**ORIGINAL PAPER**

# Effect of high levels of $CO_2$ and $O_2$ on membrane fatty acid profile and membrane physiology of meat spoilage bacteria

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## Abstract

The membrane is the major protective barrier separating the cell from the environment and is thus important for bacteria to survive environmental stress. This study investigates changes in membrane lipid compositions and membrane physiology of meat spoiling bacteria in response to high $CO_2$ (30%) and $O_2$ (70%) concentrations, as commonly used for modified atmosphere packaging of meat. Therefore, the fatty acid profile as well as membrane fluidity, permeability and cell surface were determined and correlated to the genomic settings of five meat spoiling bacteria *Brochothrix (B.) thermosphacta*, *Carnobacterium (C.) divergens*, *C. maltaromaticum*, *Leuconostoc (L.) gelidum* subsp. *gelidum* and *L. gelidum* subsp. *gasicomitatum* cultivated under different gas atmospheres. We identified different genomic potentials for fatty acid adaptations, which were in accordance with actual measured changes in the fatty acid composition for each species in response to $CO_2$ and/or $O_2$, e.g., an increase in saturated, *iso* and cyclopropane fatty acids. Even though fatty acid changes were species-specific, the general physiological responses were similar, comprising a decreased membrane permeability and fluidity. Thus, we concluded that meat spoiling bacteria facilitate a change in membrane fatty acids upon exposure to $O_2$ and $CO_2$, what leads to alteration of membrane fluidity and permeability. The observed adaptations might contribute to the resistance of meat spoilers against detrimental effects of the gases $O_2$ and $CO_2$ and thus help to explain their ability to grow under different modified atmospheres. Furthermore, this study provides fundamental knowledge regarding the impact of fatty acid changes on important membrane properties of bacteria.

**Keywords** Fatty acid composition · Membrane fluidity · Membrane permeability · Modified atmosphere · GC–MS · Fluorescence assays

## Introduction

Fresh meats are packaged under an artificial atmosphere to decelerate bacterially induced spoilage. This technique, called modified atmosphere packaging (MAP), is mainly based on the protective action of the gases oxygen and carbon dioxide, which generally suppress bacterial growth and thus prolong the shelf life of meat [1–4].

The harmful effect of oxygen and carbon dioxide on bacterial cells can be attributed to the fact that these molecules are rather non-polar and small, enabling them to easily penetrate the cell membrane by simple diffusion [5]. The mechanistic way of action of carbon dioxide within the cell has not been examined in detail yet, but it is supposed to decrease the intracellular pH and thus lead to essential loss of function of enzymes [6–9]. Furthermore, a mass action effect of carbon dioxide has been supposed, as specific enzymes such as the iso-citrate and malate dehydrogenase as well as decarboxylases, e.g. the phosphoenolpyruvate decarboxylase were inhibited due to high concentrations of carbon dioxide [6, 10, 11]. This can result in energetically decoupling, a phenomenon called futile cycle. Contrary, high oxygen concentrations are known to induce production of reactive oxygen species (ROS) within the cell, resulting in DNA, protein and lipid damage [12–14]. Thus, it can be hypothesized that survival of bacteria under these conditions is linked to their

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ability to adjust the biophysical properties of their membrane to enhance impermeability of these gases.

Membrane fluidity and permeability are two main properties of the membrane, which can be regulated by the cell. Both properties are mainly depending on the content of distinct fatty acids in the cell membrane [15]. Fatty acids differ according to the carbon chain length (long and short chain fatty acids), the interconnection of carbon atoms (saturated and unsaturated fatty acids (SFA and UFA)), the steric conformation of double bonds (*cis* and *trans*-UFAs) and the location of methyl groups (*iso* and *anteiso*-branched fatty acids) [15]. By modifying the structures of existing fatty acids as well as regulating biosynthesis of new fatty acids, bacteria are capable to alter physiological membrane properties resulting in regulation of transport processes, protein–protein interactions and passive permeability of hydrophobic molecules [15, 16]. Nevertheless, little is known regarding changes in the fatty acid profile providing lower permeability of the gases $O_2$ and $CO_2$ as used upon MAP.

In our previous full proteomic study targeting to investigate the effect of MAP on the metabolism of meat spoilage bacteria [9], we had also found certain proteins of e.g. biosynthesis of distinct fatty acids to be upregulated. Driven by this indication, the present study aimed to investigate if gases applied in modified atmosphere packaging influence bacterial fatty acid composition and to study the impact of these changes on physiological properties of the membrane.

## Materials and methods

### Bacterial strains and cultivation

Five major meat spoilage bacteria were selected in this study. One representative strain of each species was chosen from the TMW strain collection, according to its dominant abundance on high-oxygen packaged meat. *L. gelidum* subsp. *gelidum* TMW2.1618 and *L. gelidum* subsp. *gasicomitatum* TMW2.1619 where isolated from beef steaks by Hilgarth et al. [17], *C. divergens* TMW2.1577 and *C. maltaromaticum* TMW2.1581 from skinless chicken breast by Höll et al. [18] and *B. thermosphacta* TMW2.2101 from minced beef by Hilgarth et al. [19].

### Comparative genome analysis

To evaluate bacteria's potential to change their fatty acid profile in response to carbon dioxide and high oxygen exposure, a genomic prediction of all genes needed for biosynthesis, conversion and degradation of fatty acids was performed. Genomes of the five species haven been published in a previous study [20] and are available on NCBI with the accession numbers: CP017196 (*L. gelidum* subsp.

*gelidum* TMW2.1618), CP017197 (*L. gelidum* subsp. *gasicomitatum* TMW2.1619), RSDV000000001 (*C. divergens* TMW2.1577), CP016844 (*C. maltaromaticum* TMW2.1581) and RSDU000000001 (*B. thermosphacta* TMW2.2101). All genes listed were additionally blasted manually to ensure correct annotation.

