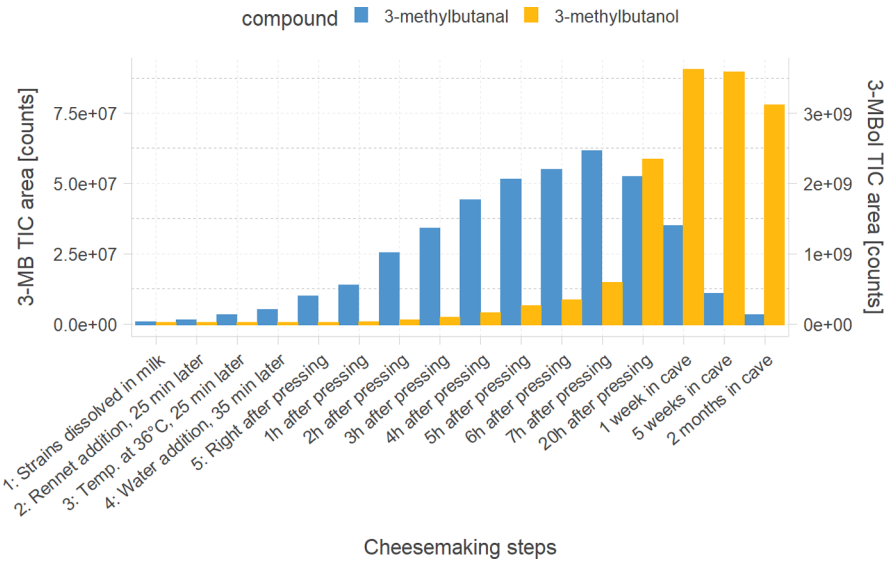

Figure 3. Kinetics of 3-methylbutanal (m/z 86) and 3-methylbutan-1-ol (m/z 70) during cheesemaking given by the HS-SPME-GC-MS extracted ion count (EIC) ($N_{replicates}$ = 1).

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However, as depicted in Figure 4a, cultivation of the bacteria in MRS broth did not build up as much 3-MB and 3-MBol as in RSM, though bacteria grew very well in both media with a similar trend and growth (Figure 5).

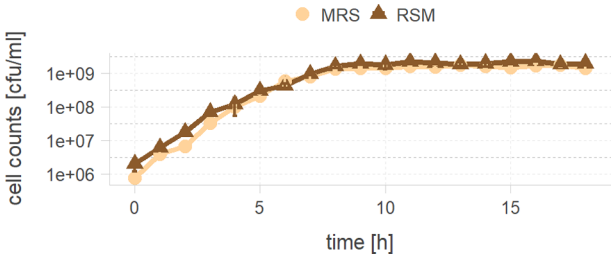

Figure 5. Viable cell counts of *L. lactis* subsp. *cremoris* FAM-17841 in RSM and filter-sterilized MRS in the logarithmic scale.

Influence of the Addition of α-Ketoisocaproic Acid, α-Ketoglutaric Acid, and L-Leucine on 3-MB and 3-MBol Formation. To study their influence on the production of 3-MB and 3-MBol by *L. lactis*subsp. *cremoris* FAM-17841, the fermentation media (RSM and MRS) were spiked with α-ketoisocaproic acid (α-KIC), α-ketoglutaric acid (α-KG), leucine, and $^{13}C_6$-leucine (Figures 6 and 7 and Table 3). Spiking concentrations were chosen so that the detectors would not be saturated but an effect on the production could still be observed. Non-spiked samples were used as the control.

