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**Membrane fatty acid composition as a determinant of *Listeria*
monocytogenes sensitivity to trans-cinnamaldehyde**

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Abstract

Trans-cinnamaldehyde, the major compound of cinnamon essential oil, is a potentially interesting natural antimicrobial food preservative. Although a number of studies have addressed its mode of action, the factors that determine bacterial sensitivity or tolerance to trans-cinnamaldehyde are poorly understood. We report the detailed characterization of a *Listeria monocytogenes* Scott A trans-cinnamaldehyde hypersensitive mutant defective in IlvE, which catalyzes the reversible transamination of branched-chain amino acids to the corresponding short-chain α -ketoacids. This mutant showed an 8.4 fold extended lag phase during growth in sublethal concentrations (4 mM), and faster inactivation in lethal concentrations of trans-cinnamaldehyde (6 mM). Trans-cinnamaldehyde hypersensitivity could be corrected by genetic complementation with the *ilvE* gene and supplementation with branched-chain α -ketoacids. Whole-cell fatty acid analyses revealed an almost complete loss of anteiso branched-chain fatty acids (BCFAs), which was compensated by elevated levels of unbranched saturated fatty acids and iso-BCFAs. Sub-inhibitory concentrations of trans-cinnamaldehyde induced membrane fatty acid adaptations predicted to reduce membrane fluidity, possibly as a response to counteract the membrane fluidizing effect of trans-cinnamaldehyde. These results demonstrate the role of IlvE in BCFA production and the role of membrane composition as an important determinant of trans-cinnamaldehyde sensitivity in *L. monocytogenes*.

Keywords: amino acids, branched chain; food preservatives; oils, volatile; antimicrobial agents

1. Introduction

Minimally processed chilled foods are one of the strongest growing and innovative segments in the food industry since at least two decades. This expansion has stimulated research into mild food processing and preservation techniques that allow a maximal retention of the food fresh-like properties and at the same time provide a healthy image and a long shelf-life to the food. In this context, natural preservatives such as plant essential oils and their components are being studied to improve the safety and extend the microbiological shelf-life of foods without the use of traditional ‘chemical’ preservatives [1, 2].

Dozens of essential oil compounds displaying a wide variety of properties like reactivity, water and fat solubility, volatility and smell and aroma have been documented to have antimicrobial activity. These compounds generally have a wide activity spectrum including both Gram-positive and Gram-negative bacteria, yeasts and molds, and this makes them potentially useful for food applications. On the other hand, many compounds can only be applied at concentrations that are not very effective because of their low water solubility and strong sensory impact. An attractive approach to overcome this problem is the use of hurdle technology, which is the combination of multiple gentle processing and preservation techniques to obtain maximal control of microbial safety and stability with a minimal impact on food quality [3]. Hurdle technology is most effective when it combines techniques that reinforce each other in a synergistic fashion. In the case of essential oil compounds, this can make them effective at lower concentrations and thus reduce their sensory impact. However, combined treatments can also have an additive or even an antagonistic effect, and it is currently not understood what determines the outcome of the interaction. This question can probably only be answered by acquiring a more detailed insight in the cellular or molecular mode of action of individual treatments. Studies on the mode of action of essential oil

compounds are therefore essential as a basis for the development of new intelligent applications of hurdle technology using these compounds.

Our research group recently investigated the inactivation of different Gram-negative and Gram-positive food pathogens by the combination of high hydrostatic pressure and different small molecule antimicrobials, mostly essential oil compounds [4]. For less than half of the tested compounds this resulted in synergy with the high pressure treatment, the strongest synergy being observed with α , β -unsaturated aldehydes (t-cinnamaldehyde, t-2-hexenal, dimethylfumarate), isothiocyanates (allyl isothiocyanate, sulforaphane) and reuterin. These compounds are all electrophilic and show reactivity towards thiol groups [5, 6], and it was therefore hypothesized that thiol reactivity might be the cause of synergy with high pressure treatment. In addition, since thiol-reactive compounds from plant essential oils like t-cinnamaldehyde, t-hexenal and isothiocyanates are hydrophobic, they are also predicted to partition into the bacterial cell membrane, thereby disturbing its structure and increasing its permeability [1]. Besides combinations with processes, the mutual combination of two or more essential oil compounds or the combination with other natural antimicrobials can also result in a synergistic effect. For example, the combination of t-cinnamaldehyde and carvacrol has been found to result in both a synergistic growth-inhibitory effect on a set of Gram-positive and Gram-negative food-borne bacteria, and a synergistic bactericidal effect on *Escherichia coli* and *Staphylococcus aureus* [7]. Also the combination of a nisin variant (nisin V) in the form of a fermentate with thymol, carvacrol and t-cinnamaldehyde elongated the lag phase of *Listeria monocytogenes* in a synergistic way [8].

Among the thiol-reactive compounds, t-cinnamaldehyde (t-CIN), the major constituent of cinnamon bark essential oil, is one of the most investigated both with regard to its mode of action and its potential food applications. Although the antimicrobial properties of t-CIN generally result from its overall electrophilic and lipophilic character, a number of studies

have looked at the effects on bacterial cells in more detail. Based on the observation that t-CIN induced filamentation by inhibiting cell separation in *Bacillus cereus* [9], it was demonstrated that t-CIN binds with the *E. coli* cell division protein FtsZ with an affinity constant of $1.0 \pm 0.2 \mu\text{M}^{-1}$ and inhibits its GTP-dependent polymerization [10]. Several phenylpropanoid compounds structurally related to t-CIN but lacking an aldehyde group (e.g. cinnamic acid, caffeic acid, chlorogenic acid) similarly inhibited FtsZ polymerization [11]. In addition, t-CIN also inhibited ATPase activity of isolated membranes from *E. coli* and *L. monocytogenes* [12], and this effect was suggested to be the cause of the reduced growth rate observed at sublethal concentrations. Genome-wide transcriptional profiles of *E. coli* O157:H7 after 2 h of exposure to a sublethal concentration of t-CIN showed elevated expression of oxidative stress-related genes and reduced expression of DNA, protein, O-antigen and fimbrial synthetic genes. Moreover *E. coli* O157:H7 was able to metabolize t-CIN to less toxic cinnamic alcohol after 4 h of exposure by using dehydrogenase/reductase enzymes [13]. Proteomic analysis of *Cronobacter sakazakii* exposed to a sub-inhibitory dose of t-CIN showed disrupting effects on the carbohydrate, amino acid and lipid metabolism, motility, invasion and attachment ability and cellular defenses against oxidative stress [14]. These omic studies indicate that t-CIN affects many essential bacterial processes and pathways, but most of these effects are likely to be secondary, and it is not possible to identify the primary target(s) of t-CIN based on these studies. A different, complementary approach that has not yet been applied to study t-CIN, but that may provide additional insights in its mode of action is the use of genetic analysis to identify genes that affect bacterial t-CIN sensitivity. Therefore, we have constructed and screened a transposon mutant library in *L. monocytogenes* and we report here the detailed characterization of a t-CIN sensitive mutant with a defect in the synthesis of branched-chain fatty acids. *L. monocytogenes* was chosen for this work because it is one of the most important pathogens in minimally processed chilled

