International Journal of Food Microbiology 241 (2017) 173–180

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

# Isolation and characterization of *Lactobacillus helveticus* DSM 20075 variants with improved autolytic capacity

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ARTICLE INFO

*Article history:*
Received 22 June 2016
Received in revised form 27 September 2016
Accepted 15 October 2016
Available online 22 October 2016

*Chemical compounds studied in this article:*
Benzaldehyde (PubChem CID: 240)

*Keywords:*
Adjunct
Starter
Cheese
Autolysis
Ripening
Aroma

ABSTRACT

*Lactobacillus helveticus* is widely used in dairy fermentations and produces a range of enzymes, which upon cell lysis can be released into the cheese matrix and impact degradation of proteins, peptides and lipids. In our study we set out to explore the potential of *Lb. helveticus* DSM 20075 for increased autolytic capacity triggered by conditions such as low pH and high salt concentrations encountered in cheese environments. *Lb. helveticus* DSM 20075 was subjected to varied incubation temperatures (ranging from 37 to 50 °C). High-temperature incubation (in the range of 45 to 50 °C) allowed us to obtain a collection of six variant strains (V45-V50), which in comparison to the wild-type strain, showed higher growth rates at elevated temperatures (42 °C–45 °C). Moreover, variant strain V50 showed a 4-fold higher, in comparison to wild type, autolytic capacity in cheese-like conditions. Next, strain V50 was used as an adjunct in lab-scale cheese making trials to measure its impact on aroma formation during ripening. Specifically, in cheeses made with strain V50, the relative abundance of benzaldehyde increased 3-fold compared to cheeses made with the wild-type strain. Analysis of the genome sequence of strain V50 revealed multiple mutations in comparison to the wild-type strain DSM 20075 including a mutation found in a gene coding for a metal ion transporter, which can potentially be linked to intracellular accumulation of $Mn^{2+}$ and benzaldehyde formation. The approach of high-temperature incubation can be applied in dairy industry for the selection of (adjunct) cultures targeted at accelerated cheese ripening and aroma formation.



## 1. Introduction

Adjunct cultures are used in cheese production to accelerate ripening and support specific flavor development (Fox et al., 1998). Both the acceleration of ripening and flavor development can be affected by the addition of an adjunct culture due to its enzymatic activity (Khalid and Marth, 1990). Adjunct cultures are usually inoculated at cell counts together with the starter culture and they can survive and sometimes even grow in cheese (Antonsson et al., 2002; Briggiler-Marcó et al., 2007). Despite this fact, improvement of adjunct cultures is often focused on obtaining strains with increased autolytic capacity. Autolysis of adjunct cultures leads to the release of intracellular enzymes, which can improve the formation of specific aroma compounds. Previous attempts to modify adjunct cultures of *Lactobacillus helveticus* I or *Lactobacillus casei* T included a variety of sublethal physical treatments such as freeze shock, heat shock or spray drying (Madkor et al., 2000).

*Lb. helveticus* strains are widely used as adjuncts for accelerated ripening in different types of cheeses (Cheddar, Swiss-type). This application of *Lb. helveticus* is based on the fact that this lactic acid bacterium has a potent collection of enzymes including cell-envelope bound proteinases (CEPs) and intracellular peptidases (Griffiths and Tellez, 2013) which upon cell lysis can be released into the cheese matrix and impact proteolysis, as previously shown in Cheddar cheese (Hannon et al., 2003, 2007).

Recently, Smith et al. (2012) isolated heat-resistant variants of *Lactococcus lactis* MG1363 with increased autolytic capacity. The observed autolysis was found to correlate with salt hypersensitivity. Genome analysis of these variants of *L. lactis* MG1363 revealed mutations in gene *llmg_1816* encoding a membrane-bound stress signaling protein of the GdpP family.

In our study we used high-temperature incubation of *Lb. helveticus* DSM20075 to select naturally occurring heat-tolerant and salt sensitive variants with higher autolytic capacity compared to the wild type (WT) in cheese-like conditions. We demonstrated that application of the variants in a cheese ripening model leads to a significant increase of one specific aroma compound (benzaldehyde). Finally, the complete genomes of the WT and of one the heat-tolerant variants were compared to find explanations for the industrially relevant phenotype.

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http://dx.doi.org/10.1016/j.ijfoodmicro.2016.10.020
0168-1605/

## 2. Materials & methods

### 2.1. Strains, growth conditions and growth rate determination

*Lactobacillus helveticus* DSM 20075 (WT) was incubated at temperatures ranging from 37 °C to 50 °C. Incubation of the cultures was performed in test tubes placed in a PCR machine (Veriti® Thermal Cycler, Applied Biosystems, Bleiswijk, The Netherlands), which allowed six different temperatures to be set up in the same run – an increasing temperature gradient every two lanes. The cultures were incubated in MRS broth (0.5% (*w*/*v*) lactose) and at different time points during the incubation (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 36 h) samples were taken, diluted in phosphate buffered saline (PBS) and plated on MRS agar (1.5%) supplemented with 0.5% (w/v) lactose.