In the presence of a 1 mM excess of free leucine in RSM, levels of 3-MB and 3-MBol increased by a factor of three compared to the control. Nevertheless, the amount of leucine consumed did not account for the total molar amount of 3-MB and 3-MBol as more 3-MB and 3-MBol were produced than leucine consumed. The addition of ^{13}C-labeled leucine allowed us to follow the actual amount of leucine being degraded during the 18 h fermentation. Both ^{13}C-labeled 3-MB and 3-MBol were detected in RSM and quantitated (Figure 6). In this case, the total amount of ^{13}C-labeled 3-MB and 3-MBol (455 μM) was lower than the amount of ^{13}C-leucine consumed, 1.35 mM (Table 3). In MRS samples, an excess of leucine did not significantly change the concentrations of 3-MB and 3-MBol after fermentation. By following the ^{13}C-

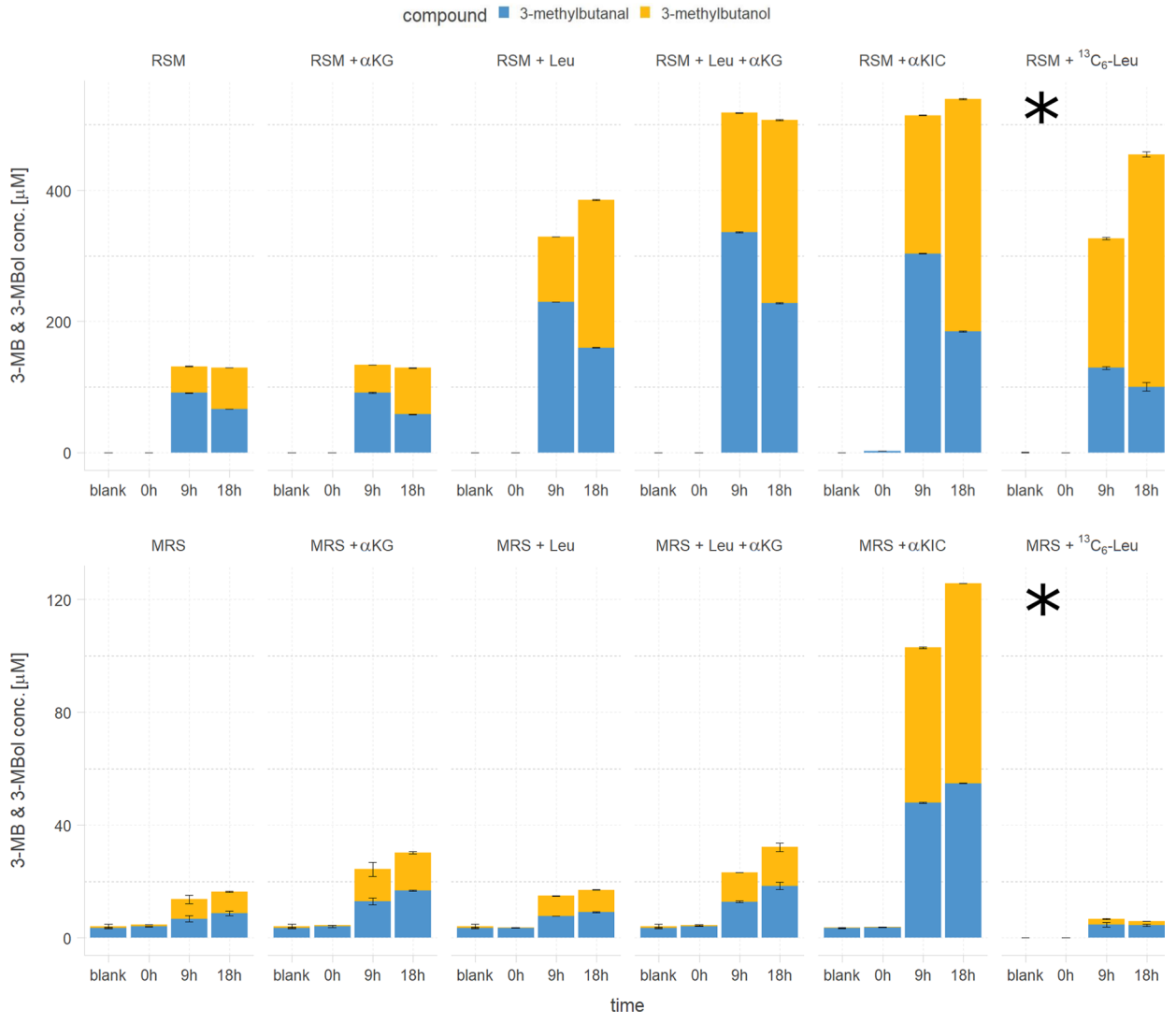

Figure 6. Influence of α-ketoglutaric acid (αKG), leucine (Leu), and α-ketoisocaproic acid (αKIC) on the formation of 3-methylbutanal (3-MB) and 3-methylbutan-1-ol (3-Mbol) in μM produced by *Lactococcus lactis* subsp. *cremoris* FAM-17841 at 30 °C in RSM (top) and MRS (bottom) media. Note: graphs with an asterisk (*) are ^{13}C-labeled compounds ($N_{replicates}$ = 2).

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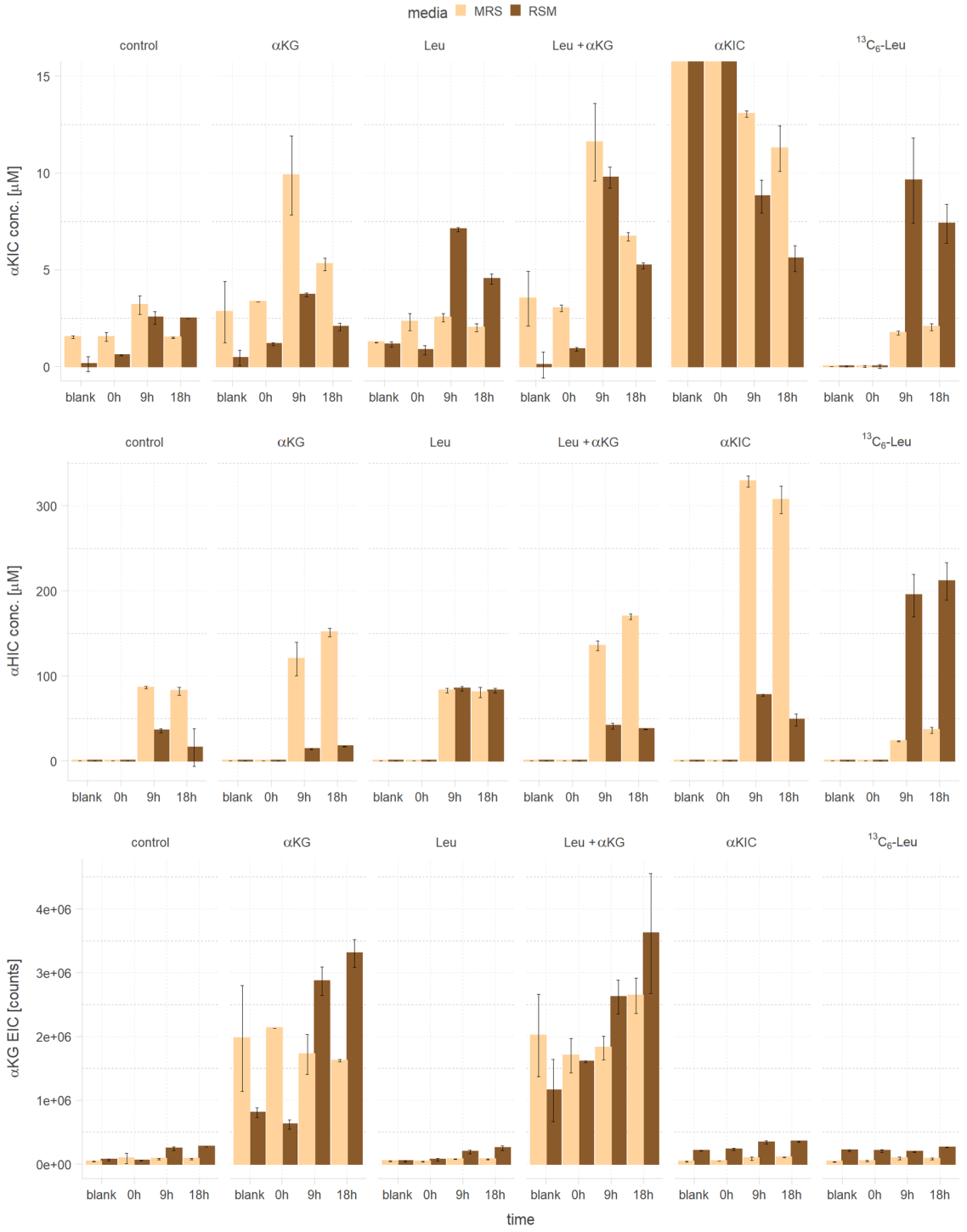

