3-Hydroxypropionic acid contributes to the antibacterial activity of glycerol metabolism by the food microbe *Limosilactobacillus reuteri*

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Abstract

Strains of *Limosilactobacillus reuteri* are used as starter and bioprotective cultures and contribute to the preservation of food through the production of fermentation metabolites lactic and acetic acid, and of the antimicrobial reuterin. Reuterin consists of acrolein and 3-hydroxypropionaldehyde (3-HPA), which can be further metabolized to 1,3-propanediol and 3-hydroxypropionic acid (3-HP). While reuterin has been the focus of many investigations, the contribution of 3-HP to the antimicrobial activity of food related reuterin-producers is unknown.

We show that the antibacterial activity of 3-HP was stronger at pH 4.8 compared to pH 5.5 and 6.6. Gram-positive bacteria were in general more resistant against 3-HP and propionic acid than Gram-negative indicator strains including common food pathogens, while spoilage yeast and moulds were not inhibited by ≤640 mM 3-HP. The presence of acrolein decreased the minimal inhibitory activity of 3-HP against *E. coli* indicating synergistic antibacterial activity. 3-HP was formed during the growth of the reuterin-producers, and by resting cells of *L. reuteri* DSM 20016 in buffer. Taken together, this study shows that food-related reuterin producers strains synthesize a second antibacterial compound, which might be of relevance when strains are added as starter or bioprotective cultures to food products.

Keywords

Reuterin, 3-hydroxypropionic acid, *Limosilactobacillus reuteri*, antibacterial, antifungal

Introduction

Strains of *Limosilactobacillus reuteri* are used as starter cultures in cereal fermentations and have been suggested as bioprotectants in milk products (Avila et al., 2017; Ortiz-Rivera et al., 2017). *L. reuteri* contributes to the preservation of fermented food through the production of fermentation metabolites lactic and acetic acid, and of reuterin. The antimicrobial activity of reuterin was first described in the 1980s as a compound produced during glycerol metabolism of *Limosilactobacillus reuteri* (Axelsson et al., 1989; Talarico et al., 1988). Reuterin formation was later linked to the *pdu* cluster, which encodes for the enzymes converting glycerol and 1,2 propanediol, and a *cbi-cob-hem* operon, which contributes to the biosynthesis of the cofactor cobalamin (Morita et al., 2008). Diol metabolism has been described for food, gut and silage-related taxa of the phyla *Firmicutes* including *Anaerobutyricum hallii, Blautia obeum,* and *Ruminococcus gnavus*, and *Proteobacteria* including *Klebsiella* and *Citrobacter* species (Hao et al., 2008; Lindlbauer et al., 2017; Zhang et al., 2019). In lactobacilli, strains of *L. reuteri, Furfurilactobacillus rossiae, Loigolactobacillus coryniformis* as well as *Secundilactobacillus* spp., *Levilactobacillus* spp. and *Lentilactobacillus* spp. metabolise glycerol (Zheng et al., 2020). Glycerol is first dehydrated to 3-hydroxypropionaldehyde (3-HPA) in a reaction catalyzed by glycerol/diol dehydratase PduCDE (also called GdhCDE) (**Figure 1**). 3-HPA can either form reuterin, a multi-compound system consisting 3-hydroxypropionaldehyde (3-HPA), 3-HPA hydrate, 3-HPA dimer and acrolein (Engels et al., 2016), or is further metabolized to 1,3-propanediol (1,3-PDO) and 3-hydroxypropionic acid (3-HP) by the enzymes encoded by the diol/propanediol-utilization (*pdu*) operon (**Figure 1**).

Reuterin exhibits broad spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria including food-related pathogens, yeasts, molds, and protozoa both *in vitro* and *in situ* (Stevens et al., 2011). Reuterin depletes free thiol groups of glutathione (GSH), and proteins, resulting in an imbalance of the cellular redox status, which leads to bacterial cell death (Schaefer et al., 2010). The highly reactive, double unsaturated aldehyde acrolein was suggested as the main component responsible for the antimicrobial activity of reuterin (Engels et al., 2016).

Organic acids are weak acids that typically show pH-dependent antimicrobial activity, which relates to their importance in the preservation of fermented food products. A major parameter determining antimicrobial activity is the pK_A, as mainly the undissociated form enters the cell and acidifies the cytoplasm. Export of protons consumes metabolic energy

while a drop of the cytoplasmic pH modifies enzyme activity and damages structural proteins and nucleic acids; in addition, anion concentration can increase to toxic levels (Mani Lopez et al., 2012). The antifungal propionic acid is used as food preservative and is formed in propionibacteria-driven food fermentations, for example in Emmental cheese. Propionic acid is produced *in situ* in silage or cereal fermentations with *Lentilactobacillus* species as starter cultures (Zhang et al., 2010, Zheng et al., 2020). In contrast, only few studies reported on the antibacterial potential of 3-HP (Chun et al., 2014; Warnecke et al., 2012; Sankaranarayanan et al., 2014), which could contribute to antimicrobial potential of *L. reuteri* when used as starter culture or bioprotectant. It was, therefore, the aim of this study to test the antimicrobial activity of 3-HP against selected Gram-positive and Gram-negative bacteria including common food pathogens, and against selected spoilage yeast and mycelial fungi, and to compare to propionic acid. We investigated the cooperative activity of 3-HP with acrolein, the second antimicrobial derived from glycerol metabolism, against selected bacteria, yeast and fungal strains, and tested whether selected reuterin forming lactobacilli produced 3-HP during growth in the presence of glycerol, or in a resisting state mimicking a starter or bioprotective culture, respectively.