### Experimental setup

Bacterial cultivation was performed in gas tight glass bottles filled with 0.4 L of a specifically developed meat simulation medium (MSM). The composition and species specific adaptation of the media is described in detail in a previous study by Kolbeck et al. [21]. Briefly, main components of MSM comprise meat extract, glycerol, tween80 and heminchlorid. Inoculation of bacteria in MSM was performed with a defined optical density ($OD_{600\,nm}=0.01$). During cultivation time (48 h), MSM was aerated constantly with one of the following atmospheres, atmosphere1: $21\%\_O_2/0\%\_CO_2/79\%\_N_2$ (air), atmosphere2: $21\%\_O_2/30\%\_CO_2/49\%\_N_2$, atmosphere3: $70\%\_O_2/30\%\_CO_2/0\%\_N_2$. Atmosphere 3 represents the composition of commonly used high-oxygen MAP, while atmosphere 1 (air) acted as a control and atmosphere 2 was selected to study the effect of $30\% CO_2$ as employed in MAP while retaining the oxygen concentration of air. By performing a pairwise comparison of the amount of each fatty acid identified in atmosphere 1 *vs.* atmosphere 2, we were able to uncover the effect of $30\% CO_2$ on the fatty acid profile. The effect of $70\% O_2$ was uncovered by comparing atmosphere 2 and atmosphere 3. Biological replicates were performed for each strain and gas mixture in three independent culture bottles. MSM was stirred constantly and temperature was set to $25\ °C \pm 2\ °C$. Bacteria were harvested at exponential growth phase for fatty acid analysis and physiological cell membrane assays.

### Measurement of fatty acids

Harvested cells were frozen at -80 °C and freeze dried. The dry weight of each sample was determined afterwards. To detect losses during sample preparation, glyceryl trinonanoate was added as an internal standard. Lipid extraction was performed as described by Santivarangkna et al. [22]. Methylation of fatty acids was done by dissolving the extracted fat in 200 µl tetrahydrofuran (THF) and 400 µl of $0.5\ mol\ l^{-1}$ sodium methylate (in Methanol). Samples were incubated for 15 min at 80 °C. Afterwards, 200 µl water and 200 µl hexane was added. Finally, the hexane layer was transferred into a vial for analysis.

Samples were determined using a gas chromatograph coupled to a mass spectrometer (GCMS-TQ8040, Shimadzu) with a split of 10 and injection volume of 1 µl. Sample separation was performed on a Zebron

ZB-5MSplus column (30 m × 0.25 mm × 0.25 μm) from Phenomenex. Helium was chosen as a carrier gas. The following temperature gradient was run: 35 °C for 2 min, a rise of 10 °C min$^{-1}$ until 140 °C, 140 °C for 10 min, a rise of 2 °C min$^{-1}$ until 240 °C and finally 240 °C for 10.5 min. Data analyzation was performed with the software LabSolution—GCMSsolution (Version 4.30, Shimadzu). For fatty acid identification, the bacterial acid methyl ester standard (BAME Mix, Sigma-Aldrich, St. Louis, Missouri) as well as a mixture of fatty acid methyl esters (Supelco 37 FAME mix, Supelco, Bellefonte, PA, USA) was used. Furthermore, peak identification was confirmed by running a mass spectra search against the Nist11 database. Quantification was done by correlating the peak area of each fatty acid to the peak area of the used internal standard (C9:0) and its known concentration. Afterwards, total amounts of fatty acids were calculated per gram of cell mass and finally expressed in percentage of total fatty acids per single cell. All further calculations are based on this values which were calculated for all species in replicates under the three gas atmospheres.

## Measurement of membrane fluidity

Membrane fluidity of cells was determined using the fluorescence dye Laurdan (6-Dodecanoyl-2-Dimethylaminonaphthalene). Sample were prepared as described by Behr et al. [23]. A LS 50B luminescence spectrometer (Perkin-Elmer, Rodgau-Jügesheim, Germany) was used for measuring of dyed samples (excitation wavelength 360 nm, emission wavelengths 380 to 550 nm, 5 nm steps). For comparing the membrane fluidity of a strain grown at different gas atmospheres, the general polarization (GP), defined as

$$GP = \frac{I_{440\,nm} - I_{490\,nm}}{I_{440\,nm} + I_{490\,nm}}$$

was calculated for each replicate, where $I_{440}$ is the relative fluorescence at 440 nm and $I_{490}$ is the relative fluorescence at 490 nm [24]. To compare calculated GP values between different samples, the general polarization was calculated per cell. Therefore, GP values where divided by the viable cell count (CFU) of each sample, which was determined after fluorescence measurement.

## Measurement of membrane permeability

A fluorescence assay using the dye propidium iodide (PI; Sigma-Aldrich) was performed to detect changes in membrane permeability [25]. Samples were prepared as described by Klotz et al. [26]. In detail, cells suspensions were adjusted to an $OD_{600} = 1.0$, washed with PBS, mixed with PI to a final concentration of 2.9 μM, incubated for 10 min at room temperature and washed in PBS. Fluorescence was determined at an excitation wavelength of 544 nm and an emission wavelength of 620 nm with a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Afterwards, CFU was determined for each sample, and determined fluorescence intensity values where calculated per cell to ensure proper comparisons between different samples.

## Measurement of cell surface

Cell sizes were determined of 50 cells from exponential growth phase per replicate by an Axiostar plus microscope (Zeiss, Jena, Germany) according to Sun, Liu [27]. The surface of the spheric-shaped *Leuconostoc gelidum* bacteria were calculated based on the following mode (shape code 1-H):

$$\text{Surface}(A_{\text{sphere}}) = \pi \times a^2.$$

with a representing the diameter of the sphere. The surface of the rod-shaped bacteria *B. thermosphacta*, *C. divergens* and *C. maltaromaticum* were calculated based on the models (shape code 5-H):

$$\text{Surface}(A_{\text{rod}}) = \pi \times a \times b$$

with a representing the length and b the wide of the rod-shaped bacteria.

## Statistical data and growth curve analysis

Data of fatty acid analysis and physiological assays (fluidity, permeability) were performed in three independent replicates and checked for statistical significance by conducting a variance and T-test (double side confidence interval 95%). Significant differences between variances or means where defined as $p < 0.05$. Microscopy analyses were performed in 50 replicates per strain and gas. Statistically significant differences of microscopy data where analyzed like described above. Growth curves were analyzed using the open source software RStudio ver. 3.3.0 (RStudio, Inc., Boston, MA, United States) running the CRAN package grofit ver. 1.1.1–1 with default settings. This package calculates the lag-Phase, maximal optical density ($OD_{max}$) and maximal growth rate ($\mu_{max}$) for each replicate by applying a fitting model to the data. Significant differences of these parameters between the three replicates and gas mixtures were calculated for each strain by performing an one-way analysis of variances (ANOVA). A post hoc Tukey test was connected to the ANOVA, assigning significant differences between means by applying a confidence interval of 95% ($p = 0.05$).