Figure 7. Comparison of the formation of α-ketoisocaproic acid (α-KIC) in μM (top), α-hydroxyisocaproic acid (α-HIC) in μM (middle), and α-ketoglutaric acid (α-KG) in EIC m/z 101 (extracted ion count) (bottom) produced by *L. lactis* subsp. *cremoris* FAM-17841 at 30 °C in RSM and MRS when spiked with α-KG, L-leucine, L-leucine, and α-KG, α-KIC, and $^{13}C_6$-L-leucine, respectively. Note: graphs with an asterisk (*) are ^{13}C-labeled compounds ($N_{replicates}$ = 2).

labeled products in MRS, a maximum concentration of 6 μM of $^{13}C_5$−3-MB and $^{13}C_5$−3-MBol was measured, which is 75 times lower than in RSM. To understand the inhibited production of 3-MB in MRS samples, α-KG was added to both RSM and MRS media to promote the catabolism of leucine.

No increase in 3-MB or 3-MBol was observed in RSM with the addition of α-KG (Figure 6, top). However, the addition of leucine combined with α-KG produced 1.5 times more 3-MB and 3-MBol than RSM spiked with leucine only. In RSM, an excess of α-KG alone did not influence the production of 3-MB and 3-MBol. Qualitative analyses of α-KG showed that α-KG itself is produced during fermentation, even when an excess is added. In MRS, however, a 2-fold increase of both 3-MB and 3-MBol could be observed when spiked with α-KG only. Interestingly, there was no influence of the addition of leucine and α-KG in comparison with the addition of α-KG only.

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Table 3. Total Glutamic Acid and Leucine Analyzed by LC-UV and $^{13}C_6$-Leucine Analyzed by LC−MS in mmol L^{-1} ($N_{replicates}$ = 2)