After a certain period of exposure to elevated incubation temperatures, non-diluted cultures were plated (with the exception of variant 45 (V45) which was isolated from 100 times diluted culture) and colonies of survivors with increased heat resistance were collected (for details see Table A.2, Supplementary material). The collected isolates were subsequently grown in MRS broth (0.5% lactose) and preserved in glycerol (20% (*v*/v)) at −80 °C for further analysis. Specific growth rates of six isolates (designated as variants V45, V46, V47, V48, V49 and V50) were determined using modified Gompertz model (Zwietering et al., 1990) (Fig. 1). Strain V50, showed the highest growth rate at elevated temperature of 45 °C and was chosen for further analysis of autolytic capacity and the milli-cheese trial (see below).

The caseinolytic *Lactococcus lactis* subsp. *Cremoris* TIFN1 (Erkus et al., 2013) was used as a starter during milli-cheese preparation (see below). *L. lactis* TIFN1 was plated on LM17 (Oxoid, Landsmeer, The Netherlands) agar supplemented with 0.5% (*w*/*v*) lactose and incubated at 30 °C for 24 h to further pick a single colony for inoculation of LM17 broth (0.5% (w/v) lactose). The culture was incubated for 24 h at 30 °C before use in milli-cheese preparation.

### 2.2. Lactate dehydrogenase activity measurement

The lytic capacity of *Lb. helveticus* strains was determined by measuring the activity of lactate dehydrogenase (LDH) released from cells suspended in a lactate buffer (100 mM sodium lactate, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 5 mM $KH_2PO_4$, pH 5) in the absence (control conditions) and presence of 0.5 M NaCl. LDH activity in the supernatant was determined using the LDH cytotoxicity test (Cytotoxicity Detection Kit, Roche, USA) executed according to the manufacturers' protocol. The low pH and high NaCl concentration were chosen to mimic the cheese environment (Weimer, 2007). Cells grown at 37 °C in MRS broth (0.5% lactose *w*/*v*) were harvested, collected by centrifugation at 6000 ×*g* for 10 min, washed twice and finally re-suspended in the lactate buffer.

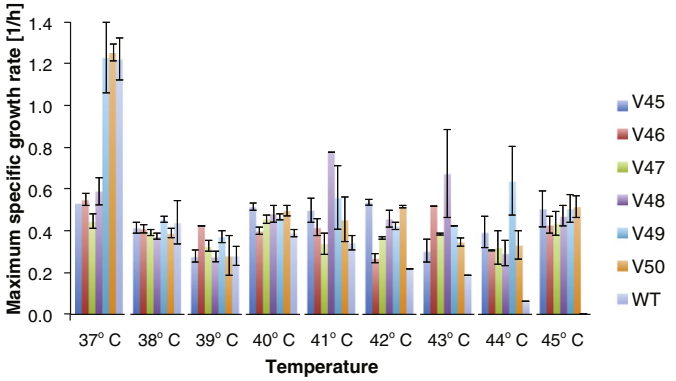


**Fig. 1.** Maximum specific growth rates ($\mu_{max}$) of wild-type (WT) *Lactobacillus helveticus* DSM 20075 and obtained variants (V45-V50) at different elevated suboptimal temperatures in MRS broth (0.5% lactose). Error bars indicate the standard deviations of the twelve independent replicates.

Optical density (determined at 600 nm–$OD_{600}$, path length 1 cm) of cell suspensions was adjusted to 0.35 ± 0.05 resulting in approximately $10^8$ cfu/mL. Samples of 1 mL were collected during the salt stress intervention at the following time points: 0, 4, 6, 9, 24, 48, 72, 96 and 120 h. After centrifugation at 13,000 ×*g* at 4 °C the supernatant was used directly in LDH activity test. The measurement of each sample was performed in triplicate using absorbance microplate reader (SpectraMax® Plus384, Molecular Devices, CA, USA). Cell lysis was expressed in percentage as relative cell lysis. The maximum cell disruption percentage was determined using the homogenizer (FastPrep®-24, MP BIOMEDICALS, CA, USA) and 2 mL tubes with 0.1 mm silica spheres (Lysis Matrix B, MP BIOMEDICALS, CA, USA) at varied cycles of cell disruption. Generally, the amount of active LDH released from cells of V50 and the WT into lactate buffer (pH 5) was growing with increasing cycle numbers until a plateau level was reached followed by a drop in LDH activity with further increase in the disruption cycle count. Results of cycle three were used to ensure the maximal release of LDH from both V50 and the WT strain. Viable plate count data confirmed the cell disruption efficiency at cycle three to be 98% (see Fig. A1, Supplementary material).