# Results

## Comparative genomic analysis for prediction of fatty acid metabolism

Bacterial genomes were analyzed focusing on the presence and absence of all genes needed to establish a membrane associated pathway using the database KEGG and own literature research. All bacteria in this study were able to predictively perform fatty acid *de-novo* biosynthesis and fatty acid membrane incorporation (Table 1). Regarding conversion of existing fatty acids, both *L. gelidum* subspecies and *C. divergens* TMW2.1577 are predictively able to build cyclopropane fatty acids as they exhibit the cyclopropane fatty acid synthase gene (*Cfas*). Direct fatty acid analysis also revealed the production of the corresponding fatty acid (*C19:0 delta*) for this species. Although, conjugated and unsaturated fatty acids could also be directly determined for all species (e.g., *18:2 conj.*), no genes for unsaturation or conjugation of single steps leading to conversion of those fatty acids could be found in the five genomes. Genes facilitating degradation of short chain fatty acids were present in the genomes of all bacteria, whereas genes facilitating degradation of saturated medium and long chain fatty acid were only found in the genomes of both *Carnobacterium* species and *B. thermosphacta* TMW2.2101. Furthermore, both *Carnobacterium* spp. can predictively degrade polyunsaturated fatty acids. A detailed list of enzymes needed for membrane lipid metabolism is given in Table S1.

## Revealing the effects of carbon dioxide and high oxygen by comparison of different gas compositions

The aim of this study was to uncover the effects of carbon dioxide and high oxygen on bacterial cell membrane properties. Therefore, bacteria were grown under atmosphere1: $21\%\_O_2/0\%\_CO_2/79\%\_N_2$ (air), atmosphere2: $21\%\_O_2/30\%\_CO_2/49\%\_N_2$ and atmosphere3: $70\%\_O_2/30\%\_CO_2/0\%\_N_2$. This experimental approach allowed uncovering the effects of carbon dioxide by comparing results obtained from atmosphere1 and atmosphere2 and

**Table 1** Summary of metabolic pathways needed to build up and maintain a physiological active cell membrane

| | Genes and Gen clusters | *B. thermosphacta* TMW2.2101 | *C. divergens* TMW2.1577 | *C. maltaromaticum* TMW2.1581 | *L. gelidum* subsp. *gelidum* TMW2.1618 | *L. gelidum* subsp. *gasicomitatum* TMW2.1619 |
|---|---|---|---|---|---|---|
| Fatty acid de-novo biosynthesis | | | | | | |
| SFA and UFA biosynthesis by FASII | *Acc, Fab cluster* | ✓ | ✓ | ✓ | (✓) | (✓) |
| Branched chain fatty acid biosynthesis | *BCKDC, Ilv cluster, Leu cluster* | ✓ | ✓ | ✓ | (✓) | (✓) |
| Exogenous fatty acid incorporation | *FakA, FakB* | ✓ | ✓ | ✓ | ✓ | ✓ |
| Fatty acid incorporation into membrane | *PlsX, PlsY* | ✓ | ✓ | ✓ | ✓ | ✓ |
| Regulation of fatty acid biosynthesis | *SpoT, FabR, FabR* | ✓ | ✓ | ✓ | ✓ | ✓ |
| Conversion of existing fatty acids | | | | | | |
| Cyclisation of fatty acids | *Cfas* | – | ✓ | – | ✓ | ✓ |
| *Cis/Trans* isomerization of fatty acids | *Cti* | – | – | – | – | – |
| Conjugation of fatty acids | *Cla cluster* | – | – | – | – | – |
| Desaturation of fatty acids | *DesA, DesB* | – | – | – | – | – |
| Fatty acid degradation | | | | | | |
| Saturated medium and long chain fatty acid degradation (beta-oxidation) | *Fad cluster* | (✓) | ✓ | ✓ | – | – |
| Saturated short chain fatty acid degradation | *Ato cluster* | ✓ | ✓ | ✓ | ✓ | (✓) |
| Polyunsaturated fatty acid degradation | *FadH, FadB* | – | ✓ | ✓ | – | – |

All data are based on genomic analysis (NCBI annotation and manual blast search). ✓ = whole metabolic pathway found in the genome, (✓) = single genes of the pathway could not be found, nevertheless the pathway is considered to be present. – = pathway is not present in the genome

**Table 2** Atmospheres used for the study and comparisons chosen to define the effect of carbon dioxide and high oxygen on the fatty acid composition and growth of each species

| Nr | Atmospheres | Atmosphere comparisons/Read out |
|----|-------------|---------------------------------|
| 1 | $21\%\_O_2/0\%\_CO_2/79\%\_N_2$ (air) | **1–2**/Effect of 30% carbon dioxide |
| 2 | $21\%\_O_2/30\%\_CO_2/49\%\_N_2$ | **2–3**/Effect of 70% high oxygen |
| 3 | $70\%\_O_2/30\%\_CO_2/0\%\_N_2$ | |

the effects of high oxygen comparing results obtained from atmosphere2 and atmosphere3 (Table 2). Subsequently, all results obtained from different experiments where analyzed in this comparative manner and all differences stated are statistically significant.

## Cell growth

Growth of bacteria in MSM under the different atmospheres was monitored over 48 h by measuring their optical density (Figure S1). Furthermore, growth analyses were performed, calculating the lag-Phase, division rate ($\mu_{max}$) and maximal OD for each replicate and atmosphere (Table S2).

Effect of $CO_2$ (0% compared to 30%): A significant prolongation of the lag-phase could be determined for *L. gelidum* subsp. *gelidum* TMW2.1618, *C. maltaromaticum* TMW2.1581, *C. divergens* TMW2.1577 and *B. thermosphacta* TMW2.2101 but not for *L. gelidum* subsp. *gasicomitatum* TMW2.1619. The maximal OD was not affected by carbon dioxide for any species. We further detected an increase in the growth rate of *C. maltaromaticum* TMW2.1581 and *L. gelidum* subsp. *gelidum* TMW2.1618. The other species did not exhibit changes in their growth rate.

Effect of high $O_2$ (21% compared to 70%): *B. thermosphacta* TMW2.2101 was the only species exhibiting a shortened lag phase due to high oxygen exposure. We further detected a decrease in maximal OD for *L. gelidum* subsp. *gasicomitatum* TMW2.1619 and an increase for *C. maltaromaticum* TMW2.1581. The other species were not affected in their maximal OD. Both *L. gelidum* subspecies exhibit a significant lower growth rate due to high oxygen exposure, whereas the growth rate of *C. maltaromaticum* TMW2.1581 was increased.

