Effect of drying on oxidation of membrane lipids and expression of genes encoded by the Shiga toxin prophage in *Escherichia coli*

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ABSTRACT

Drying processes do not eliminate pathogenic *Escherichia coli* in foods but induce sublethal injury, which may also induce the Shiga toxin (Stx) prophage. This study investigated the effect of drying on membrane lipid oxidation and *stx* expression in *E. coli*. Lipid peroxidation was probed with C_{11}-BODIPY$^{581/591}$; and *stx* expression was assayed by quantification of GFP in *E. coli* O104:H4 Δ*stx2a:gfp*:*amp*^{r}. Treatment of *E. coli* with H_2O_2 oxidized the probe; probe oxidation was also observed after drying and rehydration. Lipid oxidation and the lethality of drying were reduced when cells were dried with trehalose under anaerobic condition; in addition, viability and probe oxidation differed between *E. coli* AW1.7 and *E. coli* AW1.7Δ*cfa*. Desiccation tolerance thus relates to membrane lipid oxidation. Drying also resulted in expression of GFP in 5% of the population. Overexpression of *gfp* and *recA* after drying and rehydration suggested that the expression of Stx prophage was regulated by the SOS response. Overall, C_{11}-BODIPY$^{581/591}$ allowed investigation of lipid peroxidation in bacteria. Drying causes lipid oxidation, DNA damage and induction of genes encoded by the Stx prophage in *E. coli*.

KEYWORDS C_{11}-BODIPY$^{581/591}$, membrane lipid oxidation, STEC, oxidative stress, desiccation tolerance, Stx, prophage induction, cyclopropane fatty acid, Trehalose

1. Introduction

The survival of pathogenic bacteria in low water activity (a_w) foods is a threat to food safety; low-infectious dose pathogens including *Salmonella* and Shiga toxin producing *Escherichia coli* are of particular concern. Bacteria in the dry state resist heat and other interventions, which makes the decontamination of low a_w foods challenging (Beuchat et al., 2013; Finn et al., 2013; Gruzdev et al., 2011). STEC caused numerous outbreaks associated with low a_w foods including nuts, fenugreek seeds and dry cured meats (Beuchat et al., 2013). The production of Stx by STEC results in severe illness, such as hemolysis, kidney failure, and neurological symptoms (O'Loughlin EV, 2001; Trachtman et al., 2012). Stx is encoded in the late region of prophages (Iii et al., 1999). Induction of prophage and production of Stx are repressed by the prophage repressor CI but induced by environmental stress including oxidative stress (Fang et al. 2017; Licznerska et al., 2015; Waldor and Friedman, 2005).

The accumulation of compatible solutes mediates tolerance of eukaryotic and bacterial cells to desiccation (Crowe, 2001). Among compatible solutes, trehalose is particularly effective; it stabilizes not only cytoplasmic proteins but also the lamellar structure of the phospholipid membrane (Crowe and Hoekstra, 1992; Leslie et al., 1995). The generation of reactive oxygen species (ROS) after drying induces oxidative stress in dehydrated cells (França et al., 2007). In metabolically active cells, ROS are rapidly reduced by enzymes or antioxidants, such as catalase, superoxide dismutase, glutathione, or thioredoxin (Cabiscol Català et al., 2000). Dehydration compromises the permeability barrier of the cytoplasmic membrane and inactivates enzymes, which promotes the accumulation of ROS (França et al., 2007). The accumulation of ROS in dehydrated yeast and plant cells contributes to protein denaturation, DNA damage, and lipid peroxidation (França et al., 2007, Garre et al., 2010).

Membrane fluidity and integrity are crucial to protect macromolecules in dry cells. Unsaturated fatty acids in the membrane are prone to oxidation by ROS (Castro et al., 1996; Crowe et al., 1989). Upon lipid peroxidation, ROS are formed and sustain the autocatalytic oxidation of membrane lipids, which compromises survival during desiccation (Castro et al., 1996; Laguerre et al., 2007). Cyclopropane fatty acids (CFA) are more stable to ROS than unsaturated fatty acids as the double bond is converted to the cyclopropane ring (Grogan and Cronan, 1997). Conversion of unsaturated fatty acids to CFA maintains the membrane fluidity in stress conditions and increases bacterial resistance to heat, high pressure, oxidative stress, and freeze-drying (Chen and Gänzle, 2016; Muñoz-Rojas et al., 2006; Zhang and Rock, 2008). Desiccation induces oxidative stress, which also relates to induction of Shiga-toxin prophages in STEC (Licznerska et al., 2015); however, the role of oxidative stress in the survival of dehydrated bacteria and particularly its effect on the expression of Stx in STEC are poorly documented.