### 2.3. Milli-cheese model system

The MicroCheese model system for starter bacteria screening developed by Bachmann et al. (2009) was adapted to a 24-deep well plate format and will be further referred to as the milli-cheese model system. 40 mL of full-fat pasteurized milk (Friesland Campina, The Netherlands) was used to manufacture each of the control milli-cheeses (without the adjunct) and the milli-cheeses with adjunct. The sterile 24 deep-well plate was covered with an adhesive seal (Microseal®, BIO-RAD, CA, USA) and placed in an incubator at 37 °C for approximately 30 min (pre-heating). Subsequently, 10 μL of rennet, 16 μL of 33% (*w*/*v*) of $CaCl_2$, 400 μL (8.7 log cfu/mL) of the starter (TINF1 strain) and 400 μL of the adjunct (8.6 log cfu/mL) were added to 40 mL of milk and mixed thoroughly. Furthermore, the 24-deep well plate was filled with 5 mL per well of the milk-rennet-$CaCl_2$-starter/adjunct mixture. The plate was sealed with the adhesive seal and incubated in thermomixer (Eppendorf, USA) at 32.5 °C. After 40 min, the coagulated milk (curd) was cut with a custom-made sterile stirring device. This stirring device is made from stainless steel, and consists of a plate with a handle at the top and 24 pins attached to the bottom. The pins are aligned according to the shape of the 24-deep well plate. Manual stirring was carried out with horizontal and vertical movements for 20 min at defined intervals (20 s stirring and 3 min rest). Next, the curd was kept untouched for 5 min and the plate was sealed again for a centrifugation step (Centrifuge 5804 R, Eppendorf, USA) at 500 ×*g* for 30 min at 30 °C. Subsequently, 1.9 mL of whey was removed and replaced with 1.8 mL of sterile demi water (pre-heated at 45 °C). After addition of water, the plate was placed in thermomixer at 35.5 °C, followed by manual cutting and stirring, as described above, for 40 min. Then, the plate was rested at the same temperature for 20 min without stirring. To remove the remaining whey and liquid, the plate was centrifuged at 2250 ×*g* for 2 h at 30 °C. The supernatant was discarded well-by-well using a pipet. Finally, the plate was sealed with a gas-permeable seal (BREATHseal™, Greiner Bio One, Frickenhausen, Germany) and incubated overnight at 30 °C. After overnight incubation, 50 μL of a sterile 19% (*w*/*v*) sodium chloride solution was added to each well, followed by a centrifugation step at 75 ×*g* for 5 min. Each batch was sealed with gas-permeable seal, placed in a jar under anaerobic conditions and incubated at 12 °C. Finally, the milli-cheeses were ripened for 2 and 6 weeks.

### 2.4. Volatile compounds analysis

Samples of milli-cheeses at different ripening time points (2 and 6 weeks) were collected and transferred to GC–MS vials. Vials were

sealed and kept at −20 °C until the GC–MS analysis was performed. Volatile organic compounds (VOCs) were extracted and detected using headspace solid-phase micro-extraction gas chromatography–mass spectrometry (HS-SPME GC–MS) with fiber (carboxen/polydimethylsiloxane, CAR/PDMS, Supelco Inc. USA) (Gamero et al., 2013). The procedure entails pre-incubation for 2 min at 60 °C without agitation. Then the fiber was exposed to the sample headspace for 5 min at 60 °C. Volatiles were injected into the GC column by desorption of the fiber for 10 min. For GC–MS analysis we used the Finnigan Trace GC Ultra (Thermo Fisher Scientific, USA) with a Stabilwax®-DA Crossband® acid deactivated Carbowax® polyethylene-glycol column (30 m length, 0.25 mm internal diameter, 0.5 μm internal thickness; Restek, Bellefonte, PA, USA). As an injection device we used a TriPlusTM autosampler (Thermo Fischer Scientific, USA) in PTV Split-less mode for 5 min at 250 °C. Helium was used as mobile phase flowing at a constant rate of 10 mL/min. First, the temperature in the GC oven was set at 40 °C for 2 min, then it was gradually raised to 250 °C (10 °C/min) and then kept for 5 min at the final temperature of 250 °C. We collected the mass spectral data over a range of *m/z* 33–250 in full scan mode with 0.5 s of scan time.

### 2.5. HS-SPME GC–MS data analysis

Chromatograms were analyzed first manually in AMDIS tool integrated in NIST MS Search Program (http://chemdata.nist.gov/mass-spc/ms-search/; 10.03.2016) to determine a retention times list of VOCs found in milli-cheeses. Then the mass spectral data were processed in MetAlign (Lommen, 2009; Lommen and Kools, 2012) using parameters optimized for the Finnigan Trace GC Ultra. The resulting dataset was subjected to VOCs identification in MetAlignID (Lommen et al., 2012) using the prepared retention times list and an in-house compounds library. Final dataset contained total ion counts (TICs) of 23 compounds found in 2-week ripened milli-cheese samples and 27 compounds found in 6-week ripened milli-cheese samples. TIC of each compound in each sample was presented as percentage of the total TICs found (Fig. 1).

### 2.6. Sequencing and genome data analysis

Wild-type strain of *Lb. helveticus* DSM 20075 and the heat resistant variant V50 were both sequenced using Illumina HiSeq 2500 technology. The Illumina reads were assembled using Ray assembler, using standard settings (Boisvert et al., 2010). The assembled sequences were annotated using RAST (Aziz et al., 2008). Then, the reads of the WT and the V50 strains were mapped onto the annotated contigs of the WT using breseq (Deatherage and Barrick, 2014). This resulted in two SNP/INDEL analyses: i) WT reads against WT annotated contigs and ii) V50 reads against WT annotated contigs. As a crosscheck the raw reads of the WT were mapped against the assembly of the V50 variant, although this analysis did not result in the identification of any new mutations.