measured AA	medium	time	control	αKG	Leu	Leu + αKG	αKIC	$^{13}C_6$-Leucine[a]
leucine	RSM	blank	30.01 ± 0.12	30.38 ± 0.39	30.68 ± 0.43	30.55 ± 0.2	30.16 ± 0.12	3.16 ± 0.21
		0 h	30.25 ± 0.02	29.99 ± 0.1	30.85 ± 0.24	30.49 ± 0.23	30.24 ± 0	2.6 ± 0.26
		9 h	30.05 ± 0.1	29.99 ± 0.05	30.74 ± 0.82	30.17 ± 0.39	29.92 ± 0.07	2.25 ± 0.04
		18 h	29.69 ± 0.29	29.84 ± 0.28	30.41 ± 0.29	30.36 ± 0.23	29.73 ± 0.31	1.81 ± 0.17
	MRS	blank	8.11 ± 0	8.17 ± 0.02	9.05 ± 0.02	9.12 ± 0.07	8.11 ± 0.01	3.05 ± 0.02
		0 h	8.29 ± 0.03	7.98 ± 0.19	9.19 ± 0	9.15 ± 0.14	8.18 ± 0.09	2.91 ± 0
		9 h	8.18 ± 0.09	8.13 ± 0.07	8.85 ± 0.11	9.02 ± 0.15	8.12 ± 0.06	3.21 ± 0
		18 h	8.02 ± 0.03	7.98 ± 0.16	8.92 ± 0.24	9 ± 0.08	8.13 ± 0.04	3.13 ± 0.04
glutamic acid	RSM	blank	58.41 ± 0	58.83 ± 0.45	57 ± 0.29	57.3 ± 0.55	58.3 ± 0	
		0 h	59.16 ± 0.04	58.4 ± 0.07	58 ± 0.52	57.52 ± 0.06	58.92 ± 0.21	
		9 h	58.85 ± 0.46	58.05 ± 0	58.16 ± 1.33	57.8 ± 0.68	58.37 ± 0.33	
		18 h	58.13 ± 0.49	57.94 ± 0	57.66 ± 1.5	57.89 ± 0.42	57.37 ± 1.36	
	MRS	blank	18.82 ± 0.04	18.99 ± 0.03	18.9 ± 0.06	18.97 ± 0.15	18.88 ± 0.13	
		0 h	19.25 ± 0.06	18.76 ± 0.31	18.81 ± 0	18.96 ± 0.21	18.86 ± 0.38	
		9 h	19.37 ± 0.17	19.27 ± 0.14	18.83 ± 0.4	19.02 ± 0.19	19.05 ± 0.13	
		18 h	19.12 ± 0.06	19.2 ± 0.13	18.82 ± 0.24	19.15 ± 0.03	18.92 ± 0.04	

[a]These results stem from the analysis of $^{13}C_6$-leucine.

Nevertheless, an increase in the production of α-KG was observed when leucine and α-KG were spiked in the media, but when α-KG was spiked alone, a slight depletion of the α-KG was observed (Figure 7, bottom). In addition, the concentrations of 3-MB and 3-MBol in MRS did not reach the concentrations observed in RSM, even with excess α-KG.

To observe if the decarboxylation of α-ketoisocaproic acid to 3-MB and 3-MBol occurred, α-KIC was spiked in the culture media. A clear influence on the formation of 3-MB and 3-MBol with an increase in both RSM and MRS could be observed (Figure 6). In RSM at 0 h, 2.58 μM 3-MB was present, but no 3-MB was detected in other 0 h samples, whether it was spiked with reactants or in MRS samples. Although the same amount of α-KIC was added to RSM as to MRS, the total concentration of 3-MB and 3-MBol in RSM after 18 h fermentation was higher than in MRS with a concentration of 539 μM in RSM and 125 μM in MRS. The amount of α-KIC spiked was mostly depleted with 5 μM in RSM and 10 μM in MRS left at the end of the fermentation, indicating that α-KIC was consumed in both cases (Figure 7, top).

A side reaction to the decarboxylation of α-KIC can occur, which is hydroxylation giving α-hydroxyisocaproic acid (α-HIC). Quantitative analyses of α-HIC showed that the acid was generally found in higher concentrations in MRS than in RSM (Figure 7, middle).

DISCUSSION

The compounds identified by GC-MS-O as malty were previously associated with malty aroma in other cheeses, such as Camembert,[37] blue cheese,[38] Swiss cheese,[1] Parmigiano Reggiano,[39] and even Swiss Gruyère cheese,[40] but they were identified here for the first time as potent odorants for malt in Swiss Raclette. These compounds are formed due to the activity of starter cultures added to the cheese manufacture.[11] One way to control the formation of the flavor compounds is the development of specific starter cultures since it is known that cheese flavor can be altered depending on the bacteria used.[41,42]

Figure 2 shows that individual strains of RdV 27 can develop different concentrations of the compounds responsible for the malty aroma. This can be explained by different enzymatic activity or expression of the gene of each strain. Interestingly, however, the combination of different strains can modify the formation of 3-MB and 3-MBol. One specific strain, *L. lactis* subsp. *cremoris* FAM-23219, showed a potency of producing 3-MB and 3-MBol either when fermented alone or when combined with other strains. This strain could potentially be used in cheesemaking where a malty flavor is appreciated. These preliminary results confirm that aroma formation can be complex when a mixture of cultures is involved; the careful selection of strains is therefore important when specific aromas are desired during cheese manufacture.