## Changes of the fatty acid composition and predicted effect on membrane permeability and fluidity

The fatty acid composition of each species grown under the three atmospheres was analyzed and quantified in triplicates. The effects of carbon dioxide and high oxygen on single fatty acid regulation are listed in Table S3. An overview of the

effects of carbon dioxide and high oxygen on fatty acids with similar properties e.g. chain length, branched methyl groups or double bounds is to be seen in Table 3. The predicted effect of those fatty acid changes on the membrane properties fluidity and permeability are summarized in Table 4 and marked by "FA".

Effect of $CO_2$ (0% compared to 30%): We determined an increase in *iso-* and short chain fatty acids and a decrease in long chain fatty acids for *B. thermosphacta* TMW2.2101, which is predicted to result in a decrease of the membrane fluidity. *C. divergens* TMW2.1577 and *L. gelidum* subsp. *gelidum* TMW2.1618 exhibit an increase in saturated and a decrease in unsaturated fatty acids, predictively leading to a less fluid and permeable cell membrane. Similar was also detected for *L. gelidum* subsp. *gasicomitatum* TMW2.1619, who exhibit a decrease in *iso-*, long chain and unsaturated fatty acids as well as an increase in short chain, saturated and total fatty acids, predictively resulting in a decreased membrane fluidity and permeability.

Effect of high $O_2$ (21% compared to 70%): *B. thermosphacta* TMW2.2101 exhibits an increase in *iso-* and unbranched fatty acids and a decrease in *anteiso-* and branched fatty acids, predictively resulting in a reduced membrane fluidity. Contrary, *C. divergens* TMW2.1577 exhibits an increase in unsaturated, branched and total fatty acids and a decrease in saturated and unbranched fatty acids, leading to an increase in membrane fluidity and permeablity. We further determined an increase in total fatty acids for *C. maltaromaticum* TMW2.1581, which is predictively decreasing the membrane fluidity. *L. gelidum* subsp. *gelidum* TMW2.1618 increases its long chain and concomitantly decrease its short chain fatty acids under oxygen exposure, having no predicted effect on membrane fluidity or permeability. At least, we determined a slight decrease in *iso-*fatty acids for *L. gelidum* subsp. *gasicomitatum* TMW2.1619, presumably leading to an increase in membrane fluidity.

## Measured membrane fluidity, permeability and cell surface

Physiological assays were performed for each species by measuring the effect of carbon dioxide and high oxygen on membrane fluidity (Fig. 1) and membrane permeability (Fig. 2). Furthermore, the cell surface was determined for all species cultivated under carbon dioxide and oxygen conditions (Fig. 3). Cell surfaces sizes where significantly different for most species and gases but absolute values do not exhibit strong differences (less than twofold regulation). Thus, determined surface values were only taken into account for interpretation of species significantly changing their total fatty acid content, e.g. an increase in total fatty acid content concomitant with an increase in cell surface does not affect cell membrane properties, whereas an

**Table 3** Effect of the gases carbon dioxide and high oxygen on the fatty acid composition of the five meat bacteria *B. thermosphacta* TMW2.2101, *C. divergens* TMW2.1577, *C. maltaromaticum* TMW2.1581, *L. gelidum* subsp. *gelidum* TMW2.1618 and *L. gelidum* subsp. *gasicomitatum* TMW2.1619

| | *B. thermosphacta* TMW2.2101 | *C. divergens* TMW2.1577 | *C. maltaromaticum* TMW2.1581 | *L. gelidum* subsp. *gelidum* TMW2.1618 | *L. gelidum* subsp. *gasicomitatum* TMW2.1619 | *B. thermosphacta* TMW2.2101 | *C. divergens* TMW2.1577 | *C. maltaromaticum* TMW2.1581 | *L. gelidum* subsp. *gelidum* TMW2.1618 | *L. gelidum* subsp. *gasicomitatum* TMW2.1619 |
|---|---|---|---|---|---|---|---|---|---|---|
| | x-fold upregulation by $CO_2$ (30%) | | | | | x-fold upregulation by high $O_2$ (70%) | | | | |
| Iso | **1.18**\* | 0.24 | 1.07 | 1.09 | **0.45**\* | **1.20**\* | 3.25 | 1.51 | 0.72 | **0.67**\* |
| Anteiso | 0.96 | 0.16 | 1.10 | 1.18 | 1.23 | **0.83**\* | 4.93 | 1.27 | 0.61 | 0.70 |
| Long chain | **0.63**\* | 0.90 | 1.00 | 1.00 | **0.81**\* | 0.96 | 1.03 | 1.03 | **1.03**\* | 1.02 |
| Short chain (< C16) | **1.13**\* | 1.14 | 1.01 | 0.99 | **1.74**\* | 1.01 | 0.96 | 0.91 | **0.93**\* | 0.97 |
| Saturated | 1.02 | **1.28**\* | 0.97 | **1.11**\* | **2.08**\* | 0.98 | **0.78**\* | 0.99 | 1.05 | 0.98 |
| Unsaturated | 0.85 | **0.87**\* | 1.01 | **0.89**\* | **0.67**\* | 1.16 | **1.15**\* | 1.00 | 0.94 | 1.02 |
| Branched | 1.02 | 0.93 | 1.06 | 0.97 | 1.56 | **0.95**\* | **1.23**\* | 1.09 | 1.01 | 0.96 |
| Unbranched | 0.92 | 1.02 | 0.99 | 1.00 | 0.95 | **1.19**\* | **0.95**\* | 1.00 | 1.00 | 1.01 |
| Total Fatty acid content [µg FA/g cell] | 0.77 | 1.23 | 0.83 | 0.75 | **1.31**\* | 1.13 | **1.29**\* | **1.42**\* | 1.16 | 0.98 |

X-fold upregulated by $CO_2$; Differences in regulation of fatty acids between atmosphere1 and atmosphere2. X-fold upregulated by high $O_2$; Differences in regulation of fatty acids between atmosphere2 and atmosphere3. \*Significant different regulations between gas atmospheres were identified by a two-sided *T*-test with $p < 0.05$ (highlighted in bold)