117 foods in view of its ability to grow at low temperature [15], its relatively high tolerance to
118 elevated CO₂ concentrations [16] as used in modified atmosphere packaging, to salt and to
119 preservatives like nitrite [17, 18]. *L. monocytogenes* is widespread in the environment and a
120 frequent contaminant in food production facilities. Although human listeriosis is rare, it has a
121 high hospitalization and mortality rate, and is particularly dangerous for pregnant women and
122 elderly people [19].

2. Materials and Methods

2.1. Bacterial strains and growth conditions

L. monocytogenes Scott A, obtained from the International Life Sciences Institute North America (ILSI NA) *L. monocytogenes* strains collection [20], was used in this work as the wild-type (WT) strain. Cultures of both the WT and transposon mutants were routinely grown at 30 °C in Brain Heart Infusion (BHI, Oxoid, Hampshire, Great-Britain) broth or on BHI agar (BHI with 15 g/l agar). *E. coli* S17-1 λ pir [21] was used as a donor for conjugational plasmid transfer to *L. monocytogenes* Scott A, and was grown in lysogeny broth (LB; 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) or on LB agar (LB with 15 g/l agar) at 37 °C. Growth media were supplemented with the following antibiotics: chloramphenicol (Cm, 50 µg/ml) for *E. coli* DH5 α -pMC38, erythromycin (Em, 50 µg/ml) for *L. monocytogenes* transposon mutants, kanamycin (Km, 50 µg/ml) for *E. coli* S17-1 λ pir-pIMK2 and the combination of Em and Km for genetically complemented *L. monocytogenes* transposon mutants. For long-term storage at -80 °C, stationary-phase bacterial cultures were supplemented with 25% glycerol. Experiments were always performed with cultures obtained from single colonies from plates that were not older than one week.

2.2. Random transposon mutagenesis, screening and mutant identification

Random *HimarI* transposon mutants were generated using the temperature-sensitive pMC38 delivery plasmid [22]. After temperature shift, Em-resistant colonies were isolated and purified and stored as BHI cultures in 96-well plates at -80 °C. In about 25 independent experiments, a random transposon mutant library of 7680 mutants, corresponding to a predicted gene coverage of >99 % was created and used for screening for t-CIN sensitivity. Randomness of transposition was verified on about 15 colonies per experiment using a Y-linker PCR protocol (see below). Stationary cultures of the total amount of 7680 mutants were

diluted 1000x to a final concentration of approximately 10^6 /ml and grown at 30 °C in 200 μ L BHI with 4 mM of t-CIN ((2E)-3-phenylprop-2-enal, 99%, Acros Organics, New Jersey, USA) in 96-well microplates sealed with an oxygen impermeable cover foil (EASYseal™, Greiner Bio-One, Frickenhausen, Germany). t-CIN sensitivity was scored based on optical density (OD) measurement at 600 nm after approximately 45 h, which was the time needed for the WT strain to reach stationary phase (OD ~ 0.6). Mutants were considered to be t-CIN sensitive when the OD was below 0.100 after 45 h. Sensitivity of selected mutants was subsequently confirmed by growth assays with three independent cultures (see below). In mutants for which t-CIN sensitivity could be confirmed, the transposon insertion sites were identified by PCR amplification of the flanking DNA sequences with the Y-linker protocol of Kwon and Ricke (2000) [23] using primers Y-linker and Marq269 and subsequent sequence analysis with primer Marq269 (primer sequences specified in Table 1).

2.3. Genetic complementation

Genetic complementation was performed using the pIMK2 integration plasmid with a highly expressed constitutive promotor [24]. The WT *ilvE* gene was amplified using primers *ilvE*_NcoI and *ilvE*_SalI (Table 1), and cloned as an NcoI - SalI fragment into pIMK2, which was linearized with the same restriction enzymes. The resulting plasmid was conjugated from *E. coli* S17-1 λ pir to *L. monocytogenes ilvE::HimarI*, using selection for Km (pIMK2 marker) and Em (transposon marker). The pIMK2 plasmid does not replicate in *L. monocytogenes*, but is designed to integrate specifically in the tRNA^{Arg} gene, which is the unique integration site of the phage PSA in the genome of *L. monocytogenes* Scott A [24, 25]. Integration in this site was confirmed via PCR using primers *ilvE*_NcoI and NC16, which anneals just outside the phage integration site [25]. Additionally, the presence of the intact *ilvE* copy in the integrants was confirmed via PCR and Sanger sequencing analysis (MacroGen Europe, Amsterdam, Netherlands) using primers pIMK_FW and pIMK_REV,

which anneal at both sides just outside the cloning site of the plasmid (Table 1). Finally, complemented transposon mutants were phenotypically analyzed by growth assays.

2.4. Growth assays

Growth experiments were conducted in 200 μ L BHI cultures in 96-well microplates covered with an oxygen-impermeable cover foil in an automated microplate reader (Multiskan Ascent, Thermo Labsystems, Helsinki, Finland) at 30 °C. Every 15 minutes the plates were shaken at 960 rpm and the OD_{630nm} was measured. The inoculum was prepared by washing stationary overnight cultures once with potassium phosphate buffer (PPB, 10 mM, pH 7.0), resuspending the cells in fresh BHI medium and further diluting them to a start cell concentration of about 10⁶ cfu/ml in BHI or BHI with t-CIN. Lag phase duration (λ), maximum growth rate ($\mu_{\max}$) and maximal OD value at stationary phase (OD_{max}) were determined by fitting the data according to the model of Baranyi and Roberts [26], using the Excel add-in package DMFit (Institute of Food research, Norwich, United Kingdom). In some experiments, cultures were supplemented with precursors of branched-chain fatty acid synthesis, including the branched-chain amino acids (BCAAs) L-leucine (Leu, $\geq$ 98.5%, Sigma-Aldrich, Steinheim, Germany), L-isoleucine (Ile, 99%, Acros Organics), L-valine (Val, $\geq$ 98.5%, Fisher Scientific, Leicestershire, Great-Britain) and the branched-chain α -ketoacids (α -KAs) α -ketoisocaproic acid sodium salt (KIC, $\geq$ 98%, Sigma-Aldrich), α -ketomethylvaleric acid sodium salt (KMV, $\geq$ 98%, Sigma-Aldrich) and α -ketoisovaleric sodium salt (KIV, $\geq$ 98%, Acros Organics).