Material and methods

Strains and growth conditions. Bacterial strains were obtained from the strain collection of the Department of Microbiology, Nutrition and Dietetics (CZU, Czechia), from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany), and from the Czech Collection of Microorganisms (CCM, Masaryk University Brno, Czechia) (**Table 1**). The identity of cultures was confirmed with MALDI-TOF MS (Bruker Daltonik GmbH, Germany) as described (Modrackova et al., 2019). Bifidobacteria and *Listeria monocytogenes* were routinely cultured in Wilkins-Chalgren broth (Oxoid, UK) supplemented with soy peptone (5 g L^{-1}, Oxoid), L-cysteine (0.5 g L^{-1}, Sigma-Aldrich, USA), and Tween 80 (1 mL L^{-1}, Sigma-Aldrich) (WSP broth) in an oxygen-free carbon dioxide environment (bifidobacteria) or aerobically (*Listeria*) at 37 °C for 24 h. Lactobacilli were grown using MRS (Sigma-Aldrich) at 37 °C for 24 h, while *Escherichia coli* and *Salmonella* spp. were cultivated aerobically in Nutrient Broth Nr. 2 (Thermo Scientific, Czechia) at 37° C for 24 h. Stock cultures were stored at –80 °C with 30% glycerol addition. To obtain working cultures, strains were streaked on agar plates, lactobacilli were incubated under anaerobic conditions created by GENbag anaerobic (Biomerieux, France). Single colonies were picked, incubated in broth and subcultured once. Acrolein

formation of lactobacilli was qualitatively tested using the colorimetric assay described by Lüthi-Peng et al. (2002a) using *L. reuteri* DSM 20016, *L. reuteri* FUA 3400, and *F. rossiae* as positive controls (Engels et al., 2016; Fekry et al., 2016; Lin and Gänzle, 2014).

Yeast and fungi were obtained from strain collection of the Food Microbiology lab at the University of Alberta. *Aspergillus niger* FUA 5001 and *Penicillium roqueforti* FUA 5004 were cultured on malt extract agar (MEA) at 25 °C for 7 d. *Zygosaccharomyces* spp. FUA 4034 and *Saccharomyces cerevisiae* FUA 4011 were cultured on yeast extract peptone dextrose (YPD) agar at 30 ° C for 2 d. A single colony of yeast was picked to make a 2-day sub culture at 30 ° C, 200 rpm.

Preparation of 3-hydroxypropionate and propionic acid, and acrolein solutions. The pH dependent antibacterial effect of organic acids was determined in media after adjusting the pH to 6.5, 5.5 and 4.8 using 5N HCl. 3-HP (Sigma-Aldrich, Czechia, 30% (w/v, 6 M solution) and propionic acid (Sigma-Aldrich, 13.4 M) were diluted to a stock solution of 60 mM in the cultivation broths. To test the inhibition of yeast and fungi, stock solutions (1.92 M) of lactate, sodium propionate and 3-HP were prepared in mMRS broth, followed by pH adjustment to 4.8 (all Sigma-Aldrich, Canada). Acrolein (Sigma-Aldrich, 15 M) was first 100-fold diluted in BifiBuffer (K_2HPO_4 1.2 g L^{-1}, KH_2PO_4 0.333 g L^{-1}; Lachner, Czechia), and was further diluted to a working concentration of 1.5 mM in cultivation media. Acrolein solutions were always prepared fresh.

Broth dilution assay to determine the minimal inhibitory concentration (MIC) of 3-HP and propionic acid against bacterial indicators. The antibacterial activity of 3-HP and propionic acid was tested using two-fold broth dilution assay in 96-well sterile microtiter plates (VWR, Czechia). Cells from overnight cultures were harvested by centrifugation at 6000 x g for 5 min, and were resuspended in the same volume of fresh medium (pH 6.5). A two-fold dilution series of the organic acids was prepared in microtiter plates, the maximum concentration of 3-HP and propionic acid tested was 30 or 60 mM. Cultures (10%) were added, and plates were incubated under aerobic, or anaerobic conditions created by GENbag anaerobic, at 37 °C for 24 h. Positive controls (cultivation medium without organic acid), as well as blanks (sterile media instead of cell suspension), were included in each assay. Bacterial growth was detected by measuring the optical density at 600 nm after 24 h of incubation. A sigmoidal (four parameter logistic) equation was used to fit the data (Sigma Plot version 13, Systat Software, USA); the inflection point of the resulting curve represents the MIC_{50} value, which was defined

as the concentration that reduced final optical density of the test strains to 50% compared to optical density without inhibitors. Every strain was analysed at least three times unless otherwise indicated.