Fluorescence-based methods to measure the ROS are commonly used (Gomes et al., 2005; Laguerre et al., 2007), but only a few studies determined oxidation of bacterial membranes. C_{11}-BODIPY$^{581/591}$ is a membrane soluble fluorescein lipid analog which is oxidised by peroxide radicals (Laguerre et al., 2007). BODIPY$^{581/591}$ includes a phenyl moiety and a conjugated diene, and displays red fluorescence (Laguerre et al., 2007). Oxidation of the diene groups shifts its fluorescence from red to green (Borst et al., 2000; Drummen et al., 2002; Gomes et al., 2005; Laguerre et al., 2007; Yoshida et al., 2003). This study aimed to develop a flow cytometry-based method to evaluate lipid oxidation in cells of *E. coli* after staining with C_{11}-BODIPY$^{581/591}$. The method was used to explore the effect of drying, compatible solutes and CFA on the oxidation of membrane lipids. The expression of *stx* at low a_w environment was investigated with a strain of *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r}, which was constructed with green fluorescence protein (GFP) as

a reporter of *stx2a* (Fang et al. 2017). The effect of drying on the oxidative stress and prophage induction during drying was further confirmed by relative gene expression.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Strains of *E. coli* used in this study included *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r}, an outbreak strain with *stx2a* replaced with *gfp* (Fang et al. 2017; Muniesa et al., 2012); *E. coli* AW1.7, a non-pathogenic and heat resistant beef isolate (Dlusskaya et al., 2011) and *E. coli* AW1.7 Δ*cfa*, which has an in-frame unmarked deletion of the CFA synthase (Chen and Gänzle, 2016). Strains of *E. coli* were streaked from frozen stock cultures, sub-cultured in Luria-Bertani (LB) (BD, Mississauga, ON, Canada) broth and incubated overnight at 37 °C and 200 rpm. The protocol for preparing dry cells was established for *Salmonella enterica* (Uesugi et al., 2006). Bacterial culture (100 µL) was plated on the surface of LB agar and incubated overnight at 37 °C. Cells were removed from the surface of the agar by washing with 1 mL of 0.85% NaCl (Fisher Scientific, Ottawa, ON, Canada). The resulting cell suspension had a cell count of 10-11 $\log_{10}$ CFU/mL.

2.2. Preparation of dried bacterial cells

Bacterial culture was washed twice with 0.85% NaCl and re-suspended in 0.1% peptone water (BD) or solutions of 1 or 10% (w/v) trehalose (Fisher Scientific) in water. Bacterial suspensions (20 µL) were transferred into sterilized glass vials (12 mm by 30 mm by 4.6 mm; Sigma-Aldrich, St Louis, MO, USA) and dried under vacuum in a desiccator for 3-4 h, or air dried in an anaerobic system (model 1025/1029, ThermoForma, Fisher Scientific). After drying, samples were transferred to an air-tight container containing saturated NaCl solution to achieve a_w of 0.75 and incubated at 37 °C for 18 h (Mathlouthi, 2001). Samples dried in the anaerobic chamber were transferred in an airtight container with two BD GasPak™ (Fisher Scientific).