## 3. Results

### 3.1. Selection of heat-resistant variants

Smith et al. (2012) obtained stable heat-resistant and salt hypersensitive variants of L. *lactis* MG1363 after a high-temperature incubation step. We adapted this approach in an attempt to isolate *Lb. helveticus* DSM 20075 variants with increased heat resistance. *Lb. helveticus* DSM 20075 cultures were incubated at different high (non-optimal) temperatures ranging between 38 °C and 50 °C with 1 °C intervals for up to 36 h. Afterwards, the incubated cultures were plated to isolate survivors. In total six single colony isolates designated as V45, V46, V47, V48, V49 and V50 were obtained from cultures exposed to 45, 46, 47, 48, 49 and 50 °C, respectively. The probability of occurrence of such heat resistant variants at a given temperature was estimated based on the initial cell number, plate counts after the incubation and dilution used (for details see Table A.2, Supplementary material). We calculated that the frequency of occurrence of surviving variants ranges between $2.3 \times 10^{-6}$ and $7 \times 10^{-7}$ at temperatures between 46 °C to 50 °C and approximates $1.1 \times 10^{-4}$ at 45 °C.

### 3.2. Growth characteristics of the wild type and the heat-resistant variants

The specific growth rates of WT and heat-resistant variants were determined at optimal growth temperature for *Lb. helveticus* DSM 20075 (37 °C) as well as at elevated temperatures ranging from 38 °C to 45 °C with 1 °C intervals (Fig. 1). As expected, the WT strain showed the highest growth rate at the optimal 37 °C ($\mu_{max} = 1.22$ 1/h). Compared with the WT, four variants, namely V45 ($\mu = 0.53$ 1/h), V46 ($\mu = 0.55$ 1/h), V47 ($\mu = 0.45$ 1/h) and V48 ($\mu = 0.59$ 1/h), showed significantly reduced growth rates at 37 °C. On the other hand, variants V49 ($\mu = 1.23$ 1/h) and V50 ($\mu = 1.25$ 1/h) had comparable ($p = 0.88$ and $p = 0.54$, respectively) growth rates to the WT at 37 °C.

As expected, the growth rate of the WT culture decreased with increasing temperature. This was not the case for the heat-resistant variants, which showed higher growth rates at elevated temperatures, especially above 41 °C. All heat-resistant variants showed higher growth rates at 45° than the WT (Fig. 1). These results show that, compared to the WT, heat resistant variants grow better at elevated temperatures while variants V45, V46, V47 and V48, but not V49 and V50, show lower growth rates at 37 °C. Based on these results we selected variant V50 for further characterization.

### 3.3. Lytic behavior of the heat-resistant variant

To test the hypothesis that heat resistant variants of *Lb. helveticus* DSM 20075 also show more cell lysis in cheese-like conditions we determined the relative lysis of the selected variant V50 at pH 5 and in the presence of 0.5 M NaCl (Fig. 2). Already after 2 h of incubation in lactate buffer with salt, the relative cell lysis of strain V50 was 4.5-fold higher compared to the WT. Cell lysis of the suspensions of WT and V50 progressed during incubation but was always at least 4-fold higher for strain V50 (Fig. 2). In control suspensions without salt addition, we also observed higher lysis of V50 (up to 2.9-fold at 120 h) compared to the WT, although the values were lower both for the WT (between 1.7% at 2 h and 15.5% at 120 h) and V50 (between 4.5% at 2 h and 40.8% at 120 h) (Fig. 2). These results illustrate that, under conditions mimicking the cheese matrix, strain V50 has a higher capacity to lyse compared to the WT.

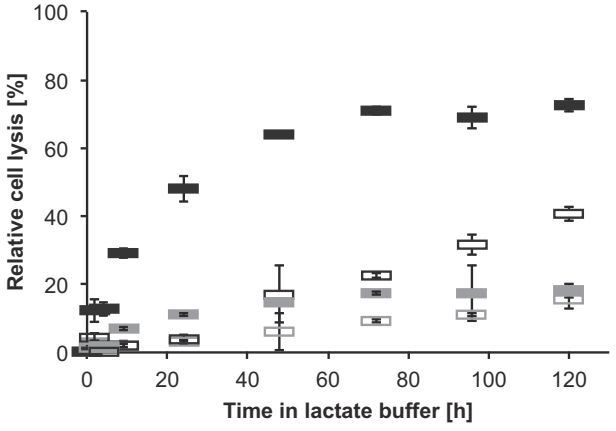


**Fig. 2.** Relative cell lysis in lactate buffer (pH 5). *Lactobacillus helveticus* DSM 20075 (wild type; grey boxes) and *Lactobacillus helveticus* DSM 20075 variant strain V50 (black boxes) with (filled boxes) or without (open boxes) 0.5 M NaCl addition. Error bars indicate the standard deviations of three independent replicates.