Cooperative antibacterial activity of 3-HP and acrolein. The cooperative antibacterial activity of 3-HP and acrolein was tested using a modified broth dilution assay in 96-well sterile microtiter plates (VWR). Two-fold broth dilution assays were prepared horizontally (3-HP) and vertically (acrolein) with concentrations ranging from 0.1-16.5 mM and 5-750 μM, respectively.

Working cultures of *E. coli* ATCC 25922, or *L. reuteri* DSM 20016 were centrifuged, and the cell pellet was resuspended in the same volume of fresh medium (pH 6.5), and was inoculated at 10%. Plates were incubated aerobically (*E. coli*) or anaerobically (*L. reuteri*) using Genbag anaerobic at 37 °C for 24 h. Positive controls as well as blanks were included in each assay. Bacterial growth was detected by measuring the optical density at 600 nm after 24 h of incubation. A sigmoidal (four parameter logistic) equation was generated for each acrolein concentration as described above. Every strain was analysed at least three times.

Broth dilution assay to determine the MIC of yeast and fungi. The antifungal activities of 3-HP, lactic and propionic acids, were measured as described (Liang et al., 2017). In brief, organic acid stock solutions (100 μL) were added into 100 μL of mMRS broth (pH=4.8), and a series of two-fold dilution was made. Suspensions of fungal conidiospores or yeast cells were adjusted to a cell count of 10^4 cells / mL after counting with a haemocytometer, and 33.3 μL of this suspension was added to each well. Positive controls, and blanks were included in each assay. Cultures were incubated at 25 °C for molds and 30°C for yeasts. Growth of mycelia and vegetative cells, respectively, was observed after one day for the positive control, and the minimum concentration of compound of interest that inhibited growth were recorded as MIC. Every strain was tested three times.

Production of 3-HP and fermentation metabolites during growth in the presence of glycerol or by resting cells. To investigate 3-HP formation during growth, we grew selected reuterin-positive and reuterin-negative, heterofermentative lactobacilli in MRS, which contained 110 mM glucose, and in MRS additionally supplied with of 55 or 110 mM glycerol (MRS + 55 mM glycerol and MRS + 110 mM glycerol). Working cultures were inoculated (2%) in 10 mL MRS in Hungate tubes and were incubated at 37 °C. Cell density was measured hourly for 9 h, and after 24 h of incubation, using a McFarland DEN1-B densitometer (Biosan, Latvia).

Supernatant was collected at t=0 and 24 h for metabolite analysis. Experiments were conducted with three biological replicates unless otherwise indicated.

We additionally tested the formation of 3-HP by resisting cells. Overnight cultures of *L. reuteri* DSM 20016 were centrifuged, washed once using Bifibuffer, and concentrated 10-fold. Cells were inoculated (1%) in 200 µL glycerol solution (55 or 110 mM in Bifibuffer) and were incubated for 4 h at 37 °C. Supernatants were used for metabolite analysis as described below.

Metabolite formation analysis using ion chromatography with suppressed conductivity detection (IC-SCD) and high performance chromatography with refractive index detector (HPLC-RI)

As lactate and 3-HP, and lactate and glycerol eluted at the same retention time using IC-SCD and HPLC-RI, we used two chromatographic systems to determine metabolite formation. The concentration of main fermentation metabolites lactate and acetate, and of 3-HP was determined using capillary high-pressure ion-exchange chromatography with suppressed conductivity detection on a Dionex ICS 4000 (Thermo Scientific) system equipped with IonPac AS11-HC 4 µm (Thermo Scientific) guard and analytical columns. Eluent composition was as follows: 0–10 min isocratic: 1 mM KOH; 10–20 min linear gradient: 1–60 mM KOH; and 20–25 min isocratic: 60 mM KOH. The flow rate was set to 0.012 mL min^{-1}. An ACES 300 suppressor (Thermo Scientific, USA) was used to suppress eluent conductivity, while a carbonate Removal Device 200 (Thermo Scientific) was implemented to suppress carbon dioxide baseline shift. The concentrations of 3-HP and 1,3-PD were determined HPLC-RI were quantified using a 1260 Infinity II (Agilent, Denmark) and a Hi-Plex H column (Agilent). H_2SO_4 (50 mM) was used as eluent at a flow rate of 0.6 mL min^{-1} at a column temperature of 40 ˚C.

Chromatograms were processed with Chromeleon 7.20 (Thermo Scientific). Standards were prepared from 1 g L^{-1} stock solutions (Analytika, Czechia; Inorganic Ventures, USA). Deionised water (conductivity <0.055 µS cm^{-1}; Adrona, Latvia) was used for eluent and standard preparation (0.1–40 mg L^{-1}).

Statistical analysis. The statistical packages implemented in SigmaPlot 13 was used. The impact of pH on the MIC of 3-HP or propionic acid, and lactic and acetic acid concentrations formed after incubation in different media was compared by One-Way Analysis of Variance with all pairwise multiple comparison procedures (Holm-Sidak method). A value $p<0.05$ was considered significant.