2.5. Growth assay at 10 °C

Cells from three independent stationary-phase overnight cultures of each strain were washed once with PPB, diluted to approximately 10⁴ cfu/ml in 4 ml of BHI broth in 10 ml

glass tubes with loose caps, and incubated shaking at 10 °C. Every 24 h, samples were taken for plate counting on BHI agar.

2.6. Whole-cell fatty acid analysis

The whole-cell fatty acid composition was determined for WT and *ilvE* mutant stationary- and early-exponential-phase cultures grown in the absence of t-CIN, and for exponential-phase cultures growing in 2 mM t-CIN. Each analysis was performed with three independent cultures, grown shaken at 200 rpm in 50 ml BHI medium in closed 100 ml Erlenmeyers at 30 °C. At the desired growth stage, cultures were cooled on ice for 10 min, cells were washed 3 times with sterile cold deionized water, and finally pelleted by centrifugation at 15000 rpm. All manipulations were performed rapidly at 4 °C to avoid any further change in the fatty acid composition. The cell pellets were frozen with liquid nitrogen and finally lyophilized. Lipid extraction and fatty acid methyl ester (FAME) analysis were performed directly on the lyophilized pellets using the method recommended by the Microbial ID System (MIDI Inc., Delaware, USA). Samples were analyzed using an Agilent Technologies 6890N gas chromatograph (Santa Clara, CA, USA) and the peaks were identified using the TSBA50 identification library version 5.0 (MIDI). This analysis was performed by the Belgian Co-ordinated Collections of Micro-organisms (BCCM/LMG, Ghent, Belgium). The cell concentration of the cultures used for this analysis was determined by colony counts on BHI agar.

2.7. Headspace - solid phase micro-extraction - gas chromatography - mass spectrometry (HS-SPME-GC-MS) analysis

Stationary-phase overnight cultures grown in BHI broth at 30 °C were washed once with PPB (10 mM, pH 7.0), diluted 1000x to approximately 10^6 cfu/ml in 80 ml BHI broth supplemented with t-CIN in a 100 ml Erlenmeyer closed tightly with a screw-cap with teflon

219 seal, and incubated at 30 °C. Samples from the cultures and controls without cells were taken
220 at several time points during the lag phase and exponential phase. The cell concentration of
221 each sample was determined by plate count on BHI agar. For quantification of t-CIN, cells
222 were eliminated from the samples by passage over a 0.2 µm cellulose acetate syringe filter
223 (VWR International, Radnor, USA) and the filtrate was frozen with liquid nitrogen and stored
224 at -80 °C. Four hours before HS-SPME-GC-MS analysis, samples were thawed at 4 °C. One
225 ml of the thawed sample was mixed with 1 ml saturated NaCl solution in a 10 ml amber glass
226 vial. The vials were tightly closed using screw-caps with a PTFE/silicon septum seal and
227 hundred µl 0.01 mM ethyl benzoate ($\geq 99\%$, Sigma Aldrich, Steinheim, Germany) was added
228 to each sample as an internal standard. Headspace fingerprinting was conducted on a gas
229 chromatography system (6890N, Agilent Technologies, Diegem, Belgium), coupled to a mass
230 selective detector (5973N, Agilent Technologies, Diegem, Belgium) and equipped with a
231 CombiPAL auto-sampler (CTC analytics, Zwingen, Switzerland). Volatiles were directly
232 extracted from the headspace with a HS-SPME fiber coated with 30/50 µm
233 divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (StableFlex, Supelco,
234 Bellefonte, PA, USA) at 40 °C during 10 min. The SPME fiber was then inserted into the
235 heated (230 °C) GC-injection port for 2 min to thermally desorb the volatiles.
236 Chromatographic separation was carried out on a HP-5MS capillary column (30 m x 0.25 mm
237 i.d., 0.25 µm film thickness, Agilent Technologies J&W, Santa Clara, CA, USA) having a
238 5%-phenyl-methylpolysiloxane stationary phase and using a constant helium flow of 1.5
239 ml/min. The oven temperature was initially 40 °C for 2 min, then ramped to 160 °C at 14
240 °C/min, to 300 °C at 70 °C/min and held at 300 °C for 2 min before cooling back to 40 °C.
241 Mass spectra were obtained in electron ionization mode at 70 eV with a scanning range of 35
242 to 400 m/z and a scanning speed of 3.8 scans/s. MS ion source and quadrupole temperatures

were 230 °C and 260 °C, respectively. The experiment was performed for three independent WT cultures (biological replicates). Each sample was analyzed twice (technical repetitions).

2.8. Bacterial inactivation by t-CIN

Stationary-phase overnight cultures grown in BHI broth at 30 °C were washed twice with PPB (10 mM, pH 7.0) and diluted to approximately 10^7 cfu/ml in PPB supplemented with 6 mM t-CIN. Cell suspensions were then kept in Eppendorf tubes at 22 °C in a heating/cooling block and sampled every hour for determination of survivors by plate count on BHI agar.

2.9. Statistical analysis

Means of the parameter estimates of the Baranyi and Roberts growth model (λ , $\mu_{\max}$ and $OD_{\max}$ [26]), mean percentages of the different fatty acids, mean t-CIN percentage losses determined by HS-SPME-GC-MS, mean cell concentrations during growth at 10 °C and mean cell concentrations during the inactivation at different time points, were statistically compared by the Tukey-Kramer HSD test with the JMP[®] Software (JMP, Version Pro12, SAS Institute Inc., Cary, USA), and considered significantly different when a p -value < 0.05 was obtained.