**Table 4** Summary, highlighting the effects of carbon dioxide and high oxygen on the physiological properties membrane fluidity and permeability as well as cell surface and the total fatty acid content of the species *B. thermosphacta* TMW2.2101, *C. divergens* TMW2.1577, *C. maltaromaticum* TMW2.1581, *L. gelidum* subsp. *gelidum* TMW2.1618 and *L. gelidum* subsp. *gasicomitatum* TMW2.1619

| | Membrane fluidity | Membrane permeability | Cell surface | Total fatty acid content | Membrane fluidity | Membrane permeability | Cell surface | Total fatty acid content |
|---|---|---|---|---|---|---|---|---|
| | Effect of $CO_2$ (30%) | | | | Effect of high $O_2$ (70%) | | | |
| *B. thermosphacta* TMW2.2101 | ⇩ FA | | ⇧ M | | ⇩ FA | | ⇩ M | |
| *C. divergens* TMW2.1577 | ⇩ FA | ⇩ FA | ⇩ M | | ⇧ FA | ⇧ FA | ⇧ M | ⇧ M |
| *C. maltaromaticum* TMW2.1581 | ⇩ M" | | ⇧ M | | ⇩ FA/M | ⇧ M | ⇩ M | ⇧ M |
| *L. gelidum* subsp. *gelidum* TMW2.1618 | ⇩ FA | ⇩ FA | | | | | ⇧ M | |
| *L. gelidum* subsp. *gasicomitatum* TMW2.1619 | ⇩ FA | ⇩ FA | ⇧ M | ⇧ M | ⇧ FA | ⇧ M | | |

Arrows indicate a significant rise or decrease of the analyzed property of the cell based on a two-sided *T*-test with $p < 0.05$, M = conclusion drawn out of measurements (physiological assays), FA = conclusion drawn out of fatty acid analysis
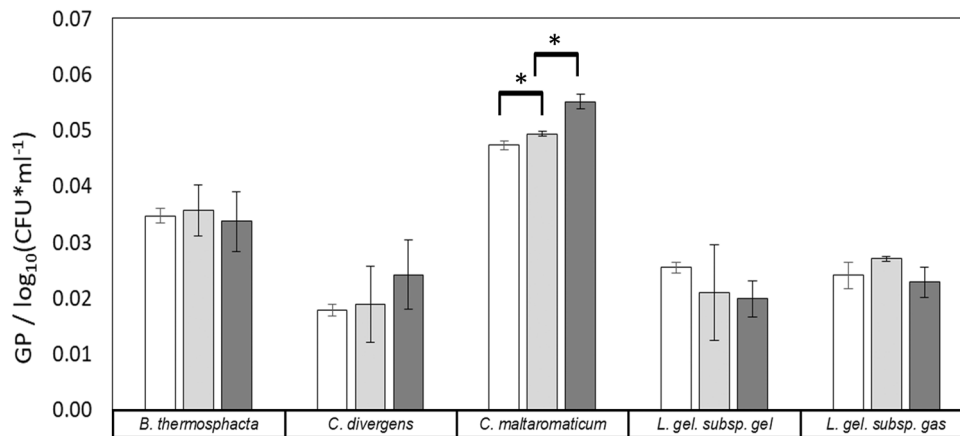


**Fig. 1** Measurement of the cell membrane fluidity of the five meat bacteria *B. thermosphacta* TMW2.2101, *C. divergens* TMW2.1577, *C. maltaromaticum* TMW2.1581, *L. gelidum* subsp. *gelidum* TMW2.1618 and *L. gelidum* subsp. *gasicomitatum* TMW2.1619 under the following atmospheres: (white square) atmosphere1: 21%_$O_2$/0%_$CO_2$/79%_$N_2$ (air); (light grey square) atmosphere2: 21%_$O_2$/30%_$CO_2$/49%_$N_2$; (dark grey square) atmosphere3: 70%_$O_2$/30%_$CO_2$/0%_$N_2$. Bars represent mean values of three independent experiments, error bars represent standard deviations. * indicate significant differences between gas atmospheres identified by a two-sided *T*-test with $p < 0.05$

increase in total fatty acids concomitant with an unchanged or even reduced cell surface implies a tighter and denser packaged cell membrane with reduced fluidity and permeability. Results from the physiological assays are also listed in Table 4 and marked by an "M".

Effect of $CO_2$ (0% compared to 30%): *B. thermosphacta* TMW2.2101 exhibits an increase in cell surface, but was not affected in its total fatty acid amount, thus having no effect on membrane fluidity or permeability. Similarly, *C. divergens* TMW2.1577 exhibited a decrease in cell surface without any regulation in total fatty acids. We measured a significant decrease in membrane fluidity for *C. maltaromaticum*

TMW2.1581 due to $CO_2$ exposition as well as an increase in cell surface which does not affect membrane properties, as no regulation of total fatty acids could be determined. Furthermore, we measured an increase in the cell surface as well as total fatty acid for *L. gelidum* subsp. *gasicomitatum* TMW2.1619, what in combination is predictively not affecting the membrane fluidity.

Effect of high $O_2$ (21% compared to 70%): We determined a decrease in cell surface for *B. thermosphacta* TMW2.2101, which does not affect membrane properties, as no changes in total fatty acids could be detected. *C. divergens* TMW2.1577 exhibits an increase in cell
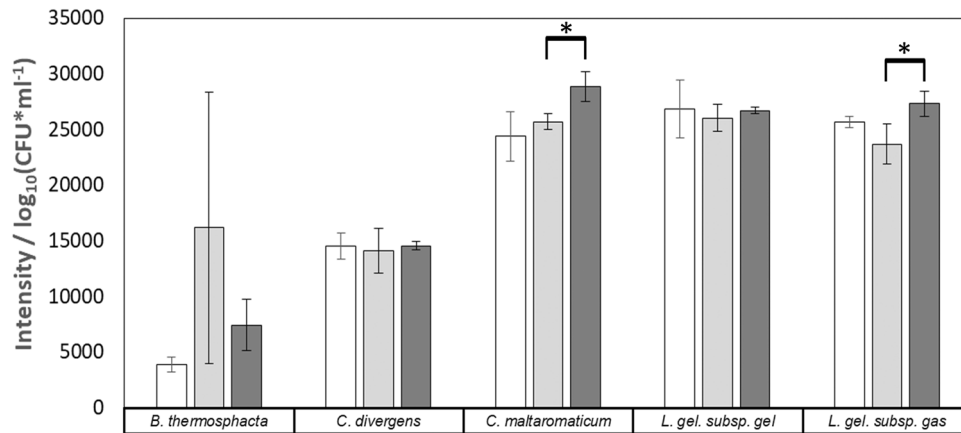
**Fig. 2** Measurement of the cell membrane permeability of the five meat bacteria *B. thermosphacta* TMW2.2101, *C. divergens* TMW2.1577, *C. maltaromaticum* TMW2.1581, *L. gelidum* subsp. *gelidum* TMW2.1618 and *L. gelidum* subsp. *gasicomitatum* TMW2.1619 under the following atmospheres: (white square) atmosphere1: $21\%\_O_2/0\%\_CO_2/79\%\_N_2$ (air); (light grey square) atmos- phere2: $21\%\_O_2/30\%\_CO_2/49\%\_N_2$; (dark grey square) atmosphere3: $70\%\_O_2/30\%\_CO_2/0\%\_N_2$. Bars represent mean values of three inde- pendent experiments, error bars represent standard deviations. * indi- cate significant differences between gas atmospheres identified by a two-sided *T*-test with $p < 0.05$

