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

Maciej Spus [a,b], Hua Liu [b], Michiel Wels [a,c], Tjakko Abee [b], Eddy J. Smid [a,b,*]

[a] TI Food and Nutrition, P.O. Box 557, 6700 AN Wageningen, The Netherlands
[b] Wageningen University, Laboratory of Food Microbiology, P.O. Box 17, 6700 AA Wageningen, The Netherlands
[c] NIZO Food Research, P.O. Box 20, 6710 BA Ede, The Netherlands

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.

* Corresponding author at: P.O. Box 17, 6700AA Wageningen, The Netherlands.
E-mail addresses: maciej.spus@gmail.com (M. Spus), hualiu.wageningen@outlook.com (H. Liu), michiel.wels@nizo.com (M. Wels), tjakko.abee@wur.nl (T. Abee), eddy.smid@wur.nl (E.J. Smid).

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.

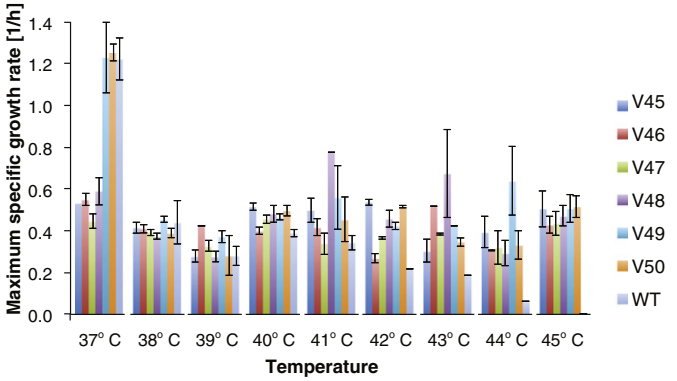

Fig. 1. Maximum specific growth rates (μ_{max}) of wild-type (WT) *Lactobacillus helveticus* DSM 20075 and obtained variants (V45-V50) at different elevated suboptimal temperatures in MRS broth (0.5% lactose). Error bars indicate the standard deviations of the twelve independent replicates.

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