3. Results and discussion

3.1. Isolation of *ilvE* mutant from screening a genome-wide random transposon mutant library for t-CIN sensitivity

The screening of the 7680 transposon mutants initially yielded 12 putative t-CIN sensitive mutants (data not shown). One of the most sensitive mutants with reproducible t-CIN sensitivity was selected for further analysis. The transposon was found to be located in the *ilvE* gene of this mutant, at 792 base pairs from the start codon (Fig. 1). IlvE is a branched-chain amino acid aminotransferase (PATRIC accession number VBILisMon199232_1061 [27], Kegg Enzyme EC 2.6.1.42) that reversibly catalyzes the transamination of the branched-chain amino acids (BCAAs) L-leucine, L-isoleucine and L-valine to the corresponding short-chain α -ketoacids (α -KAs), α -ketoisocaproate (KIC), α -ketomethylvalerate (KMV) and α -ketoisovalerate (KIV), respectively. The latter in turn serve as substrates for the synthesis of branched-chain fatty acids (BCFAs) [28, 29] (Fig. 2).

3.2. Characterization of the t-CIN sensitivity of *L. monocytogenes ilvE::HimarI*

Fig. 3 shows the growth curves and the growth parameters of *L. monocytogenes* Scott A and its *ilvE::HimarI* transposon mutant in BHI broth with and without the addition of 4 mM of t-CIN. In absence of t-CIN, the mutant showed a slightly attenuated growth in BHI, with a lower $\mu_{\max}$ and $OD_{\max}$ (Fig. 3b). Plate counting showed that the stationary-phase cell concentration was about 2.5 times higher for the WT than for the mutant $((4.0 \pm 0.5) \times 10^9$ cfu/ml compared to $(1.6 \pm 0.2) \times 10^9$ cfu/ml). Because initial cell concentration may affect the inhibitory effect of t-CIN, the stationary-phase cultures used as inoculum for the growth experiments in the presence of t-CIN were diluted 2.5 fold more for the WT than for the mutant, to ensure that the amount of available t-CIN per cell was equal for both strains. It should be noted that the inoculum may also have been in a somewhat different physiological

state because both strains reached stationary phase at different cell density and because the *ilvE* mutation may have altered cellular metabolism. Both the WT and the mutant showed a significant extension of the lag phase and a significant reduction of $\mu_{\max}$ and $OD_{\max}$ in the presence of t-CIN (Fig. 3b). Similar effects on the growth parameters were also observed by Siroli *et al.* (2015) for *L. monocytogenes* Scott A in BHI supplemented with sub-lethal concentrations of trans-2-hexenal and citral, two other small hydrophobic α , β -unsaturated aldehydes like t-CIN [30]. The t-CIN sensitivity of the *ilvE* mutant is particularly reflected in the lag phase. While the reduction in $\mu_{\max}$ and $OD_{\max}$ caused by t-CIN were of similar magnitude in the WT and *ilvE* mutant, the lag phase was extended much more in the mutant (8.4-fold) than in the WT (3.6-fold).

Several possible explanations can be proposed for the extended lag phase and the subsequent resumption of growth: (i) the requirement of cellular adaptation before cells can grow in 4 mM t-CIN, (ii) chemical or bacterial degradation of t-CIN followed by growth at a lower t-CIN concentration, (iii) the outgrowth of a small subpopulation of mutants with increased t-CIN resistance, or (iv) a combination of these factors. Plate counting at different time points of the lag phase showed stable cell numbers for both the WT and *ilvE* mutant, indicating that no transient inactivation occurred upon exposure to t-CIN. The formation of t-CIN resistant mutants could be excluded since the growth parameters of cultures of the WT and the *ilvE* mutant grown in BHI broth with 4 mM t-CIN did not alter when they were reinoculated in the same medium (data not shown). Finally, chemical degradation of t-CIN could also be ruled out as an explanation because BHI with 4 mM t-CIN that had been pre-incubated at 30 °C for 44 h still caused an extended lag phase in both WT and mutant (data not shown). Therefore, we hypothesize that the resumption of growth after a long lag phase results from bacterial adaptation to t-CIN and/or bacterial degradation of t-CIN.

3.3. Genetic and chemical complementation of *L. monocytogenes ilvE::Himar1*

3.3.1. Genetic complementation

As can be seen from Fig. 1, it cannot be excluded that *ilvE* is co-transcribed with two downstream ORFs and that the *Himar1* insert in *ilvE* also knocks out the production of the corresponding proteins. However, this is unlikely, since *ilvE* was shown to be separately transcribed in *L. monocytogenes* strain EGD-e, which has the same gene organization in this region [31]. To confirm that it is indeed disruption of *ilvE* that is responsible for t-CIN sensitivity, the mutant was genetically complemented with the *ilvE* gene using the overexpression integration vector pIMK2 [24]. This restored the OD_{max} to WT level, and the lag phase even became slightly shorter than that of the WT (Fig. 3). These results, together with the results of chemical complementation below, allow us to conclude that loss of IlvE activity results in increased t-CIN sensitivity in *L. monocytogenes* Scott A.

3.3.2. Chemical complementation

IlvE reversibly catalyzes the transamination between the BCAAs Leu, Ile and Val and their corresponding α -ketoacids KIC, KMV and KIV, respectively. As such it has a dual metabolic role (Fig. 2). First, it catalyzes an essential step in the biosynthesis of BCAAs when these are in limited supply in the medium. Second, when BCAAs are abundant, IlvE can deaminate them to KIC, KMV and KIV, which are subsequently converted by the branched-chain ketoacid dehydrogenase complex (BCKD) to isovaleryl-CoA, 2-methylbutyryl-CoA and isobutyryl-CoA, which serve as direct precursors for the synthesis of the BCFAs iso-C15:0/iso-C17:0, anteiso-C15:0/anteiso-C17:0 and iso-C14:0/iso-C16:0, respectively [28, 29, 32]. To find out whether t-CIN sensitivity of the *ilvE* mutant in BHI broth was due to their inability to synthesize BCAAs or to convert BCAAs from the medium to BCFAs, we supplemented the medium with an equimolar mix (10 mM each) of the above three α -KAs or

BCAAs. The α -KAs fully reversed the t-CIN sensitivity of the mutant to WT level, while the BCAAs had no effect (Fig. 4a, 4c). Supplementation of the WT cultures with α -KAs also significantly shortened the lag phase in the presence of t-CIN. These results lead us to conclude that the t-CIN sensitivity of the *ilvE* mutant is due to its inability to use BCAAs from the medium for the production of BCFAs. It should be noted that the full pathway to synthesize BCFAs *de novo* without IlvE (Fig. 2) is predicted to be present in *L. monocytogenes* Scott A based on its genome sequence, and the fatty acid analysis of the *ilvE* mutant indicates this (see below). Therefore our results suggest that BCFA production via this pathway may be kinetically limited. This would also explain the much reduced growth rate of the *ilvE* mutant at 10 °C (Fig. 5), since *L. monocytogenes* requires BCFAs for maintaining its membrane fluidity under these conditions [29]. The growth defect at 10 °C rate was also completely restored by genetic complementation (Fig. 5).