Results and discussion

Antibacterial activity of 3-HP and propionic acid was pH-dependent. During a lactic acid bacteria driven food fermentation, the pH drops from near neutral to pH<5 enhancing the antimicrobial activity of organic acids. We therefore tested pH dependent activity of 3-HP and propionic acid using two-fold dilution assay in media adjusted to pH 6.5, 5.5, and 4.8. Surprisingly, both 3-HP and propionic acid were inhibitory against the tested *Enterobacteriaceae* and *Bifidobacterium* spp. even at the near-neutral pH of 6.5, indicating that pH is not the only parameter responsible for antimicrobial activity of organic acids. It was shown before that lactic acid (pK_A 3.86) likewise reduced growth and modified transcript and metabolic response of *L. monocytogenes* at pH 6.1 (Stasiwiecz et al., 2011).

Antibacterial activity of both organic acids increased with decreasing pH, as reported before for lactic, acetic and propionic acid, likely due to dissociation of weak acids in a pH dependent manner (Buchanan et al., 1993; Gerez et al., 2009; Mani-Lopez et al., 2012) (**Table 1**). Sensitivity of *E. coli* towards 3-HP and propionic acid was strain dependent. In general, the MIC_{50} at pH 6.5 were 1.8-5.4-fold higher compared to pH 4.8. The MIC_{50} of *Salmonella enterica* Enteritidis ATCC 13076 was in the same range as for *E. coli* (**Table 1**). For *Listeria monocytogenes* ATCC 7644 and *Bifidobacterium* spp., the MIC_{50} of propionic acid and 3-HP was higher when compared to *Enterobacteriaceae,* but was also significantly lower at pH 4.8 when compared to pH 6.5 (**Table 1**), indicating the 3-HP could potentially contribute to antibacterial activity in a fermented food product.

3-HP (pK_a 4.51) is a stronger acid than propionic acid (pK_a 4.87), and it was previously observed that growth of *E. coli* was reduced by organic acids at neutral pH depending on the dissociation constant (Chun et al., 2014). We therefore expected a higher MIC of 3-HP compared to propionic acid at the same pH. However, the MIC of propionic acid and 3-HP against *E. coli*, *Salmonella,* and *L. monocytogenes* ATCC 7644 was similar (**Table 1**), suggesting

that mechanisms other than the membrane permeability of undissociated organic acids impacted bacteria inhibition.

For *Bifidobacterium breve* B13 and *Bifidobacterium longum* subsp. *infantis* DSM 20088, the MIC_{50} of 3-HP was even lower than for propionic acid, especially at pH 4.8. This might be due to higher pH sensitivity of *Bifidobacterium* spp. (Sánchez et al., 2007). Indeed, final optical density of cultures grown at pH 4.8 was 60-80% lower compared to optical densities reached at pH 6.5 and 5.5 (data not shown).

Lactobacilli and enterococci were not inhibited by the presence of 60 mM propionic acid or 33 mM 3-HP even at pH 4.8 (**Table 1**). Lactobacilli are intrinsically resistant against low pH and high acid concentrations due to several mechanisms including amino acid dependent neutralization processes and proton pumps (Gänzle, 2015, Wang et al., 2018), and their ability to lower the internal pH (Van Immerseel et al., 2006), which contributes to their suitability to be used as starter cultures. Propionic acid reduced the growth of *Lactobacillus helveticus* and *Lacticaseibacillus casei* only at concentrations around 100 mM (Perez Chaia et al., 1994).

Antifungal activity of organic acid depends on compound structure and target strain.

Selected food products, such as yogurt and bread, are sensitive towards spoilage by yeast and moulds. To investigate the impact of acidic strength and dissociation potential on antifungal activity, we compared the sensitivity of *S. cerevisiae* FUA 4011 and *Zygosaccharomyces* sp., and of the mycelial fungi *P. roqueforti* FUA 5004 and *A. niger* FUA 5001 towards of 3-HP, its isomer lactic acid and the non-OH analogue propionic acid. Only propionic acid exhibited antimicrobial activity among the organic acids tested, yeasts and fungi were not inhibited by up to 640 mM lactic acid or 3-HP. The additional hydroxyl group of 3-HP and lactic acid reduces the hydrophobicity and thus the potential to partition into the membrane. Accordingly, an increasing length of the aliphatic chain of orgaic acids, corresponding to a increasing hydrophobicity, decreased the MIC against *A. niger* (Stratford et al., 2009). Yeast (MIC of *S. cerevisiae* FUA 4011: 160 mM; MIC of *Zygosaccharomyces* sp.: ~320 mM) were more resistant towards propionic acid than mycelial fungi (40 mM). Accordingly, weak acid resistance of *S. cerevisiae* has been linked to the ability to restore the cell internal pH, and to an anion exporter system (Ullah et al., 2012).