**Fig. 3** Determination of cell surface of *B. thermosphacta* TMW2.2101, *C. divergens* TMW2.1577, *C. maltaromaticum* TMW2.1581, *L. gelidum* subsp. *gelidum* TMW2.1618 and *L. gelidum* subsp. *gasicomitatum* TMW2.1619 grown under the different atmospheres: (white square) atmosphere1: $21\%\_O_2/0\%\_CO_2/79\%\_N_2$ (air); (light grey square) atmosphere2: $21\%\_O_2/30\%\_CO_2/49\%\_N_2$; (dark grey square) atmosphere3: $70\%\_O_2/30\%\_CO_2/0\%\_N_2$. Boxes are based on 50 deter- mined values. * indicate sig- nificant differences between gas atmospheres identified by a two- sided *T*-test with $p < 0.05$. Black diamonds represent outlier of the analysis
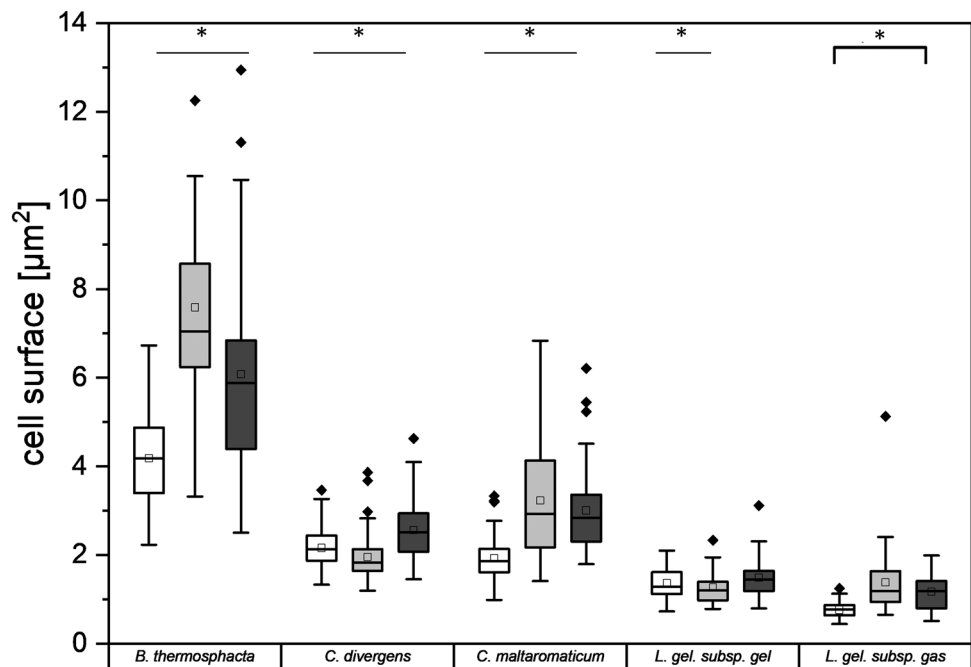


surface and total fatty acids, both in combination also not affecting membrane properties. Contrary, we detected a significant decrease in cell surface and increase in total fatty acids for *C. maltaromaticum* TMW2.1581, predic- tively resulting in a decrease of membrane fluidity, which was further confirmed by a measured decrease in mem- brane fluidity due to high oxygen exposure for this species. Furthermore, *C. maltaromaticum* TMW2.1581 exhibits a measurable increase in membrane permeability under high

oxygen exposure. *L. gelidum* subsp. *gelidum* TMW2.1618 exhibits an increase in cell surface with no change in total fatty acids, thus not affecting membrane properties, while *L. gelidum* subsp. *gasicomitatum* TMW2.1619 exhibits a measured increase in permeability under high oxygen conditions.

Figure 4 summarizes the major findings of this paper comprising the gas-depending shifts in the fatty acid pro- file and their effect on physiological membrane properties.
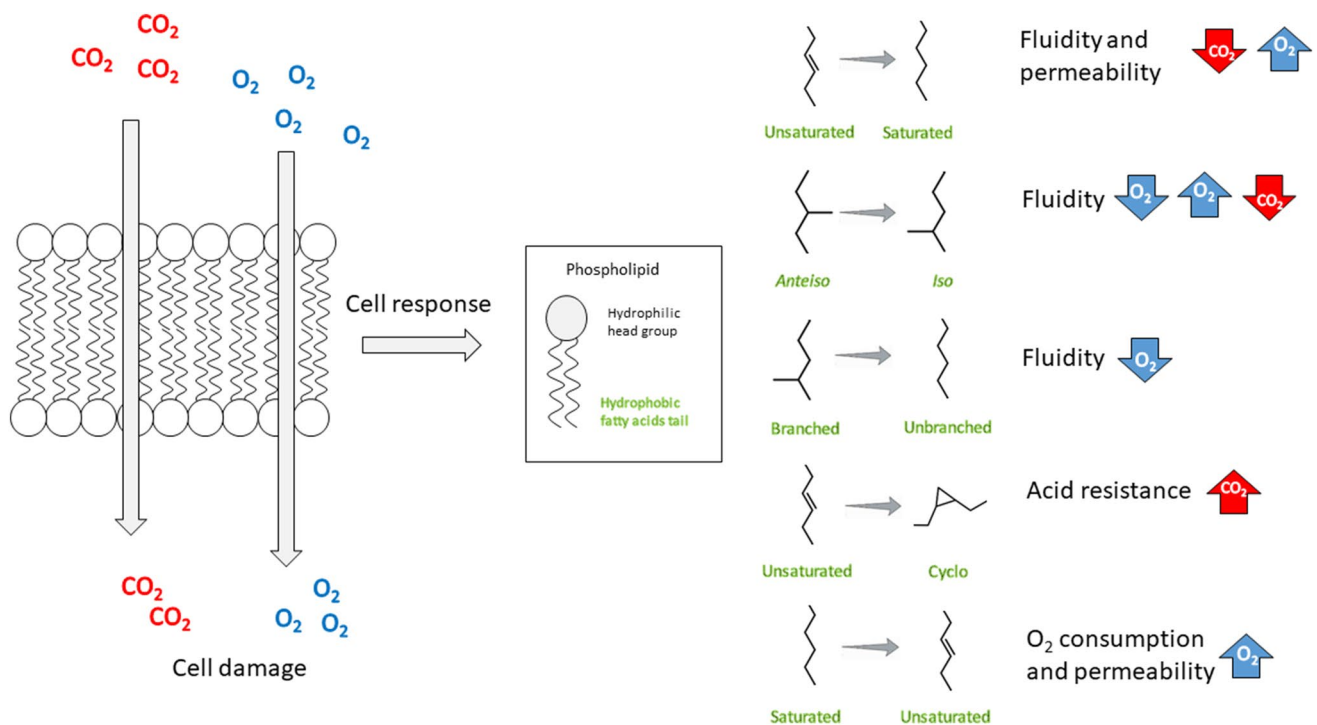
**Fig. 4** Graphical summary of the cell response to high oxygen (70%) or carbon dioxide (30%) exposure regarding fatty acid and physiological membrane changes. A shift in the fatty acid composition does influence the physiological membrane behaviour, e.g., fluidity and permeability. Arrows indicate if those membrane properties are increased or decreased in response to oxygen or carbon dioxide. Data shown are based on the overall reaction of all tested meat spoiling bacteria, which behave quite similar