Next, we investigated whether there is a difference in the ability of the three α -KAs to neutralize the t-CIN sensitive phenotype of the *ilvE* mutant (Fig. 4b, 4c). All three α -KAs significantly reduced the lag phase, but only KIV restored the t-CIN tolerance to the same level as the WT supplemented by all three α -KAs. Since KIV supports the synthesis of iso-fatty acids (especially C14:0 and C16:0, see Fig. 2), it could be that this type of BCFAs is most effective in generating t-CIN tolerance. However, this cannot be concluded with certainty, since there may be additional factors influencing the response to α -KAs, like the uptake efficiency from the medium.

3.4. Membrane fatty acid analysis

3.4.1. Stationary-phase cells grown in absence of t-CIN

The previous results suggest that the t-CIN sensitivity of the *ilvE* mutant may be due to an altered membrane composition. To provide more direct evidence for this hypothesis, we

first compared the whole-cell fatty acid composition of stationary-phase cultures of the WT and the *ilvE* mutant after growth in BHI at 30 °C (Table 2). The most notable feature of the fatty acid composition of the WT strain is the high level of BCFAs (~95%), mainly anteiso-BCFAs (~71.5%). This prominent presence of BCFAs has been reported before for the same strain, grown in buffered BHI at 30 °C to mid-log phase [33]. In comparison, and as expected, the *ilvE* mutant had strongly reduced BCFA levels (53.7%). This reduction was entirely accounted for by the anteiso fraction, which decreased from 71.5% to only 4.1 %, while the iso-BCFAs even increased from 23.5% to 49.6%. The reduced anteiso-BCFA content is further balanced by an increase in unbranched saturated fatty acids, predominantly C14:0 and C16:0. Furthermore there is a clear shift to fatty acids with lower molecular mass (< C15) in the mutant. Since the *ilvE* mutant can no longer convert BCAAs to BCFAs, its BCFAs must be derived from *de novo* synthesis or from alternative substrates from the medium like α -KAs or branched-chain carboxylic acids (Fig. 2). The increased level of iso- but not anteiso-BCFAs in this mutant may therefore indicate that BHI medium is a good source of alternative iso-BCFAs precursors (i.e. KIV, KIC, isobutyrate and isovalerate) but less so for anteiso-BCFAs precursors (KMV and 2-methylbutyrate). Alternatively, the increased iso/anteiso ratio of the mutant may reflect a kinetic limitation of the anteiso biosynthesis pathway, possibly because this pathway is much longer than the iso biosynthesis pathway (Fig. 2).

From a functional perspective, the membranes of the *ilvE* mutant are predicted to be more rigid, predominantly due to the almost complete loss of the anteiso-BCFAs. Despite the small structural difference, anteiso-BCFAs have a much lower phase transition temperature than iso-BCFAs with the same number of carbon atoms (iso-C15:0, 52.2 °C; anteiso-C15:0, 25.8 °C; anteiso-C17:0, 30.0 °C; iso-C17:0, 60.5 °C) [32, 34], and this explains why anteiso-BCFAs are much more critical for maintaining membrane fluidity and supporting growth of *L. monocytogenes* at low temperature [29, 34, 35]. Therefore, in the *ilvE* mutant, the increase

of iso-BCFAs and the shift towards less carbon atoms apparently could not fully compensate for the loss of anteiso-BCFAs in maintaining membrane fluidity, since the mutant showed a severe growth defect at 10 °C (Fig. 5). Finally, the shift towards less carbon atoms in the mutant may reflect an adaptation to maintain membrane thickness, because the replacement of BCFAs by unbranched fatty acids would otherwise lead to increased chain length, since the methyl side chain in BCFAs does not contribute to chain length.

Although our study is the first to document the role of *IlvE* in BCFA synthesis in *L. monocytogenes*, mutants in the branched-chain α -KA dehydrogenase complex (BCKD), which are unable to convert the branched-chain α -KAs to the corresponding acyl-CoA derivatives (Fig. 2) have been previously isolated as being cold-sensitive, and showed a similar decrease in total and anteiso-BCFAs as our *ilvE* mutant [28]. As opposed to *ilvE* mutants, *bckd* mutants fully depend on exogenous branched-chain carboxylic acid precursors for BCFA synthesis.

3.4.2. Exponential-phase cells grown in the presence of t-CIN

While the above findings clearly indicate that the modified membrane fatty acid composition is at the basis of the increased t-CIN sensitivity of the *ilvE* mutant, the growth curve in Fig. 3 shows that also *ilvE* mutant cells eventually start growing in the presence of t-CIN after a long lag phase. To investigate whether adaptation of the fatty acid composition during the lag phase in the presence of t-CIN could play a role in this, we analyzed the whole-cell fatty acid composition of early-exponential phase WT and mutant cells grown both in the absence and in the presence of 2 mM t-CIN. This concentration induces a lag phase in the WT of 11.0 ± 0.2 h and in the mutant a lag phase of 24.8 ± 0.6 h. Early-exponential phase cells were chosen for this experiment because if resumption of growth in t-CIN depends on an adaptation of membrane composition, it is expected to be best visible shortly after the lag phase. In addition, HS-SPME-GC-MS analysis indicated that the concentration of t-CIN in