The antibacterial activity of 3-HP and acrolein was synergistic. Glycerol metabolism of strains that harbour the *pdu* cluster can yield acrolein and 3-HP simultaneously, which could increase the antimicrobial activity in food. To investigate whether the presence of acrolein impacted

the antibacterial activity of 3-HP, we conducted two-fold dilution assays in the presence of both compounds, 3-HP (0.1-16.5 mM) and acrolein (5-684 µM) at pH 4.8 using *E. coli* ATCC 25922 and *L. reuteri* DSM 20016 as indicator strains. The maximum concentration of acrolein reduced the optical density of *E. coli* by less than 50% while the optical density of *L. reuteri* was not affected. When combined with 3-HP, higher concentrations of acrolein significantly reduced the MIC_{50} of 3-HP (**Figure 2**). The log-linear relationship of the MIC_{50} of 3-HP at different acrolein concentrations suggests that 3-HP and acrolein act synergistically likely due to different mode of action. 3-HP acts as organic acid dissociating in the cytoplasm, while acrolein causes oxidative stress and can interact with DNA and proteins (Schaefer et al., 2010). This synergistic activity enhances the antimicrobial potential of glycerol-metabolizing *L. reuteri* in food. In contrast to *E. coli,* the optical density of *L. reuteri* DSM 20016 after 24 h was not impacted by the combined application of 3-HP and acrolein likely due to higher intrinsic resistance of lactobacilli against acid and redox stress (Cleusix et al., 2007; Schaefer et al., 2010) protecting the producing cell.

3-HP was produced during growth by reuterin-positive strains

Reuterin/acrolein formation in the presence of glucose and glycerol was confirmed for growing cultures of *L. reuteri* ATCC 53608 (Lüthi-Peng et al., 2002b), and for *L. reuteri* DSM 20016, using a heterocyclic amine as scavenger molecule (Zhang et al, 2019). To investigate whether 3-HP would concurrently be formed, we compared glycerol metabolism of the reuterin-positive *L. reuteri* DSM 20016 and FUA 3400 and *F. rossiae* DSM 21584 (Fekry et al., 2016) to the reuterin-negative *L. reuteri* FUA 3400Δ*pduCDE* (Lin and Gänzle, 2014), J3 and CCM 3625, and *Levilactobacillus brevis* CCM 3805. The ability to release acrolein was qualitatively confirmed for *L. reuteri* DSM 20016 and FUA 3400, and *F. rossiae* DSM 21584.

The presence of glycerol did not affect growth profiles of reuterin-positive or reuterin-negative strains (**Figure 3**). The reuterin-positive strains formed mainly lactate in the presence of glucose and less than 10 mM acetate (**Table 2**). The presence of 110 mM glycerol significantly increased acetate formation of *L. reuteri* FUA 3400, while *L. reuteri* DSM 20016 formed higher levels of acetate with both concentrations of glycerol (**Table 2**). The formation of 1,3-PD was 1.3-2.6 times higher in the presence of 110 mM glycerol compared to 55 mM glycerol. 3-HP (4-10 mM) was detected if reuterin positive strains were grown in the presence of 110 mM glycerol, *L. reuteri* DSM 20016 also produced 3-HP (4 mM) with 55 mM glycerol, confirming the potential to release 3-HP during growth . Glycerol or 1,2-propanediol are

converted mainly to 1,3-propanediol and propanol, respectively, when glucose is present in the growth medium or in cereal fermentations. The reducing branch of the pathway, which supports conversion of acetyl-phosphate to acetate, generating additional ATP (Cheng et al., 2020, Gänzle, 2015; Lin and Gänzle, 2014).

Likewise, during growth, *L. reuteri* produces only low concentrations of propionic acid, the analogue compound of 3-HP formed from 1,2-PD (Cheng et al., 2020; Zhang et al., 2019). In addition, the precursor of 3-HP and 1,3-PD, 3-HPA, spontaneously degrades to acrolein at fermentation conditions and is thereby lost for further transformation through the PDU cluster (Engels et al., 2016).

Resting cells are able to produce 3-HP in the absence of fermentable sugars

It was suggested previously to use of *L. reuteri* as bioprotectant in dairy products (Avila et al., 2017; Ortiz-Rivera et al., 2017). We therefore tested whether *L. reuteri* DSM 20016 resting cells (about 10^7-10^8 cells mL^{-1}) produced 3-HP, and indeed, after 4 h incubation in glycerol buffer at 37 °C, around 0.5 mM 3-HP were formed from 55 (0.5±0 mM 3-HP) and 110 (0.5±0.2 mM 3-HP) mM glycerol. Despite the low concentration of 3-HP formed, these results demonstrate the potential of *L. reuteri* resting cells to produce 3-HP in the absence of fermentable sugars. Higher levels of 3-HP were formed by other strains of *L. reuteri* and reuterin positive *Lactobacillaceae* at different conditions. In a recent study, the reuterin-positive *L. reuteri* PTA-F13 produced 4-5 mM 3-HP during incubation in CAMH broth, which lacked glucose but was supplied with 28 mM glycerol (Asare et al., 2020). Brugé et al. (2015) reported the formation of up to about 30 mM during glycerol conversion (200 mM) of 10^{10} cells mL^{-1} of *L. reuteri* DSM 20016, DSM 17938 and ATCC 53608. These studies together with our results suggest that 3-HP can be formed in an environment that provides no or low concentrations of fermentable hexoses, and allows longer fermentation periods.