### 3.4. Specific mutations found in V50

Using comparative genome analysis, we identified seven mutations present in V50 but absent in WT (Table A.1, Supplementary material). Four SNPs were present in the annotated genes: glycerol kinase (EC 2.7.1.30) – resulting in G151D substitution; site-specific recombinase (DNA invertase Pin related protein) – mutation in stop codon (TAA -> GAA); a silent mutation in a putative pheromone cAM373 precursor lipoprotein CamS; and in a gene encoding multi-domain ZnuB, an ABC-type $Mn^{2+}/Zn^{2+}$ transport system – resulting in I102L substitution. The other two SNPs were identified in an intergenic region in a close vicinity (40 bp distance) to each other. We studied the position of these two intergenic region mutations on the genome to determine if they possibly can impact neighboring genes. In fact, these mutations are located in the upstream region of a gene encoding for glycerophosphoryl diester phosphodiesterase (E.C. 3.1.4.46), an enzyme involved in converting glycerol 1-phosphate into glycerylphosphocholine. Prior to that glycerol is phosphorylated to glycerol 1-phosphate by the glycerol kinase. Interestingly, as described above, the gene encoding a glycerol kinase in strain V50 also carries a mutation. To predict any possible promoter elements and regulons we applied the PePPER online tool (de Jong et al., 2012) using as input the sequence of a scaffold in which the two mutations were identified. However, this analysis did not predict any promoter elements or regulons in the query sequence suggesting no impact of these two mutations on the phenotype.

The last identified mutation was a 664 bp deletion in an intergenic region, although this deletion was not confirmed by PCR suggesting a sequencing artifact (data not shown).

### 3.5. Impact of Lb. helveticus adjuncts on volatile compounds formation in milli-cheese

To compare the aroma forming capacity of the heat resistant variant *Lb. helveticus* V50 with that of WT *Lb. helveticus* DSM 20075, we produced milli-cheeses with the starter culture (*L. lactis* TIFN1) and the *Lb. helveticus* strains as adjuncts and analyzed aroma formation after 2 and 6 weeks of ripening. In addition, control cheeses were made without an adjunct using only the single strain caseinolytic starter culture (*L. lactis* TIFN1). We found 23 VOCs in 2-week and 27 VOCs in 6-week ripened (Fig. 3) milli-cheeses confirming the impact of the maturation time on the development of a more complex aroma profile. The same volatiles were detected in control samples and samples of cheeses made with adjunct cultures, indicating that addition of *Lb. helveticus* DSM 20075 as adjunct does not lead to the formation of additional compounds. The most dominant compound found in both 2- and 6-week ripened cheeses was 3-hydroxy-2-butanone (acetoin) (Fig. 3). Similarly as described in a previous study by Van Leuven et al. (2008), we detected a range of free fatty acids including butanoic acid, hexanoic acid, octanoic acid and n-decanoic acid in all cheeses. In addition, fatty acid methyl esters such as 2-methyl-2-propenoic acid-methyl ester, butanoic acid-methyl ester, 2-propenoic acid-methyl ester, hexanoic acid-methyl ester, acetic acid-2-ethylhexyl ester, octanoic acid-methyl ester and decanoic acid-methyl ester, were also detected. In both milli-cheeses samples with WT and V50 2-methyl-2-propenoic acid-methyl ester was identified to be present at higher levels compared to the other fatty acid methyl esters. Moreover, a variety of alcohols was detected in our milli-cheeses, including: isooctanol, 2-ethyl-1-hexanol, (*S*)-3-ethyl-4-methylheptanol, 3-methyl-1-heptanol, 1-octanol, 6-methyl-1-heptanol and trans-4-(1, 1-dimethylethyl)-cyclohexanol.

However, the most distinguishing difference in aroma formation in the cheeses with only starter and the ones supplemented with adjunct strains was found to be the abundance of benzaldehyde (Fig. 4). The relative abundance of benzaldehyde TIC area in 2-week ripened samples with WT as adjunct increased in abundance 12-fold (Fig. 4), when compared with the control without adjunct addition. A slightly more pronounced increase in benzaldehyde TIC area relative abundance (15-fold) was found in the samples of 2-week ripened milli-cheese prepared with V50 as adjunct (Fig. 4) compared with the control. Although, the difference in benzaldehyde TIC area abundance between 2-week milli-cheese samples with WT and V50 was not significant ($p = 0.45$), we found significant differences ($p = 0.02$) in benzaldehyde levels (expressed as TIC areas) in samples of 6-weeks ripened cheeses. The TIC area abundance of benzaldehyde in 6-week ripened milli-cheeses made with either WT or V50 compared to the control was 16-, and 45-fold higher, respectively (Fig. 4).

## 4. Discussion

This study describes the successful isolation of variants of *Lactobacillus helveticus* DSM 20075 with increased autolytic capacity triggered by conditions resembling the conditions in cheese ripening (low pH and high salt concentration). As described in other studies, autolysis of lactic acid bacteria (LAB) can be induced by various environmental triggers such as nutrient depletion, high NaCl concentration and heat shock (Ardö and Pettersson, 1988; Lortal and Chapot-Chartier, 2005; Riepe et al., 1997). Dako et al. (1995) considered NaCl to be one of the key factors triggering cell autolysis and Wilkinson et al. (1994) noticed that the increase of salt in cheese was accompanied by an increase of LDH activity in the cheese matrix. The autolysis of different strains of *Lb. helveticus* was documented before by Valence et al. (2000) and they concluded that a decrease in viability in cheese is not a reliable indicator of lysis due to the possible fraction of dead but not lysed cells or non-culturable cells which could not form colonies. Measurement of the activity of released intracellular enzymes e.g. LDH is regarded as a good indicator of bacterial lysis (Hannon et al., 2003). Our results line-up with the observation of Smith et al. (2012) who used incubation at elevated temperatures to isolate variants of *Lactococcus lactis* MG1363 that showed increased cell lysis in selected conditions. Smith and colleagues suggested that the heat-resistant and salt-hypersensitive phenotype obtained by high-temperature incubation is not a strain-specific phenomenon, which indeed was confirmed in the case of our *Lb. helveticus* DSM 20075 strain.

