Identifying formation pathways for malty/chocolate flavour compounds in semi-hard cheeses by HS-SPME-GC-MS, HS-ITEX-GC-MS and LC-MS/MS

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Introduction

Hints of malt or chocolate can sometimes be perceived in Swiss Raclette cheese in various intensities. Some Agroscope strains are known to exhibit this aroma often negatively perceived. According to literature, these aromas are due to the presence of 2- and 3-methylbutanal[1][2][3]. This odour can change depending on the balance between the responsible compounds. One possible pathway for the formation of these compounds suggests isoleucine and leucine degradation by intracellular enzymes[4][5].

The compounds responsible for this aroma have been identified as part of the project by gas-chromatography mass-spectrometry olfactometry (GC-MS-O) as 2-, 3-methylbutanal, 2- and 3-methylbutanol. Kinetic studies were performed in mini-cheeses and two culturing media to understand the formation pathway of these compounds.

Methods

Agroscope strains were cultivated in an in-house produced mini-cheese and in two different media during 18 hours at 30°C: fermented milk (FM) and sterile filtered De Man, Rogosa and Sharpe (MRS) broth. Aromatic compounds were analysed using the following methods and conditions:

Headspace solid phase microextraction (HS-SPME) [6][7] **sampling:**

- T= 60 °C, $t_{extraction}$= 60 min, fiber: DVB/CAR/PDMS 50/30 µm 2 cm

Headspace in-tube extraction (HS-ITEX) [8] **sampling:**

- T= 55°C, $t_{incubation}$ = 5 min, $t_{extraction}$ = 10 min, trap: Tenax TA/Carbosieve S III

Sample analyses by LC-MS/MS:

- Column: Poroshell 120 EC C18, 2.1x100mm, 2.7µm
- Eluent A: H_2O 0.1% formic acid; Eluent B: MeOH 0.1% formic acid, gradient

Results

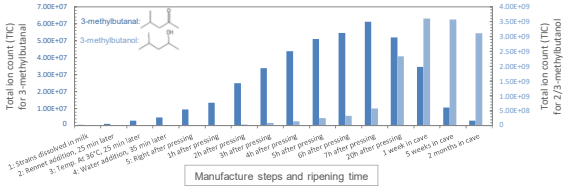

Fig. 1 Kinetics of 3-methybutanal and 2/3-methylbutanol in a mini-cheese during making and ripening given by GC-MS TIC (total ion count).

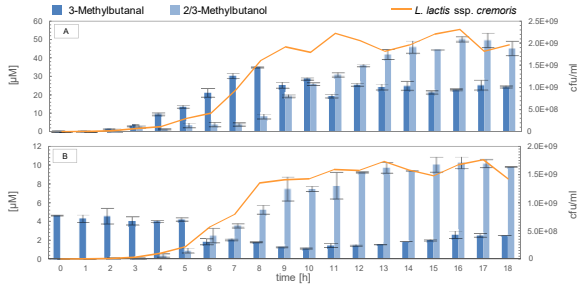

Fig. 2 Kinetics of 3-methylbutanal, 2/3-methylbutanol (in µM) and colony formation of a *Lactococcus lactis* ssp. *cremoris* (in cfu/ml) cultivated in FM (A) and MRS (B) during 18 hours.

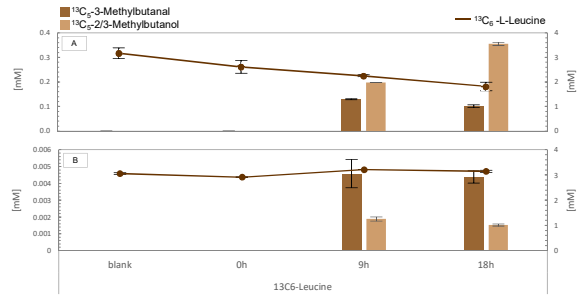

Fig. 3 Kinetics of $^{13}C_5$-3-methylbutanal, $^{13}C_5$-2/3-methylbutanol and $^{13}C_6$-L-leucine of a *Lactococcus lactis* ssp. *cremoris* cultivated in FM (A) and MRS (B) at 0h, 9h and 18 hours (in mM).

The kinetics of the three compounds identified as co-responsibles for the malt/chocolate aroma were tracked in order to know when they were formed and if their concentration remained constant from the strain culture to the cheese ripening. A mixture of strains for Raclette was used for the kinetic analyses in the mini-cheese (Fig. 1). A *Lactococcus lactis* ssp. *cremoris*, main responsible for the strong malt aroma, was used for the ones in fermented milk (FM) and in our model sterile filtered MRS medium (Fig. 2).