100 2.3. Membrane lipid oxidation assay basic on C_{11}-BODIPY$^{581/591}$

101 The stock solution of C_{11}-BODIPY$^{581/591}$ (Thermo Fisher Scientific, Waltham, MA, USA)
102 was dissolved in dimethyl sulfoxide (DMSO) to achieve a concentration of 1 mM. Cells were
103 suspended in 10 mM citrate buffer (pH 7) and incubated with 10 μM C_{11}-BODIPY$^{581/591}$ in the
104 dark for 30 min at 37 °C and 200 rpm. The outer membrane from bacteria was disrupted with
105 ethylenediaminetetraacetic acid (EDTA) and lysozyme before staining to increase the solubility of
106 C_{11}-BODIPY$^{581/591}$ into the cytoplasm lipid membrane (Gänzle et al., 1999). In brief, cells were
107 washed twice with ice-cold 50 mM Tris×HCl (pH 8.0) containing 20% (wt/vol) sucrose. Addition
108 of 0.2 mL lysozyme solution (5 mg/mL lysozyme in 0.25 M Tris×HCl, pH 8.0) and 0.4 mL EDTA
109 (0.25 M, pH 8.0) were added to the cell suspension, followed by incubation for 30 min at 37 °C
110 and 200 rpm. After incubation, cell pellets were suspended with citrate buffer and incubated with
111 C_{11}-BODIPY$^{581/591}$ for 30 min at 37 °C with shaking. To expose the cells to oxidative stress, *E.*
112 *coli* cells were treated with H_2O_2 (50 mM) for 30 min, a treatment that reduces cell counts of *E.*
113 *coli* AW11.7 by about 3 log(CFU/mL) (Chen and Gänzle, 2016), followed by staining with C_{11}-
114 BODIPY$^{581/591}$.

115 2.4. Flow cytometric determination of fluorescence

116 Flow cytometry was performed using BD LSR-Fotessa^{TM} X-20 (BD Biosciences, San Jose,
117 CA, USA) equipped with a 488 nm excitation from a blue air laser at 50 mW and a 561 nm
118 excitation from a yellow air laser at 50 mW to excite green (530±30 nm) and red fluorescence
119 (586±15 nm). Stained bacteria (1 mL) were diluted with FACS buffer (1% PBS, 2% fetal calf
120 serum [FCS], 0.02 % sodium azide) to maintain the running speeds to no more than 3000 events
121 per sec. Sample injection and acquisition were started simultaneously and stopped when about
122 10,000 events were collected. FCS files were extracted from FACSDiva 8 software and analyzed

123 by FlowJo software (Tree Star, Ashland, USA). *E. coli* cells treated with C_{11}-BODIPY$^{581/591}$ were
124 analyzed with respect to green and red fluorescence intensity. The gating of green and red
125 fluorescence intensity was manually set to include more than 99% of the cells from non-stained
126 controls as green and red fluorescence negative (Fig. 1). Gating differentiated between the
127 following four subpopulations: non-oxidized cells which display only red fluorescence; oxidized
128 cells which display green and red fluorescence, fully oxidized cells which display only green
129 fluorescence and non-stained cells which are non-fluorescent (Fig. 1). The fully oxidized
130 population was combined with oxidized C_{11}-BODIPY$^{581/591}$. Even partial oxidation of membrane-
131 bound C_{11}-BODIPY$^{581/591}$ indicates oxidative damage but whether or not non-oxidized and red
132 fluorescent C_{11}-BODIPY$^{581/591}$ remains in the membranes of oxidized cells also depends on the
133 staining protocol and the ratio of C_{11}-BODIPY$^{581/591}$ to bacterial cells.

134 2.5. Quantification of Stx prophage expression.

135 The method to quantify GFP expression in *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp* was described
136 by Fang et al. (2017). A culture of *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r} was dried in 0.1% peptone
137 water, or 10% trehalose solution in a vacuum desiccator. Dry cells were equilibrated to a_W 0.75 as
138 described above. Stationary phase *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r} in LB media served as a
139 control. After drying, *E. coli* cells were suspended in 1 mL LB broth, and analyzed by flow
140 cytometry to determine the population of cells exhibiting green fluorescence. The gating of GFP
141 fluorescence was set manually to account more than 99% of the cells in controls as GFP negative.
142 Two subpopulations consisting of GFP positive cells and GFP negative cells, respectively, were
143 differentiated; expression of GFP also indicates the expression of Stx prophage genes. Data are
144 presented as means ± standard deviation (SD) for three biological replicates.

145 2.6. Determination of viable cell counts.

Dry cells were serially diluted and surface-plated onto LB agar, followed by incubation at 37 °C for 24 h. Bacteria without drying served as controls. Reduction of cell counts after drying was expressed as $\log_{10}$ (N_0/N) with N_0 representing the cell counts of the wet cell without drying and N representing the cell counts after drying. Results are means ± SD for four biological replicates.