In *L. lactis* MG1363, high-temperature incubation resulted in spontaneous mutations in *llmg_1816* gene, which encodes for a membrane-bound stress signaling protein of the GdpP family. In our study using *Lb. helveticus* DSM20075, comparative genome sequencing identified seven mutations in variant strain V50. We calculated the frequency of occurrence of the survivor for the variant V50, which was approximately $2.3 \times 10^{-6}$. In practice it means that at least two cells per million in the culture of wild-type *Lb. helveticus* DSM20075 were able to survive prolonged incubation at 50 °C. In different studies, Curragh and Collins (1992) described a high frequency of spontaneous nitrofurazone-resistant cells in lactobacilli ($10^{-5}$ to $10^{-4}$), which suggests relatively high potential heterogeneity in the population of *Lb. helveticus* strains. Despite the identification of several mutations in V50 we were not able to link them explicitly to the phenotypes observed. Below we speculate on the possible impact of the identified mutations on the phenotype of V50 (heat-resistance and salt induced lysis).

One of the detected SNPs was located in the gene encoding for a glycerol kinase, an enzyme, which plays a role in degradation of sugar alcohols in *Corynebacterium glutamicum* (BRENDA pathway), glycerol degradation (MetaCyc) in enterococci (Charrier et al., 1997) and in glycerolipid metabolism (KEGG). According to the BRENDA database a mutation found in glycerol kinase was not previously investigated and the effect on the functionality cannot be determined without use of genetic tools for *Lb. helveticus*, which was out of the scope of present work.

We found another SNP in the homologue of *znuB* in *E. coli* (Patzer and Hantke, 1998), encoding the membrane-embedded protein of an ABC-type $Mn^{2+}/Zn^{2+}$ transport system. It is well-documented that metal ions are essential for the growth of LAB, probably because of their role as catalytic centers of many enzymes (Boyaval, 1989). Moreover, $Mn^{2+}$ was found to be accumulated by *Lb. plantarum* cells as a

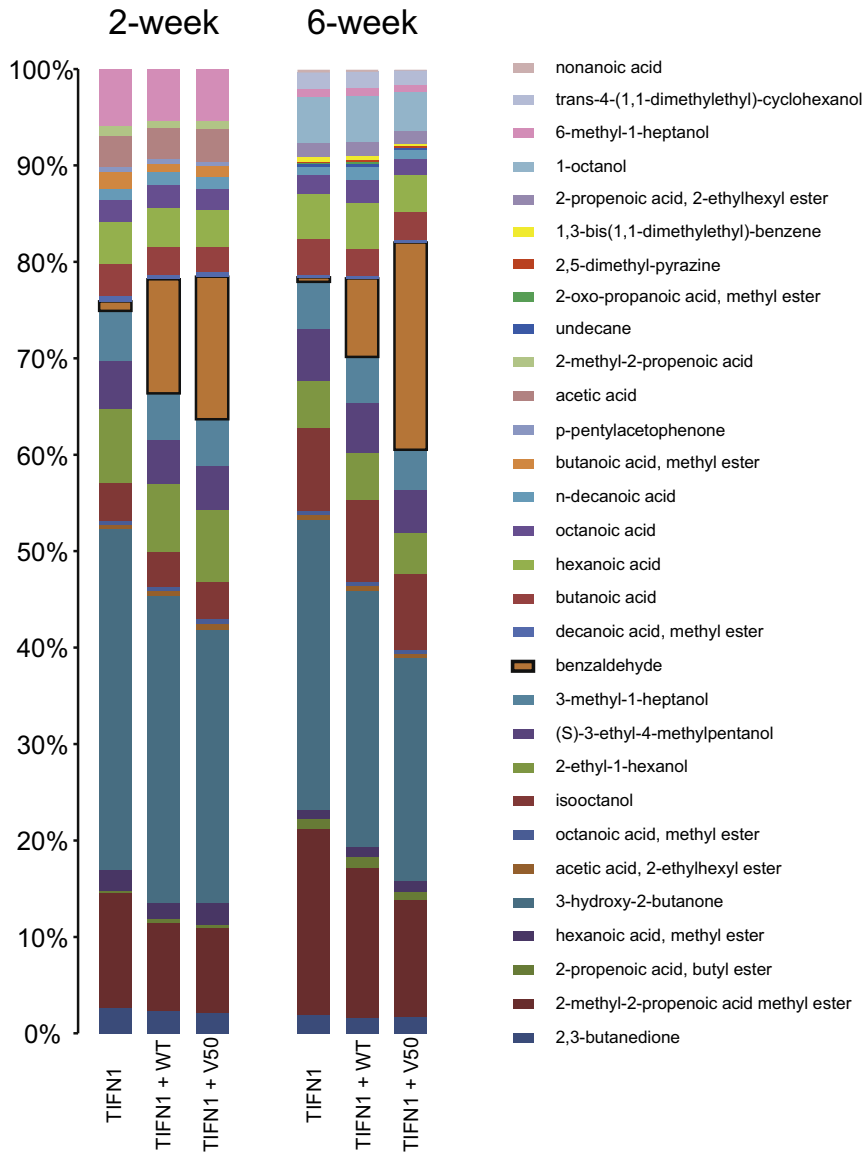


**Fig. 3.** Relative abundance of VOCs identified in milli-cheese samples at 2 and 6 weeks of ripening. The percentage was calculated using total ion counts (TICs) obtained from 6 replicates (in case of samples without the adjunct, TIFN1), 3 replicates (in case of samples with WT, TIFN1 + WT) and 9 replicates (in case of samples with V50, TIFN1 + V50). Legend: * — compounds found exclusively in 2-week ripened samples; ** — compounds found exclusively in 6-week ripened samples.