the WT culture had only slightly decreased by 5.3 ± 1.4 % until this stage of growth. This decrease was not significantly different ($p = 0.13$) from that in control medium without cells (12.2 ± 6.1 %), suggesting that it was due to chemical degradation or reaction with medium compounds rather than to bacterial metabolism. The results of the fatty acid analyses of the exponential cultures are presented in table 2. Compared to stationary phase, exponential-phase cells of the WT strain had less BCFAs (- 12%), and within the BCFAs there was a shift towards shorter chain length (C17 to C15). Also notable was the appearance of the unsaturated C18:1 ω 9c (6.3%). In the *ilvE* mutant, there was a similar shift towards shorter BCFAs (C15 but also C14 in this case) and the appearance of C18:1 ω 9c (9.8%). However, unlike in the WT, the proportion of BCFAs was higher in exponential than in stationary phase (+ 14%), mainly due to an increase in the anteiso fraction. Possibly, this is because the branched-ketoacid or acid precursors for BCFA synthesis in the BHI medium are not yet limiting during the initial stages of growth, while they get progressively exhausted later during growth. The presence of t-CIN induced some marked changes in the fatty acid profiles of both strains. There was a decrease in BCFAs both in the WT (from 83.5% to 66.8%) and in the *ilvE* strain (from 61.0% to 33.3%). However, whereas in the WT this net effect was the result of a decrease of the anteiso fraction (from 63.4% to 32.9%) and an increase in the iso fraction (from 20.1% to 33.9%), both fractions declined in the mutant (from 9.5% to 5.3% and from 51.5% to 28.0%, respectively). The unsaturated C18:1 ω 9c also decreased in both strains. These decreases are balanced by an increase in unbranched saturated fatty acids in both strains. Finally, both strains show a shift towards fatty acids with less carbons ($< C15$) in the presence of t-CIN. This shift is considerable in the WT strain (from 2.9% to 20.7%), and less marked in the *ilvE* mutant (from 29.1% to 34.0%), possibly because the level in the mutant is already elevated even in absence of t-CIN.

Although the changes in fatty acid composition induced by t-CIN differ in some aspects between the WT and the mutant, they are expected to result in higher phase transition temperatures (i.e. reduced membrane fluidity) in both cases. For the WT, this is mainly because of the reduction of the anteiso-BCFA fraction, while in the mutant the decrease in the iso-BCFAs and unsaturated FA fractions are likely to also play a role. Since small hydrophobic molecules like t-CIN partition into the membrane and have a fluidizing effect [36], the changes in fatty acid composition may be considered as a response of the bacteria to counteract this effect.

Some previous studies have investigated the effect of other hydrophobic α , β -unsaturated carbonyl compounds on the membrane composition of *L. monocytogenes* Scott A in similar conditions. Patrignani *et al.* (2008) reported an increase in saturated fatty acids, predominantly C14:0, C16:0 and C18:0, and a reduction in iso-C14:0, C15:0, iso-C15:0, anteiso-C15:0 and C18:1 ω 9c in late-exponential-phase cells grown at 37 °C in BHI when 20 ppm (~0.2 mM) trans-2-hexenal was added [37]. Even though this concentration was reported to be sub-inhibitory (while we used growth-inhibitory concentrations of t-CIN), these changes are also predicted to reduce membrane fluidity and are thus consistent with the idea of a bacterial response to counteract the membrane-fluidizing effect of the compound. However, remarkably, these authors did not find any anteiso-C17:0 in the control Scott A culture grown in BHI without antimicrobials, although we and others found this to be one of the dominant fatty acids in Scott A and other *L. monocytogenes* strains [33, 38]. Later work from the same group reported on the effect of sub-lethal concentrations of citral (50, 85, 125 ppm) in BHI during growth at 37 °C [30]. In this study the fatty acid composition of the control Scott A culture corresponded better to the composition reported in our work and that of others. Interestingly, much like t-CIN in our work, citral primarily affected growth by extending the lag phase. Although the changes in the membrane fatty acid composition were less

pronounced than for t-CIN, there was a small reduction, for cultures grown with 125 ppm citral, in the amount of anteiso-C15:0, anteiso-C17:0 and iso-C17:0, compensated by a increases in C16:0, C18:2 (cis-cis) and C18:1 ω 9c. Again, these changes in the fatty acid composition are predicted to make the membranes more rigid.

Finally, Di Pasqua *et al.* [39] investigated adaptive changes in the ratio of saturated to unsaturated fatty acids induced by sublethal concentrations of t-CIN and other essential oil compounds in various Gram-negative and Gram-positive bacteria. In *Escherichia coli* O157:H7, t-CIN induced a higher degree of unsaturation, and the same was observed for the other compounds tested. However, the effect of t-CIN in different bacteria was not consistent, since it had no significant effect on the degree of saturation in two other Gram-negative bacteria (*Salmonella enterica* serovar Typhimurium and *Pseudomonas fluorescens*), increased the ratio in one Gram-positive organism (*Brochothrix thermosphacta*) and decreased the ratio in another one (*Staphylococcus aureus*). Also for the other compounds, no consistent trends could be observed that would support the hypothesis of counteraction of a membrane fluidizing effect of the compounds. However, unfortunately, the study did not report changes in cyclopropane fatty acids and BCFAs, which are prominent, if not the major, players in membrane homeoviscous adaptation in Gram-negative and Gram-positive bacteria, respectively.

3.5. The role of IlvE in survival of lethal t-CIN concentrations

The effect of antimicrobial compounds can be either bacteriostatic or bactericidal depending on the applied concentration, and the effects do not necessarily rely on the same mode of action. Therefore, the elongated lag phase of the *ilvE* mutant during growth in the presence of t-CIN does not necessarily imply a higher sensitivity of this mutant to bactericidal concentrations of t-CIN. More specifically, we here investigated whether the aberrant membrane fatty acid composition of the *ilvE* mutant would result in a different inactivation

rate for stationary-phase cells in 6 mM t-CIN in potassium phosphate buffer without added nutrients (Fig. 6). Inactivation of both WT, *ilvE* mutant and the genetically complemented mutant (*ilvE::Himar/pIMK2-ilvE*) cells started after 1 h of exposure to t-CIN, but proceeded significantly faster for the mutant. Furthermore, when the mutant was grown in BHI with an equimolar mix (1 mM) of the three α -KA, resistance was restored to WT level. Supplementation with α -KA did not affect the inactivation of WT cells. These results indicate that the faster inactivation of the *ilvE* mutant at lethal t-CIN concentrations, similar to its retarded growth at sublethal t-CIN concentrations, is caused by their altered membrane composition rather than to their inability to synthesize BCAAs. This may lead to increased diffusion of t-CIN through the membrane into the cytoplasm, where it can react with cellular nucleophiles (mainly thiol and amino groups) based on its electrophilic properties, giving rise to DNA damage, malfunctioning enzymes, depletion of acetyl-CoA, and disturbance of redox homeostasis. The increased intracellular damage expected to occur in the mutant can in turn explain its increased sensitivity to lethal as well as to sublethal concentrations of t-CIN. In the latter case, the increased damage will require more time to be repaired, and thus a longer lag phase at sublethal concentrations of t-CIN. However, additional effects may play a role. For example, the altered membrane composition in the mutant may affect the amount of t-CIN that accumulates in the membrane and/or the interaction of t-CIN with membrane proteins. As a result, it could be that the functionality or permeability of the membrane is more sensitive to disturbance by t-CIN in the mutant than in the WT.