2.7. Quantification of relative gene expression of dry cells by RT-qPCR

Desiccated cells were rehydrated with 1 mL LB broth and incubated at 30 °C for 2 h. Total RNA was isolated from dry and stationary phase cultures of *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r}. RNA was reverse transcribed to cDNA using QuantiTect reverse transcription kit (Qiagen). Quantitative PCR was performed with a 7500 FastSystem (Applied Biosystems, Foster City, CA, USA) using primers target *gfp*, *recA*, *soxR*, *oxyR* and *rpos* (Table 1). Negative controls included DNase-treated RNA and no-template controls. The gene coding for glyceraldehyde-3-phosphate dehydrogenase A (*gapA*) served as the reference gene. The gene expression ratio of target genes to *gapA* in *E. coli* O104:H4 Δ*stx2a:gfp:amp*^{r} after drying and rehydration relative to the bacteria at control conditions were calculated with the $\Delta\Delta C_T$ method as described (Pfaffl, 2001). Stationary phase growing cells in LB broth serve as a control condition. The ratio of gene expression was normalized by transformation with $\log_2$. Data are presented as means ± SD for four biological replicates.

2.8. Statistical analysis

The data from membrane lipid oxidation and reduction of cell counts after drying and Stx prophage induction were analyzed by two-way and one-way analysis of variance (ANOVA), respectively. Statistical differences among treatments were determined by Tukey's test with $P<0.05$ using SAS (SAS Institute Inc., Cary, NC, USA).

169 3. Results

170 3.1. Development and validation of a C_{11}-BODIPY$^{581/591}$ based flow cytometric assay for
171 quantification of membrane lipid oxidation in *E. coli*.

172 To validate the method primary on C_{11}-BODIPY$^{581/591}$, the permeability of the outer
173 membrane to BODIPY$^{581/591}$ was assessed with cells of *E. coli*. The availability of *E. coli*
174 AW1.7Δ*cfa* allowed the comparison of membrane lipid oxidation in the wildtype strain of *E. coli*
175 AW1.7 and the mutant. The disruption of CFA synthase replaces cyclopropane fatty acids in the
176 membranes of *E. coli* AW 1.7 by an equivalent amount of unsaturated fatty acids (Chen and Gänzle,
177 2016). The lipid probe C_{11}-BODIPY$^{581/591}$ is soluble in the membrane lipid bilayer (Drummen et
178 al., 2002); however, the outer membrane of Gram-negative bacteria prevents the access of
179 hydrophobic dyes to the cytoplasmic membrane. Accordingly, less than 50% of cells of *E. coli*
180 were stained with C_{11}-BODIPY$^{581/591}$. Treatment with EDTA and lysozyme permeabilized the
181 outer membrane to lipophilic C_{11}-BODIPY$^{581/591}$. EDTA and lysozyme significantly enhanced the
182 membrane permeability to C_{11}-BODIPY$^{581/591}$ and decreased the proportion of unstained cells (Fig
183 S1).

184 Treatment with EDTA for 1 h or overnight did not enhance the permeability of C_{11}-
185 BODIPY$^{581/591}$. Specifically, 27% and 11% of the population incorporated C_{11}-BODIPY$^{581/591}$ after
186 treatment with EDTA for 1 h in *E. coli* AW1.7 and *E. coli* AW1.7Δ*cfa*, respectively. The non-
187 oxidized population of cells treated with EDTA and lysozyme was significantly higher than those
188 without EDTA and lysozyme treatments (Fig. S1). This suggested that adding lysozyme was
189 necessary to achieve a high level of incorporation of C_{11}-BODIPY$^{581/591}$ in the cells.

190 To determine the effect of oxidative stress on the fluorescence of C_{11}-BODIPY$^{581/591}$,
191 cultures of *E. coli* were treated with H_2O_2, labeled with C_{11}-BODIPY$^{581/591}$, and analyzed by flow

cytometry (Fig. 2). *E. coli* AW1.7 Δ*cfa* and *E. coli* AW1.7 contain unsaturated fatty acids and CFA in the membrane, respectively, as major components of the cytoplasmic membrane (Chen and Gänzle, 2016) and were used to determine the effect of unsaturated fatty acids and CFA on the lipid peroxidation. Without H_2O_2, the proportion of oxidized C_{11}-BODIPY$^{581/591}$ in wildtype and mutant were below 20%. After treatment with H_2O_2, oxidized C_{11}-BODIPY$^{581/591}$ was increased to 48% and 21% for *E. coli* AW1.7 and AW1.7 Δ*cfa*, respectively. Oxidation of C_{11}-BODIPY$^{581/591}$ indicated that *E. coli* AW1.7 Δ*cfa* were less sensitive to oxidation than *E. coli* AW1.7. Overall, the C_{11}-BODIPY$^{581/591}$-flow cytometry assay indicates the oxidation of membrane lipids.