defense mechanism against oxygen toxicity (Archibald and Fridovich, 1981). The putative role of $Mn^{2+}$ in aroma formation is discussed below.

The comparative genome analysis provided leads for further investigation to explain and understand the phenotype of the strain V50. To confirm the impact of the identified mutations on the phenotype further studies (i.e. making gene deletion mutants) are required.

Our study demonstrated the impact of the wild-type *Lb. helveticus* DSM 20075 and variant strain V50 on aroma formation in a lab-scale cheese model system. We confirmed the relevance of milli-scale model system adapted from Bachmann et al. (2009) by finding aroma compounds common for Gouda cheese, such as acetoin (Bintsis and Robinson, 2004; Dirinck and De Winne, 1999; Van Leuven et al., 2008), 2, 3-butandione (diacetyl), a variety of alcohols (products of amino acid metabolism (Engels and Voragen, 1997; Smit et al., 2005) and esters.

We found that the presence of *Lb. helveticus* DSM 20075 leads to significantly higher abundance of benzaldehyde in our model milli-cheeses. Moreover, in 6-week ripened cheeses made with variant V50, the abundance of benzaldehyde was significantly higher than in cheeses made with the WT. Benzaldehyde has a characteristic pleasant almond-like odor and is commonly found in dairy products, including Swiss cheeses. It was also detected in Gouda-type cheeses ripened for six weeks (Van Leuven et al., 2008). Benzaldehyde can be formed from phenylpyruvic acid, which is enzymatically derived from phenylalanine (McSweeney and Sousa, 2000). α-keto acids such as phenylpyruvic acid, can be formed from amino acids by various enzymes, including amino acid oxidases, aminotransferases, and dehydrogenases (Nierop Groot and de Bont, 1998). Gummalla and Broadbent (2001) and Nierop Groot and de Bont (1999) further demonstrated that production of benzaldehyde in LAB is associated with the conversion of phenylalanine to phenylpyruvic acid by aminotransferase and is stimulated by the accumulation of $Mn^{2+}$ in the cells. Biological effects of $Mn^{2+}$ on LAB were comprehensively studied by Raccach (1985), who concluded its association with structure/activation of enzymes, detoxification of the harmful superoxide radicals and stabilization in subcellular entities. Archibald and Fridovich (1981) further showed that manganese accumulation in LAB provided the cells with a defense mechanism against the toxic effects of oxygen. Oxidation of phenylpyruvic acid to benzaldehyde was reported by Pitt (1962), who observed the oxidation of enol