Loigolactobacillus coryniformis and *Lentilactobacillus* species convert glycerol in cider and wine (Garai-Ibabe et al., 2008); i.e. despite the low concentrations observed in our study, the metabolite may be relevant in specific applications / food systems. In agreement, the formation of 3-HPA/acrolein was confirmed in fermented milk and cheese when incubated with *L. reuteri.* In milk-based products *L. reuteri* showed little growth due to low proteolytic activity, but remained metabolically active (Avila et al., 2017; Ortiz-Rivera et al., 2017). fermentation conditions >37 °C would likely allow for the concurrent formation of both antibacterials, acrolein (Engels et al., 2016) and 3-HP, leading to synergistic effects. Likewise,

3-HP produced from glycerol may contribute to the preservative effect of *Lentilactobacillus* species growing in silage (Holzer et al., 2003).

Conclusion

We here show that 3-HP which can be produced by food-related microbes such as *L. reuteri* acts as pH-dependent antibacterial with similar activity as propionic acid, however, 3-HP did not inhibit spoilage yeast and fungi at the concentrations tested. The antibacterial activity of acrolein and 3-HP was synergistic, and both compounds could be formed concurrently by growing and resting cells of glycerol-metabolizing lactobacilli, which might be of relevance when strains are used as starter cultures, or bioprotectants for example in dairy products.

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Figure legends

Figure 1. Glycerol utilization by enzymes encoded by the *pdu* cluster. Glycerol is dehydrated to 3-hydroxypropanaldehyde (3-HPA) which can spontaneously transform to acrolein or be further metabolised to 3-hydroxypropionic acid (3-HP) and 1,3-propanediol.

Figure 2. Cooperative antibacterial activity of 3-HP and acrolein. The combined activity of acrolein and 3-HP was tested using *E. coli* ATCC 25922 as indicator strain, which was grown in Nutrient broth at pH 4.8. A modified broth dilution assay was used which concurrently tested the impact of 3-HP and acrolein. The impact of the presence of acrolein on the MIC of 3-HP was compared testing One-Way Analysis of Variance with all pairwise multiple comparison procedures (Holm-Sidak method). Different small letters indicate, that MIC of 3-HP are significantly different at different acrolein concentrations. NT not tested

Table 1. pH dependent MIC_{50} of propionic acid and 3-HP. MIC_{50} of selected Gram-negative and positive bacteria were determined using broth dilution assay. The impact of pH on the MIC_{50} of 3-HP or propionic acid was compared testing One-Way Analysis of Variance with all pairwise multiple comparison procedures (Holm-Sidak method). MIC_{50} of 3-HP or propionic acid at the same pH were compared using paired t-test. Different capital letters indicate significant ($p<0.05$) difference between MIC_{50} at different pH. NT not tested

	Minimal inhibitory concentration inhibiting 50% of growth (MIC_{50}, mM)					
Antimicrobial	**Propionic acid**			**3-HP**		
pH	6.5	5.5	4.8	6.5	5.5	4.8
Strain						
Escherichia coli Nissle	17.1±1.7^{A}	7.3±0.3^{B}	7.1±0.1^{B}	16.4±0.9^{A}	9.2±0.4^{B}	7.1±1.4^{C}
Escherichia coli ATCC 25922	12.7±0.4^{A}	6.9±0.3^{B}	5.8±1.6^{B}	14.4±1.7^{A}	7.8±0.8^{AB}	5.3±2.1^{B}
Escherichia coli GM 2163	7.0±0.5^{A}	3.6±0.6^{B}	1.8±0.2^{C}	7.3±0.2^{A}	3.4±0.6^{B}	1.4±0.4^{C}
Salmonella enterica Enteritidis ATCC 13076	8.6±1.8^{A}	3.7±0.5^{B}	1.2±1.7^{B}	9.7±2.6^{A}	6.7±0.1^{AB}	2.9±0.1^{B}
Listeria monocytogenes ATCC 7644*	NT	11.6±0.1	5.5±1.4	14.2±4.0	8.9±0	5.5±1.1
Limosilactobacillus reuteri FUA 3400	NT	NT	>60	NT	NT	>33.3
Limosilactobacillus reuteri FUA 3400 Δ*pduCDE*	NT	NT	>60	NT	NT	>33.3
Limosilactobacillus reuteri DSM 20016	NT	NT	>60	NT	NT	>33.3
Limosilactobacillus reuteri J3	NT	NT	>60	NT	NT	>33.3
Levilactobacillus brevis CCM 3805	NT	NT	>60	NT	NT	>33.3
Furfurilactobacillus rossiae DSM 21584	NT	NT	>60	NT	NT	>33.3
Limosilactobacillus vaginalis DSM 5837	NT	NT	>60	NT	NT	>33.3
Lactobacillus helveticus DSM 20075	NT	NT	>60	NT	NT	>33.3
Lactocaseibacillus rhamnosus GG	NT	NT	>60	NT	NT	>33.3
Enterococcus faecium FUA 3200	>60	>60	>60	>33.3	>33.3	>33.3
Enterococcus faecium CCM 6226	>60	>60	>60	>33.3	>33.3	>33.3
Bifidobacterium breve B13	26.1±2.9^{A}	21.6±1.6^{AB}	15.2±2.2^{B}	19.0±4.2^{A}	15.9±1.1^{A}	7.2.0±2.3^{B}
Bifidobacterium animalis Danone*	39.1±6.6	NT	20.6±5.4	25.1±1.2	NT	20.4±0.9
Bifidobacterium longum subsp. *infantis* DSM 20088*	25.4±7.8	20.5±9.7	15.6±5.0	19.3±0.6	13.8±2.4	4.6±0.3