## Discussion

Protective MAP gases, i.e., oxygen and carbon dioxide, widely employed in the food industry, penetrate the cell membrane of the initial spoilage bacteria, induce oxidation of fatty acids and reduce protein functions, resulting in irremediable cell damage [12, 28]. In our previous full proteomic study, we have investigated the effect of different (modified) atmospheres on the metabolism of different meat spoilage bacteria. We had found a proteomic upregulation of several enzymes of the fatty acid synthesis II complex in response to carbon dioxide exposure for the species *B. thermosphacta* TMW2.2101 and *C. maltaromaticum* TMW2.1581, whereas latter also upregulated those genes under oxic conditions. Furthermore, all three subunits of the branched-chain alpha-keto acid dehydrogenase complex were upregulated under high oxic conditions for both *Carnobacteria* species. No metabolic regulations of fatty acid genes could be observed for both *Leuconostoc* species, which could be explained by posttranslational modifications of the corresponding proteins [9]. Thus, our previous results hinted that adaptation of the cell membrane fatty acid composition might contribute to enhance survival of spoilage bacteria on MAP meat by e.g. preventing penetration of detrimental gases into the cell. Consequently, this study investigates the potential of different meat spoiling bacteria to adapt their fatty acid profile and thus their membrane behavior to exposure to different MAP gases. Therefore, we analyzed the genomic potential to build, degrade and convert existing fatty acids and determined their actual fatty acid profile and their direct physiological changes in membrane properties such as fluidity and permeability under exposure to 30% $CO_2$ or 70% $O_2$.

### Genomic potential for membrane fatty acid adaptation

Fatty acid degradation and interconversion are two important factors for rapidly changing of the existing fatty acid profile of bacteria. Indeed, we detected differences between those factors for the analyzed species. We predict the highest genomic potential for rapid fatty acid adaptation for the species C. *divergens* TMW2.1577 and *C. maltaromaticum* TMW2.1581 based on their extended ability to degrade different fatty acids and further produce cyclopropane fatty acids. Both *L. gelidum* subspecies are predictively also able to produce cyclopropane fatty acids but are disabled in saturated long and medium chain as well as polyunsaturated fatty acid degradation. *B. thermosphacta* TMW2.2101 is not able to produce cyclopropane fatty acids and degrade

polyunsaturated fatty acids and thus exhibits the lowest potential to adapt its membrane to environmental stress. However, genomic analysis *perse* are not sufficient to predict membrane adaptation potentials of bacteria. Thus, further analysis for actual determination of fatty acid composition, membrane fluidity and permeability measurements were conducted.

## Carbon dioxide (30%) induced membrane changes in meat spoilage bacteria

The restricted growth recorded for almost all species in this study in response to carbon dioxide exposure proves the high sensitivity of those bacteria to this gas. Even though all bacteria reached similar $OD_{max}$ after 48 h, lag phases were significantly prolonged due to carbon dioxide, assuming a long metabolic adaptation phase comprising alteration of membrane fatty acid metabolism. Up to now, there are only few reports in the literature analyzing changes in membrane properties of bacteria due to the gas carbon dioxide, whereas there are no reports on the changes in fatty acid profiles, yet. Furthermore, there is only a single study investigating the effect of carbon dioxide on the interfacial tensions between water and oil phases (benzene) by Sears, Eisenberg [29]. Affiliated from their results on model systems, a decrease of ion permeability by $CO_2$ for biological membranes was supposed [29]. Our study revealed an increase in SFAs and decrease in UFAs for *C. divergens* TMW2.1577 and both *L. gelidum* subspecies due to carbon dioxide. According to Zhang, Rock [15], SFAs lower, whereas *cis*-UFAs increase ion permeability of membranes. Thus, our results can confirm the assumption of Sears and Eisenberg, revealing a decrease of ion permeability of the cell membranes as a response to carbon dioxide exposure for these species.

In contrast to the few previous studies analyzing the direct effect of carbon dioxide on the cell membrane of bacteria, there are plenty of studies on membrane adaptations to acid stress in bacteria [21]. As one supposed way of action of carbon dioxide is the reduction of intracellular pH [6, 30, 31], membrane adaptation mechanisms of carbon dioxide and acid stress can be considered similar. In this way, an acid stress induced decrease of membrane permeability was also demonstrated for many Gram-positive bacteria, e.g. *Listeria (Li.) monocytogenes* [32] or *Streptococcus mutans* [33]. An association of acid stress and carbon dioxide is also demonstrated by our data, as the amount of lactobacillic acid (C19:0 delta), a cyclopropane fatty acid commonly found in lactic acid bacteria, was strongly upregulated due to carbon dioxide exposure for both *L. gelidum* subspecies and *C. divergens* TMW2.1577. Indeed, our genomic analysis revealed those species to be the only one encoding for the corresponding cyclopropane fatty acid synthase. According to the literature, an increase of cyclopropane

fatty acids has been described to enhance acid resistance and further reduces cell membrane permeability [15, 34, 35]. Thus, these results further confirm the assumption of reduced membrane permeability to be one major adaptation mechanism of *C. divergens* TMW2.1577, *L. gelidum* subsp. *gelidum* TMW2.1618 and *L. gelidum* subsp. *gasicomitatum* TMW2.1619. Furthermore, as the corresponding enzyme for cyclization is missing in *B. thermosphacta* TMW2.2101 and *C. maltaromaticum* TMW2.1581, we assume these two species to lack an important regulatory mechanism, which presence would enable to adapt to acid as well as carbon dioxide induced stress.