Redox potential has also previously been described as playing a role in the formation of malty compounds.[16] The change in redox potential can be due to processing parameters, chemical compounds, and also fermentation activity. With the idea that the production of these compounds by the starter culture could already be affected by redox potential, pH and redox were measured in malty and non-malty cultures during the fermentation of RSM (Supporting Information, Figures S3 and S4). However, no significant differences in either pH or redox potential could be observed between the different cultures. Therefore, redox and pH were not suitable for our purpose of using them as a possible indicator of the production of malty aroma compounds by a culture.

Kinetic analyses of 3-MB and 3-MBol in mini cheeses cultured with RdV 27 helped us to understand the formation of these molecules during cheese manufacture. The delayed increase of 3-MBol compared to 3-MB is explained by the reduction of 3-MB to 3-MBol through an alcohol dehydrogenase.[3,5] The re-conducted experiment of kinetics during cheese manufacture (Supporting Information, Table S1 and Figure S2) showed that the same trends were found, and the concentration of 3-MBol after 10 weeks of ripening was 34 times higher than 3-MB. Odor activity values (OAVs) were calculated based on the literature on odor detection thresholds of these compounds in water and oil[35] (Table 1b) and quantitated concentrations in mini Raclette cheeses after two months of ripening in the cellar (Supporting Information, Figure S2). According to the concentrations analyzed, OAVs of 71 in vegetable oil and 2000 in water for 3-MB and 321 in deodorized oil and 146 in water for 3-MBol could be

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calculated. These OAVs would estimate that both molecules can be found odorant in their concentrations. As fat content in Raclette cheeses can be found around 30%,[43] OAVs in cheese would be expected to be closer to the OAVs given in oil. However, the matrix texture, salt concentration, and pH also affect the odor threshold, which in turn affects the OAVs. Indeed, the cheese matrix contains not only fat but also protein and molecules, which are known to bind to protein, increasing their retention in the matrix and therefore increasing their odor threshold and decreasing the OAVs.[44−46] As observed during kinetic analyses, different ratios of 3-MB and 3-MBol can be found over the ripening period, which can change the perception of malt aroma, making the definition of maltiness difficult during sensory analyses. Further, studies must be conducted in order to understand their individual or combined sensorial impact in Raclette-type cheeses.

Kinetics in RSM with *L. lactis* subsp. *cremoris* FAM-17841 showed similar behaviors. Although we used here a different strain of the same species than those above, the choice for this strain stemmed from our knowledge from internal trials where this strain showed multiple-times stronger malt aroma in Raclette cheeses. The results can be compared as the activity of these *Lactococci* remains similar, and the incubation temperature was chosen so that all chosen *Lactococci* can develop. Moreover, as the trends over the first 18 h of incubation showed similar results to the mini cheeses, the results of both kinetics were comparable. The kinetics in filter-sterilized MRS broth with this strain, however, showed different progression and concentrations of 3-MB and 3-MBol compared to RSM, though the strain grew similarly in MRS as in RSM. This discrepancy could be due to an unknown reactant in MRS broth or the presence of inhibitory compounds, which would explain the lower production of 3-MB and 3-MBol.