*strains were only tested in duplicates and were not included in statistical analysis

Table 3. Main metabolites formed during cultivation. Strains were grown in MRS (control) which was additionally supplied with 55 or 110 mM glycerol. Lactate and acetate concentrations were determined after 24 h growth at 37 °C. The impact of medium composition on concentrations of lactate and acetate was compared testing One-Way Analysis of Variance with all pairwise multiple comparison procedures (Holm-Sidak method). A p-value $p<0.05$ was considered significant.

			Metabolites formed (mM)									
Medium			**MRS**		**MRS+ 55 mM glycerol**				**MRS+ 110 mM glycerol**			
Reuterin	**Strain**	**ID**	**Lactate**	**Acetate**	**Lactate**	**Acetate**	**3-HP**	**1,3-PD**	**Lactate**	**acetate**	**3-HP**	**1,3-PD**
+	*L. reuteri*	FUA 3400	88.5±7.6	8.0±11.3[1]	52.3±7.7	7.1±6.6[1]	-[2]	37.3±3.4	65.6±18.4	35.7±12.2[1]	5.2±1.6	65.1±12.7
+	*L. reuteri*[1]	DSM 20016	75.8±6.2	2.6±3.6	72.6±23.7	23.2±15.9	4.2±0.1	65.1±7.8	71.9±15.0	27.9±8.5	6.4±0.7	85.7±9.1
+	*F. rossiae*	DSM 15814	84.2±8.8	6.3±6.4	67.1±14.4	5.1±6.7	-	31.1±10.0	70.1±14.4	19.1±10.7	9.5±1.0	80.0±25.6
-	*L. reuteri*	FUA 3400Δ*pduCDE*	64.7±1.6	7.8±1.2	63.3±5.3	3.5±4.8	-	-	54.3±6.1	4.9±4.3	-	-
-	*L. reuteri*	J3	76.8±10.4	0.4±0.6	81.7±4.5	4.3±0.3	ND[3]	ND	72.7±3.3	0.3±1.2	ND	ND
-	*L. reuteri*	CCM 3625	67.7±12.6	3.9±2.7	71.2±15.6	7.5±6.6	ND	ND	71.3±0.8	2.1±0.4	ND	ND
-	*L. brevis*	CCM 3805	102.9±0.1[A]	0.8±1.1	122.8±27.0	2.3±2.2	ND	ND	125.9±23.5	5.4±0.4	ND	ND

[1]strains were only tested in duplicates and were not included in statistical analysis

[2]-, not detected

[3]ND not determined

[A]ANOVA indicated significant difference of the means, the posthoc test did not identify pairwise differences

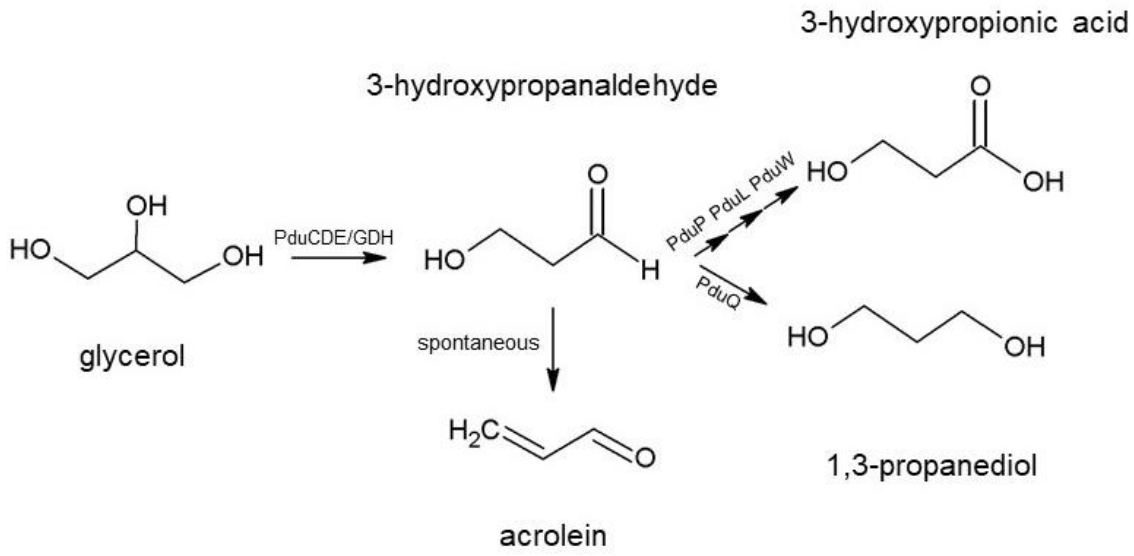
3-hydroxypropionic acid
3-hydroxypropanaldehyde
HO
OH
OH
PduCDE/GDH
HO
O
H
PduP PduL PduW
HO
O
OH
PduQ
HO
OH
glycerol
spontaneous
H2C
O
1,3-propanediol
acrolein

Figure 1.