Zhang and Rock have also demonstrated that a shift from *anteiso* to *iso*-fatty acids results in a reduction of membrane fluidity, as the methyl groups of *anteiso*-fatty acids are more distanced from the chain end and thus have a higher disruptive effect compared to *iso*-fatty acids. In our study, the named shift could be observed *B. thermosphacta* TMW2.2101 concluding a decrease in membrane fluidity for this species. Furthermore, according to Zhang, Rock [15], a shift from UFAs to SFAs also contributes to decreasing membrane fluidity [15] as demonstrated for *C. divergens* TMW2.1577 and both *L. gelidum* subspecies. We further directly measured a decreasing membrane fluidity for the species *C. maltaromaticum* TMW2.1581. Thus, all analyzed species seem to reduce their membrane fluidity in response to carbon dioxide by different changes within the membrane fatty acid profiles, concluding this mechanism to be a common bacterial response to carbon dioxide exposure.

Regarding cell surface sizes, we detected significant changes; however, the effect of carbon dioxide on cell size changes was not consistent for all species and absolute cell surface values were of comparable magnitude despite statistical differences. The elevated fatty acid content per cell determined for *L. gelidum* subsp. *gasicomitatum* TMW2.1619 could be, therefore, correlated to a bigger cell size and not to a tighter packaging of the fatty acids within the membrane. From this, we concluded fatty acid composition changes to be a more powerful tool of bacteria to respond to environmental stress than denser packaging of the fatty acids per se.

## High oxygen (70%) induced membrane changes on meat bacteria

Molecular oxygen is used by aerobic microorganisms for respiratory growth. Nevertheless, high oxygen levels also result in RNA, DNA, protein and lipid damage [12] due to generation of reactive oxygen species (ROS). Depending on the genomic equipment, bacteria can greatly vary in their oxidative stress tolerance, as can be seen for the set of our species, comprising those who exhibit higher tolerance to high oxygen exposure (*Carnobacteria* species) and

those with lower tolerance (*Leuconostoc* species). Our data further confirmed that oxygen also influences the fatty acid profile of the bacteria. We measured increased amounts of unsatured fatty acid for *C. divergens* TMW2.1577 in response to high oxygen exposure. Furthermore, the species *C. maltaromaticum* TMW2.1581 also exhibits an enhanced production of long-chain UFAs (conjugated linoleic acid (18:2 conj.), arachidonic (C20:4 Δ5,8,11,14) and eicosapentaenoic acid (C20:5 Δ5,8,11,14,17). Assuming that theses long-chain UFAs are generated by the activity of an oxygen-depending desaturation system [36–38], catabolic oxygen consumption can be seen as one adaptation mechanism of this species to high oxygen gas atmospheres [35, 39]. The increase in UFAs determined for *C. maltaromaticum* TMW2.1581 and *C. divergens* TMW2.1577, also assumed to increase membrane permeability of this species, which was confirmed by physiological assays. We further directly measured increased membrane permeability for the species *L. gelidum* subsp. *gasicomitatum* TMW2.1619. Thus, we concluded that high oxygen levels facilitate increased membrane permeability of meat spoiling bacteria, which could result in a reduction of the membrane barrier function.

We further determined a shift from *anteiso-* to *iso-*fatty acids for *B. thermosphacta* TMW2.2101 and a denser packaged cell membrane for *C. maltaromaticum* TMW2.1581. Latter was determined by an increase in total amounts of fatty acids concomitant with a decrease in cell volume. This is in accordance to our previously findings on an upregulation of several enzymes of the fatty acid synthesis II complex for *C. maltaromaticum* TMW2.1581 under high oxygen conditions [9]. Thus, *B. thermosphacta* TMW2.2101 and *C. maltaromaticum* TMW2.1581 seem to decrease their membrane fluidity in response to high oxygen stress. Contrary, *C. divergens* TMW2.1577 and *L. gelidum* subsp. *gasicomitatum* TMW2.1619 both increased their membrane fluidity by either increasing their amount of branched and unsaturated fatty acids or by reducing their amount of *iso* fatty acids. This is also in accordance to our previous proteomic study, where we observed a strong upregulation of the branched-chain alpha-keto acid dehydrogenase complex under oxic conditions for *C. divergens* TMW2.1577. Thus, adaptation of the membrane fluidity in response to exposure to high oxygen concentrations appears to be species-specific instead of a general mechanism.

This is the first study investigating potential bacterial adaptation of their cell membrane composition in response to exposure to gases applied in modified atmosphere packaging. Furthermore, this study gives insights into basic fatty acid regulatory mechanisms and their effect on changing important membrane properties of bacteria. In this manner, we first analyzed the genomic settings of our species to predict and validate their potential to adapt their

fatty acid profile to environmental stress. We predicted different genomic potentials for each species based on presence or absence of genes involved in fatty acid degradation and cyclisation pathways. In accordance to this, we identified several species-specific modifications of fatty acid profiles due to high $O_2$ and $CO_2$ concentrations by actual physiological measurements. Interestingly, those fatty acid modification result in similar physiological membrane changes comprising reduced fluidity and permeability. It has been demonstrated that decreased membrane fluidity or permeability increases bacterial resistance to organic solvent [40] as well as antibiotics [41]. Thus, it appears that those changes facilitate also an enhanced resistance to the detrimental effects of gases such as $CO_2$ or $O_2$, by e.g. decreasing the diffusion into the cell or counteracting oxidative and acid stress as well as futile cycles. Supporting this hypothesis, we further identified an increased amount of lactobacillic acid in response to carbon dioxide exposure, predictively facilitating acid resistance. However, further studies should be conducted to confirm increased gas tolerance due to reduction of membrane fluidity and permeability of Gram-positive bacteria. Nevertheless, the present findings complement the previous demonstrated adaptive metabolic responses of meat spoiling bacteria to $CO_2$ (30%) and high $O_2$ (70%) within the full proteomic expression study.

## Compliance with ethical standards

**Conflict of interest** No conflict of interest is declared by the authors.

**Compliance with ethics requirements** This article does not contain any studies with human or animal subject.

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