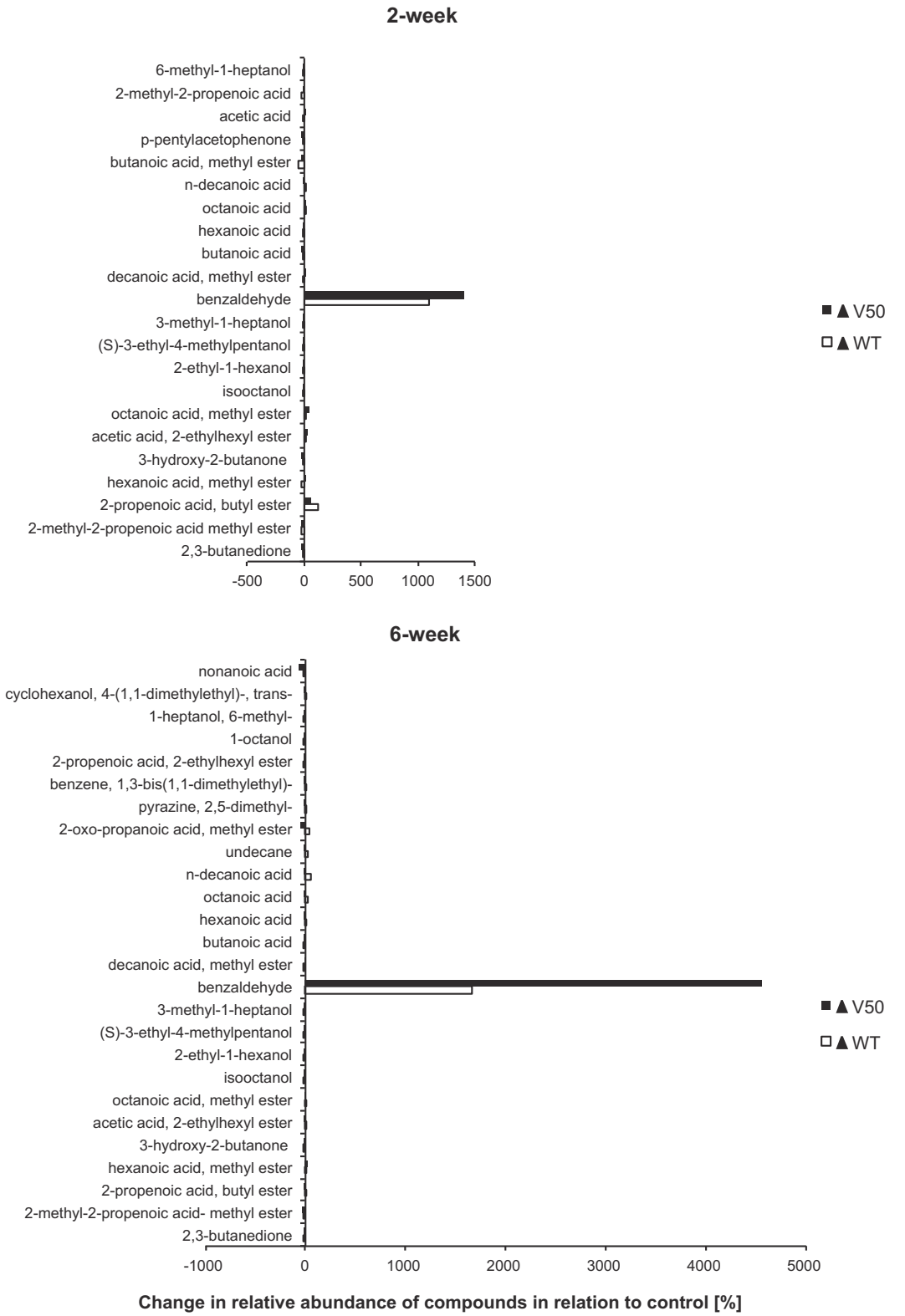


**Fig. 4.** Change in abundance of VOCs relative to the control (milli-cheese without adjunct addition). The percentage was calculated using total ion counts (TICs) of each compound identified in GC–MS run in milli-cheese model samples at 2- and 6-week ripening time points. Black bars — samples where V50 was used as an adjunct; white bars — samples where WT was used as an adjunct.

tautomer of α-keto acid instead of the α-keto acid itself, and bivalent metal ions such as $Mn^{2+}$ can accelerate this tautomerization. Based on the studies in *Lactobacillus plantarum*, Nierop Groot and de Bont (1999) proposed a mechanism for benzaldehyde formation from phenylalanine by a combination of enzymatic and chemical steps, and emphasized the required presence of an active uptake system for $Mn^{2+}$ in this bacterium. In that enzymatic-chemical pathway, phenylpyruvic acid is formed enzymatically from the phenylalanine; and the catalysing metal ions can enhance the presence of the enol tautomer of the α-keto acid, which is chemically converted to

benzaldehyde in the presence of oxygen. Notably, Klein et al. (2001) used non-treated and boiled cell free extracts of *Lactobacillus helveticus* strains LRTL 735 and LBLH2 to find that benzaldehyde was produced in both cases providing more evidence for the conclusions of Gummalla and Broadbent (2001) and Nierop Groot and de Bont (1999) for a non-enzymatic reaction (see above) involved in benzaldehyde production after the transamination of phenylalanine.

As mentioned above, we found a mutation in the gene encoding for the homologue of ZnuB, a component of an ABC-type $Mn^{2+}/Zn^{2+}$ transport system. Such putative manganese transport systems were found before in L. *plantarum* (Nierop Groot et al., 2005). Whether the higher levels of benzaldehyde in cheese with V50 can be explained by the mutation in the gene encoding ZnuB remains to be determined. As reported by Nierop Groot et al. (2005) metal ions, and $Mn^{2+}$ in particular, are essential for the formation of benzaldehyde and we speculate here that enhanced lysis of the adjunct strain leads to locally released $Mn^{2+}$ that stimulates the production of benzaldehyde in the cheese matrix.

With improved autolytic capacity of *Lb. helveticus* strain V50 derived from DSM 20075, two previously identified key factors in benzaldehyde formation, namely aminotransferase activity and intracellular $Mn^{2+}$ ions are expected to be released more rapidly, which conceivably accelerates production of benzaldehyde. Our approach of high-temperature incubation inspired by the work of Smith et al. (2012), led to the isolation of *Lb. helveticus* variants with improved growth rates at elevated temperatures combined with – in case of variant V50 – a higher autolytic capacity at low pH in the presence of NaCl. This approach can be applied in dairy industry for the selection of (adjunct) cultures targeted at accelerated cheese ripening and aroma formation.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2016.10.020.

## Acknowledgments

The project was funded by TI Food and Nutrition (TIFN), Wageningen, the Netherlands (grant number FF001). TIFN a public–private partnership on precompetitive research in food and nutrition. The public partners are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The private partners contributed to the project through regular discussion.

We would like to thank Irma van Rijkswijck, Judith Wolkers-Rooijackers (both Laboratory of Food Microbiology, Wageningen University) and Geert Meijer (Food Quality and Design Department, Wageningen University) for help with GC–MS equipment running and GC–MS data analysis. Finally, we would like to thank Ran An for her help with analyzing the predicted 664 bp deletion in strain V50.

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