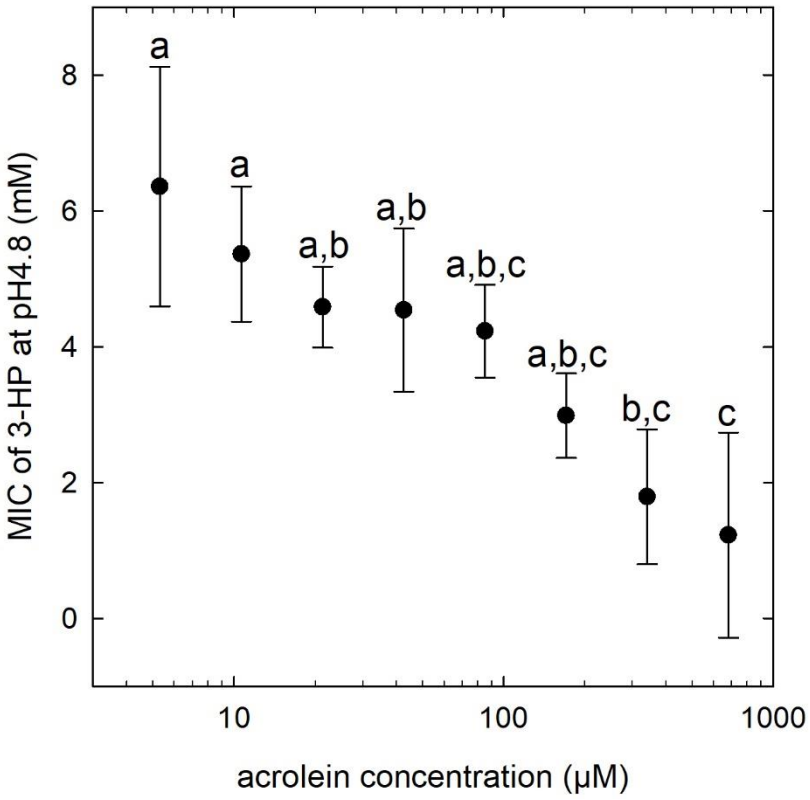
a
a
a,b
a,b,c
a,b,c
b,c
c
MIC of 3-HP at pH4.8 (mM)
0
2
4
6
8
10
100
1000
acrolein concentration (μM)

Figure 2

Supplementary Data for

3-Hydroxypropionic acid contributes to the antibacterial activity of glycerol metabolism by the food microbe *Limosilactobacillus reuteri*

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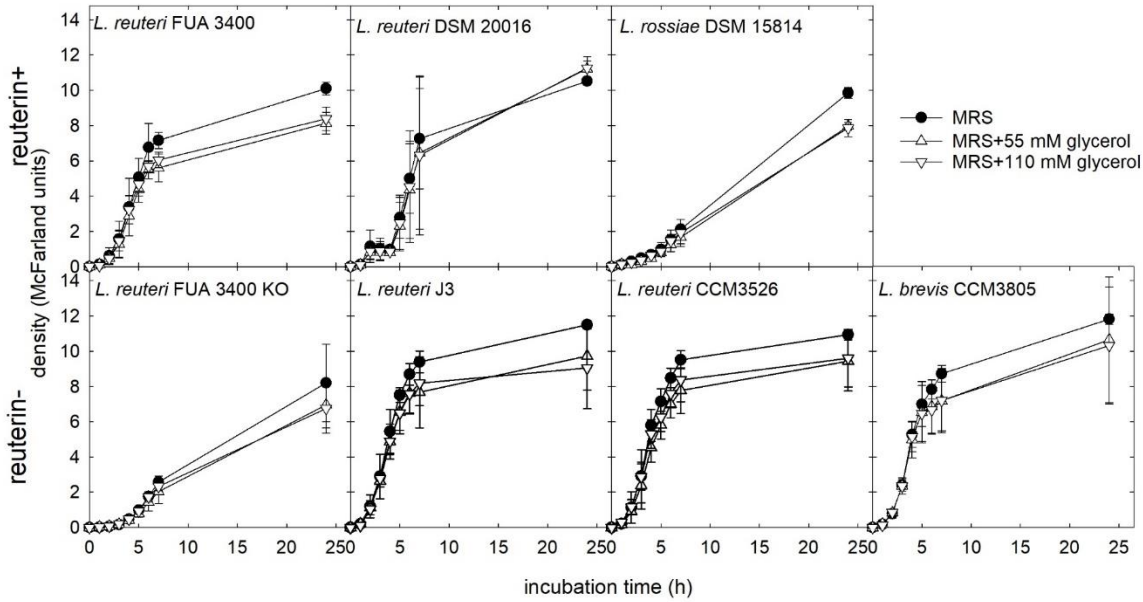

Supplementary Figure 1. Growth of reuterin-positive and negative strains in the presence and absence of glycerol. Strains were grown at 37°C in MRSm which was additionally supplied with 55 or 110 mM glycerol. Density (in McFarland units) was determined in regular intervals for 24 h.