In conclusion, the isolation and characterization of an *IlvE* deficient mutant in this work has provided novel information on the production of BCFAs and on the role of membrane composition on t-CIN sensitivity in *L. monocytogenes* Scott A. Although this strain appears to have an intact pathway for the biosynthesis of BCFAs from Krebs cycle intermediates, our experiments indicate that most BCFAs are produced from branched-chain

intermediates (BCAAs, α -KAs, organic acids) acquired from the growth medium, and that only limited amounts of BCFAs (especially the anteiso fraction) can be synthesized when these intermediates are not available or cannot be used, as is the case for BCAAs in the *IlvE* mutant. Further, our results show that *L. monocytogenes* modulates its membrane fatty acid composition in response to sub-MIC concentrations of t-CIN, confirming similar observations for t-CIN and other small hydrophobic α , β -unsaturated carbonyl compounds in a range of bacteria [30, 37, 39]. The observed changes suggest an adaptation to compensate for the fluidizing effect of t-CIN on the membrane. Our work also provides direct evidence that membrane fatty acid composition is a critical determinant of t-CIN sensitivity. Further work will be required to determine whether this sensitivity results from an increased diffusion rate of t-CIN towards the cytosol or from an increased accumulation of t-CIN in the membrane itself.

Conflict of interest

We have no conflict of interest.

Acknowledgments

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Legends to figures

Fig. 1. Organization of *ilvE* region in *L. monocytogenes* Scott A. The *Himar1* transposon (792 bp from the start codon of *ilvE*) is shown as a grey triangle, and the grey arrow indicates the orientation of the erythromycin resistance gene (*ermC*) in the transposon. In the PATRIC database the ORFs are annotated as putative acetyl esterase YjcH (EC 3.1.1.-) (1060), branched-chain amino acid aminotransferase (IlvE, EC 2.6.1.42) (1061), ABC transporter, ATP-binding protein (1062), ABC transporter, permease protein (1063) and drug resistance transporter, EmrB/QacA family (1064).

Fig. 2. Pathway of branched-chain fatty acid (BCFA) synthesis in *L. monocytogenes*. The central precursors are the α -ketoacids α -ketoisocaproate (KIC), α -ketoisovalerate (KIV) and α -ketomethylvalerate (KMV). These can be derived from the corresponding branched-chain amino acids (BCAAs) Leu, Val or Ile by the transaminase IlvE, but *L. monocytogenes* Scott A also has predicted genes for their *de novo* synthesis from pyruvate (KIC and KIV) or from Thr (KMV). Thr itself can be synthesized *de novo* from oxaloacetate. Pathways are inferred from predicted presence of enzymes based on genome sequence of *L. monocytogenes* Scott A.

Fig. 3. (a) Growth curves of *L. monocytogenes* Scott A, its *ilvE::Himar1* mutant and the genetically complemented mutant *ilvE::Himar1/pIMK2-ilvE* in BHI and BHI with 4 mM t-CIN. All curves are the average of three independent cultures. Standard deviations have been omitted for clarity. (b) Lag phases (λ), maximum growth rates ($\mu_{\max}$) and maximum optical densities ($OD_{\max}$) corresponding to the curves in (a). Values are means $\pm$ standard deviation.

Fig. 4. (a) Growth of *L. monocytogenes* Scott A (black) and its *ilvE::Himar1* mutant (grey) in BHI + 4 mM t-CIN. Full, dashed and dotted lines represent cultures without supplements, with 10 mM each of all three BCAAs, and with 10 mM each of all three α -KAs, respectively. (b) Growth of the *ilvE::Himar1* mutant in BHI + 4 mM t-CIN (—), supplemented with 10 mM each of all three α -KAs (---), 10 mM α -ketoisovalerate (KIV,), 10 mM α -ketoisocaproate (KIC, — —) or 10 mM α -ketomethylvalerate (KMV, — •). All curves are the average of three independent experiments. Standard deviations have been omitted for clarity. (c) Lag phases (λ), maximum growth rates ($\mu_{\max}$) and maximum optical densities ($OD_{\max}$) corresponding to the curves in (a) and (b). Values are means $\pm$ standard deviation.

Fig. 5. Growth of *L. monocytogenes* Scott A (black), its *ilvE::Himar1* mutant (full grey line) and the genetically complemented mutant *ilvE::Himar1/pIMK2-ilvE* (dashed grey line) in BHI at 10 °C. The asterisk indicates significant differences with the WT and genetically complemented strain at the same time point.

Fig. 6. Inactivation of WT (black lines) and *ilvE::Himar1* (grey lines) diluted stationary cultures by 6 mM t-CIN in 10 mM potassium phosphate buffer. Solid lines represent cultures grown overnight without supplementation of 1 mM each of all three α -KAs, dashed lines with supplementation. The grey dotted line represents the complemented mutant *ilvE::Himar1/pIMK2-ilvE* without supplementation of α -KAs. The asterisk indicates significant differences with the WT and genetically complemented strain at the same time point.

Table 1. PCR oligonucleotides used for complementation and mutant identification

Category	Name	Sequence ^c (5' - 3')
Complementation	ilvE_NcoI	GCAT <u>CCATGG</u> GAA GTAAAGATATTGACTGGA GTAATTTA GG
	ilvE_SalI	CACT <u>GTCGAC</u> TTATTTCACTTTATAAAATCCAACCTTC
	pIMK_REV	CCTATCACCTCAAATGGTTCG
	pIMK_FW	GAGTCAGTGA GCGA GGAAGC
	NC16 [25]	GTCAAAACATACGCTCTTATC
Y-linker identification	Ylinker [23]	CTGCTCGAATTCAAGCTTCT
	Marq269 [22]	GCTCTGATAAATATGAACATGATGATGAT

^c Restriction sites are underlined: NcoI (CCATGG) and SalI (GTCGAC)

Table 2. Percentage of total whole-cell fatty acids of cultures grown in BHI at 30 °C with and without 2 mM t-CIN. The most notable summarized differences are mentioned at the bottom of the table. Values are means $\pm$ standard deviation from three independent experiments, except for the analysis of the mutant grown in t-CIN where means are from two independent experiments. Exponential cultures were harvested at the same OD_{600nm}