It is known from the literature that α-KIC, α-KG, and leucine participate in the formation pathway of 3-MB.[4−6,16,47] The first step in the degradation of leucine starts with its transamination using α-KG as catalyst, resulting in the formation of α-KIC.[3,5,6,16] In the presence of a large excess of free leucine in RSM, levels of 3-MB and 3-MBol increased by a factor of 3 (Figure 6, top). Through the addition of ^{13}C-leucine, the discrepancy between the amount of leucine produced and the total amount of 3-MB and 3-MBol could potentially be explained by the fact that, as fermentation progresses, leucine biosynthesis occurs by regenerating leucine from other amino acids or releasing leucine from proteins through proteolysis.[48] Another explanation could be that free leucine is not only directly degraded to 3-MB but also used in the production of biomass. A previous study also found a *L. lactis* subsp. *cremoris* strain grown in M17 medium containing lactose, producing 3-MB without leucine addition required.[49] This latter pathway has not yet been explored and could partially explain the discrepancies observed as the same species was used in this experiment.

The production of 3-MB and 3-MBol in MRS cultures spiked with free leucine did not see an increase as observed in RSM cultures, which could be due to a lack of readily available α-ketoacid. Previous studies have revealed that an α-ketoacid is required for the transamination of an amino acid.[6,50] In the case of leucine, α-KG is used as the amino group acceptor and enhances the catabolism of amino acids.[47] The addition of α-KG did enhance the catabolism of free leucine to 3-MB and 3-MBol in our study. Also, even with an excess of α-KG, no or few 3-MB and 3-MBol molecules were produced if no free leucine was present. We assume that the same effect will result if the amino acids isoleucine or valine are present. However, this still needs to be confirmed. The production of 3-MB and 3-MBol in MRS did not reach concentrations observed in RSM, which, according to the currently known pathway, could be due to two suppositions. First, α-KG was not regenerated in MRS, which can be observed in Figure 7 (bottom) as the concentration of α-KG did not increase as much in MRS as in RSM. Interestingly, when α-KG was added, the abundance of α-KG itself increased by almost 3-fold over fermentation compared to the control samples. In lactic acid bacteria, α-KG is formed by deamination of glutamate, and through the conversion of leucine to α-KIC, α-KG is converted to glutamic acid. Nevertheless, no significant increase or decrease in glutamic acid (Table 3) could be observed in our case with the addition of α-KG. It is therefore most likely that, in this case, another (or other) pathway(s) are responsible for the formation of α-KG.

Second, if the α-ketoacid decarboxylase was inhibited, the decarboxylation of α-KIC to 3-MB could not occur. α-KIC has been established as the intermediate between leucine and 3-MB.[4] The decarboxylation of α-KIC to 3-MB occurs intracellularly and has also been identified as the rate-controlling step.[3,5,7] The 5-fold increase in MRS and 3-fold increase in RSM of 3-MB and 3-MBol after 9 h of incubation excludes the hypothesis of an inhibited α-KIC decarboxylation with the addition of α-KIC in excess. If the decarboxylation reaction was inhibited, the production of 3-MB would not occur according to the currently known pathway.

Although the sample was directly frozen in liquid nitrogen immediately after inoculation, the rapid conversion of α-KIC to 3-MB was unexpected. As the bacteria was not yet active, such a fast reaction to 3-MB could have been taking place extracellularly instead of intracellularly, as established in the literature.[3,16] Another possibility could be that some of the cells used for inoculation were already lysed and released the enzyme or 3-MB was already present in the cells; further study is required to confirm these hypotheses. Nevertheless, 3-MB was found to be present in the 0 h samples only when α-KIC was spiked. A similar phenomenon was discovered in cheesemaking trials, although it is not yet conclusively understood whether the discovered off-flavor is related to the malty taste. Shortly after the addition of a "malty" starter, this off-flavor was sensorially detectable. The timespan until detection was too short; therefore, bacterial activity could not be responsible for the off-flavor. It would therefore be interesting to check whether α-KIC is present in raw milk or if an analogue molecule exists.