To observe their influence on the formation of 2-, 3-methylbutanal, 2- and 3-methylbutanol, several compounds (α-ketoisocaproic acid (aKIC), α-ketoglutaric acid (aKG), L-leucine and $^{13}C_6$-L-leucine) were added to the fermentation media (Fig. 3 and 4).

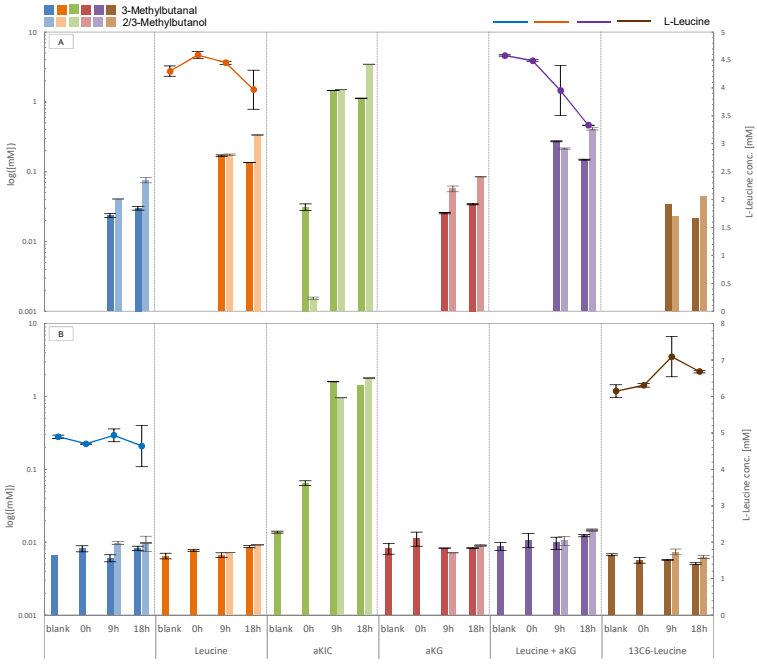

Fig. 4 Kinetics of 3-methylbutanal, 2/3-methylbutanol (in log([mM])) and L-leucine (in mM) of a *Lactococcus lactis* ssp. *cremoris* cultivated in FM (A) and MRS (B) at 0h, 9h and 18 hours. To each media was added L-leucine (orange), aKIC (green), aKG (red), L-leucine and aKG (purple) and $^{13}C_6$-L-leucine (brown).

Conclusion

Kinetic measurements showed that 2- and 3-methylbutanal were formed at the beginning of the cheese making and their concentration decreased during the ripening time. Instead, 2- and 3-methylbutanol were formed after the pressing step and their absolute concentration increased during the first hours in the cheese cellar before maintaining their concentration. The same process, with the resulting strong malt aroma, could also be observed during the 18 hours strain culturing. This led us to the hypothesis that 2- and 3-methylbutanal were reduced to 2- and 3-methylbutanol respectively.

L-leucine and aKIC, previously established as precursor and intermediate respectively of the 3-methylbutanal and 3-methylbutanol formation pathway, were confirmed here with a spiking method. ^{13}C-labelled 3-methylbutanal and 3-methylbutanol were detected with $^{13}C_6$-L-leucine spiking, therefore validating the L-leucine as a precursor and the reduction of 3-methylbutanal to 3-methylbutanol. Additionally, spiking with aKIC has strongly boosted the formation of 3-methylbutanal and 3-methylbutanol. This reaction step between aKIC and 3-methylbutanal is thought to happen extracellularly, since 3-methylbutanal is produced in such high amount and short time. This is incompatible with the low activity of the bacteria at 0h, that would not have time to transport aKIC in the cell and to then export 3-methylbutanal.

A closer look at the differences between the microbial culture in FM and MRS shows that aKIC is clearly defined as the limiting step of the pathway. The addition of non-labelled and 13C-labelled L-leucine to MRS did not show an explicit influence on the formation of 3-methylbutanal and 3-methylbutanol as observed in FM. On the contrary, aKIC addition led to an equivalent concentration of 3-methylbutanal and 3-methylbutanol formed in MRS and in FM. Therefore, a factor missing in MRS must be present in FM in the steps between L-leucine and aKIC.

These results allow us to better understand the formation pathway of compounds responsible for the malt aroma negatively perceived in order to control them during the cheese making.

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