Fatty acid	BHI		BHI		BHI + t-CIN	
	Stationary phase		Exponential phase		Exponential phase	
	Scott A	<i>ilvE::HimarI</i>	Scott A	<i>ilvE::HimarI</i>	Scott A	<i>ilvE::HimarI</i>
C10:0	0.1 $\pm$ 0.1 ^A	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A	0.3 $\pm$ 0.0 ^B
C12:0	1.4 $\pm$ 0.2 ^C	4.1 $\pm$ 0.5 ^B	0.9 $\pm$ 0.0 ^C	1.8 $\pm$ 0.2 ^C	7.0 $\pm$ 1.3 ^A	3.7 $\pm$ 0.2 ^B
Iso-C13:0	0.0 $\pm$ 0.0 ^B	0.4 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^B	0.6 $\pm$ 0.2 ^A	0.0 $\pm$ 0.0 ^B	0.0 $\pm$ 0.0 ^B
Anteiso-C13:0	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A	0.1 $\pm$ 0.1 ^A	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A
Iso-C14:0	0.6 $\pm$ 0.0 ^C	9.9 $\pm$ 1.6 ^B	0.8 $\pm$ 0.0 ^C	19.9 $\pm$ 2.9 ^A	7.5 $\pm$ 0.6 ^B	11.5 $\pm$ 0.0 ^B
C14:1 ω 5c	0.0 $\pm$ 0.0 ^B	0.1 $\pm$ 0.2 ^B	0.0 $\pm$ 0.0 ^B	0.4 $\pm$ 0.4 ^B	2.2 $\pm$ 0.4 ^A	0.4 $\pm$ 0.1 ^B
C14:0	0.5 $\pm$ 0.1 ^E	11.6 $\pm$ 0.8 ^B	1.3 $\pm$ 0.1 ^E	6.4 $\pm$ 0.4 ^C	4.0 $\pm$ 0.7 ^D	18.2 $\pm$ 0.8 ^A
Iso-C15:0	10.9 $\pm$ 0.4 ^{B,C}	17.1 $\pm$ 3.9 ^A	12.6 $\pm$ 0.2 ^{A,B}	12.5 $\pm$ 0.8 ^{A,B}	7.4 $\pm$ 0.2 ^C	1.8 $\pm$ 0.9 ^D
Anteiso-C15:0	29.9 $\pm$ 1.0 ^B	2.6 $\pm$ 0.2 ^E	36.6 $\pm$ 0.2 ^A	7.6 $\pm$ 0.7 ^D	22.4 $\pm$ 1.0 ^C	3.5 $\pm$ 0.6 ^E
Iso-C16:0	2.8 $\pm$ 0.1 ^B	16.7 $\pm$ 1.6 ^A	2.0 $\pm$ 0.1 ^B	16.9 $\pm$ 2.4 ^A	16.0 $\pm$ 1.9 ^A	14.1 $\pm$ 0.2 ^A
C16:0	2.1 $\pm$ 0.1 ^E	27.6 $\pm$ 1.3 ^B	5.5 $\pm$ 0.2 ^D	12.0 $\pm$ 1.1 ^C	10.8 $\pm$ 0.3 ^C	42.8 $\pm$ 0.7 ^A
Iso-C17:0	9.2 $\pm$ 0.5 ^A	5.5 $\pm$ 0.8 ^B	4.8 $\pm$ 0.1 ^B	1.8 $\pm$ 0.1 ^D	3.0 $\pm$ 0.3 ^C	0.7 $\pm$ 0.2 ^D
Anteiso-C17:0	41.0 $\pm$ 0.9 ^A	1.4 $\pm$ 0.2 ^D	26.8 $\pm$ 0.4 ^B	1.9 $\pm$ 0.5 ^D	10.5 $\pm$ 0.4 ^C	1.8 $\pm$ 0.6 ^D
C18:1 ω 9c	0.2 $\pm$ 0.2 ^D	0.3 $\pm$ 0.4 ^D	6.3 $\pm$ 0.3 ^B	9.8 $\pm$ 2.0 ^A	3.4 $\pm$ 0.6 ^C	0.0 $\pm$ 0.0 ^D
C18:1 ω 6c	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A	0.6 $\pm$ 0.8 ^A	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A
C18:0	0.5 $\pm$ 0.1 ^D	2.0 $\pm$ 0.2 ^{B,C}	1.0 $\pm$ 0.1 ^D	3.5 $\pm$ 0.6 ^A	2.8 $\pm$ 0.3 ^{A,B}	1.1 $\pm$ 0.3 ^{C,D}
Anteiso-C19:0	0.6 $\pm$ 0.1 ^A	0.0 $\pm$ 0.0 ^B	0.0 $\pm$ 0.0 ^B	0.0 $\pm$ 0.0 ^B	0.0 $\pm$ 0.0 ^B	0.0 $\pm$ 0.0 ^B
Iso-C17:1 ω 5c	0.0 $\pm$ 0.0 ^A	0.2 $\pm$ 0.3 ^A	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A
C18:1 ω 7c	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A	0.5 $\pm$ 0.8 ^A	0.0 $\pm$ 0.0 ^A	0.0 $\pm$ 0.0 ^A
BCFAs	95.0 $\pm$ 1.2	53.7 $\pm$ 3.8	83.5 $\pm$ 0.4	61.0 $\pm$ 3.2	66.8 $\pm$ 1.9	33.3 $\pm$ 0.9
Anteiso-BCFAs	71.5 $\pm$ 1.1	4.1 $\pm$ 0.2	63.4 $\pm$ 0.3	9.5 $\pm$ 0.8	32.9 $\pm$ 0.8	5.3 $\pm$ 0.6
Iso-BCFAs	23.5 $\pm$ 0.5	49.6 $\pm$ 3.7	20.1 $\pm$ 0.2	51.5 $\pm$ 3.1	33.9 $\pm$ 1.7	28.0 $\pm$ 0.7
Iso/Anteiso	0.3	12.2	0.3	5.4	1.0	5.3
CA <15	2.6 $\pm$ 0.2	26.1 $\pm$ 1.5	2.9 $\pm$ 0.1	29.1 $\pm$ 2.4	20.7 $\pm$ 1.3	34.0 $\pm$ 0.6
Weighted mean CA*	15.1	14.7	14.8	14.2	14.3	14.9
Weighted mean B-CA*	15.1	14.4	14.8	14.1	14.5	14.2
Weighted mean UB-CA*	14.8	15.2	16.6	16.2	15.1	15.2

^{A,B,C,D,E} Different superscripts indicate significant differences (p -value < 0.05); * CA = carbon atoms; B = branched-chain; UB = unbranched-chain

Fig. 1

ACCEPTED MANUSCRIPT

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6