Even if the same amount of α-KIC was added to RSM as to MRS, the total concentration of 3-MB and 3-MBol in RSM was higher than in MRS. This discrepancy can be explained by competition for α-KIC between hydroxyl acid dehydrogenase (HADH) and keto acid decarboxylase (KADC).[7] To observe the competition of these two reactions, the product of dehydrogenase, α-hydroxyisocaproic acid (α-HIC), was quantitatively measured. The amount of the corresponding hydroxyacid in MRS was found to be twice as high as in RSM, showing a higher rate of conversion from α-KIC to α-HIC in MRS than in RSM, which explains the difference observed with the addition of α-KIC. This trend can also be observed throughout the other spikings as well as in the control sample, indicating that the conversion of α-KIC to α-HIC is more prominent than to 3-MB in MRS. This partially confirms the

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discrepancies observed between RSM and MRS throughout the kinetic studies. The mechanisms for the reaction and the trigger for pathway selection directed toward α-HIC instead of 3-MB remain unknown.

The results obtained here give further insight into the formation of 3-MB and 3-MBol during cheese manufacture and ripening. The studied kinetics in cheese showed for the first time that these compounds are already formed during the first hours of cheese manufacture. Formation of 3-MB could be observed during the time when the bacteria were not yet active with the spiking of α-KIC. The results of these studies could further improve the selection of culture for cheese manufacture in developing a screening method involving the addition of α-KIC to push the formation of these malty compounds. Spiking studies highlighted the discrepancies of the formation of 3-MB and 3-MBol, showing that many steps of the formation pathway of these compounds still need to be clarified. Future work should focus on explaining the discrepancies between MRS broth and RSM, which could help in inhibiting or enhancing the malt aroma not only in Raclette-type cheese but also in other cheese products. Furthermore, the correlation between strain mixtures and 3-MB formation should be better understood in order to develop optimal starter cultures.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c06570.

Table S1: detailed summary of starter cultures and strains used in this study, Table S2: identification of target compounds on polar (FFAPplus) and apolar (HP-5MS) columns, Table S3: m/z 55 over m/z 57 ratio of 2-methylbutan-1-ol and 3-methylbutan-1-ol in selected malty cultures, Figure S1: olfactory signal of the malty class in cultures fermented in RSM and in mini-Raclette type cheeses obtained by DHS-VTT-GC-O using the AcquiSniff software, Figure S2: kinetics of 3-methylbutanal and 3-methylbutan-1-ol quantitated in μM by DHS-VTT-GC-MS in mini Raclette-type cheeses over the pressing and ripening period of the cheese manufacture, Figure S3: pH monitoring of five Raclette mixtures in reconstituted skim milk for 18 h with malty cultures and non-malty culture, and Figure S4: redox potential monitoring of five Raclette mixtures in reconstituted skim milk for 18 h with malty cultures and non-malty culture (PDF)

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Notes

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■ ABBREVIATION AND NOMENCLATURE

2-MB, 2-methylbutanal; 2-MBol, 2-methylbutan-1-ol; 3-MB, 3-methylbutanal; 3-MBol, 3-methylbutan-1-ol; AAbA, α-aminobutyric acid; α-HIC, α-hydroxyisocaproic acid; α-KIC, α-ketoisocaproic acid; α-KG, α-ketoglutaric acid; CE, collision energy; DHS-VTT, dynamic headspace-vacuum transfer in trap; EIC, extracted ion count; FA, formic acid; GC−MS-O, gas chromatography−mass spectrometry−olfactometry; HADH, hydroxyacid dehydrogenase; KADC, keto acid decarboxylase; HPLC, high-pressure liquid chromatography; HS-SPME, headspace-solid phase microextraction; ITEX, in-tube extraction; MRS, de Man, Rogosa, and Sharpe; ODP, odor detection port; RI, retention index; RSM, reconstituted skim milk; RT, room temperature

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