3.2. Effect of drying on the lipid peroxidation

To explore the oxidation of membrane lipids during drying, bacteria were labeled with C_{11}-BODIPY$^{581/591}$ and analyzed by flow cytometry (Fig. 3). Cultures dried in 0.1% peptone under aerobic conditions show that C_{11}-BODIPY$^{581/591}$ was extensively oxidized, and the proportion of cells with oxidized membrane lipids was above 60% in *E. coli* AW1.7 (Fig. 3A) and AW1.7 Δ*cfa* (Fig. 3B). In comparison to cultures dried in peptone solution, the percentage of cells with oxidized probe decreased in both strains of *E. coli* after drying in 1% and 10% trehalose. In the presence of 10% trehalose, drying under aerobic or anaerobic conditions did not change the proportion of oxidized probe. The presence of CFA in the membrane of *E. coli* AW1.7 did not increase level of lipid peroxidation after desiccation in comparison to *E. coli* AW1.7 Δ*cfa*. In contrast, hydrated cells of *E. coli* AW1.7 were more prone to oxidation by H_2O_2 (Fig. 2).

3.3. Effect of drying on bacterial survival

Oxidation of membrane lipids compromises the dry survival of *E. coli*. Viable cell counts were determined with *E. coli* AW1.7 and *E. coli* AW1.7 Δ*cfa* after drying at the conditions used to determine the lipid peroxidation (Fig. 4). Cultures dried with 0.1% peptone water were most

sensitive to oxidation; these cultures also showed the highest loss of viability after desiccation. Drying in the presence of trehalose, or absence of oxygen reduced the oxidation of C_{11}-BODIPY$^{581/591}$; these conditions also improved the survival of *E. coli*. Under aerobic conditions, *E. coli* AW1.7 Δ*cfa* was more sensitive to drying and re-hydration than *E. coli* AW1.7.

3.4. Induction of Stx prophage by drying

Oxidative stress induces Stx prophages in *E. coli* (Łoś et al., 2009). To determine whether drying induces Stx prophages, we determined the induction of Stx prophage and reduction of cell counts of *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r} with a GFP fusion in the prophage to report the expression of Stx (Table 2). Drying with peptone only achieved a less than 1 $\log_{10}$ (CFU/mL) reduction of cells counts of *E. coli* O104:H4. The presence of trehalose in the drying matrix enhanced the cell survival by 0.5 $\log_{10}$ (CFU/ml). In both drying conditions, GFP was expressed in *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r} in approximately 5% of the population.

3.4. Effect of drying on the expression of *gfp, recA, oxyR, soxR,* and *rpos*

Drying caused oxidation of membrane lipids in non-pathogenic *E. coli* and induced the expression of prophage-encoded genes in STEC. To explore whether this induction of Stx prophage genes is related to oxidative stress, the expression of genes coding for oxidative stress resistance and the SOS response in *E. coli* was quantified (Fig. 5). The fluorescence assay using C_{11}-BODIPY$^{581/591}$ cannot be used with *E. coli* O104:H4 Δ*stx2a*:*gfp*:*amp*^{r} because the fluorescence of GFP interferes with quantification of oxidized C_{11}-BODIPY$^{581/591}$. After drying and rehydration of STEC, the SOS response induced *recA* was overexpressed. The gene coding for the alternative sigma factor *rpoS* or *sodR* and *oxyR*, which encode for regulators of the oxidative stress response, were not overexpressed in STEC after drying.

4. Discussion

238 This study introduced a method based on C_{11}-BODIPY$^{581/591}$ as a novel tool to determine
239 the oxidative stress and lipid peroxidation in Gram-negative bacteria. In addition, this study
240 establishes a relationship between oxidative stress, SOS response, and expression of virulence
241 genes of STEC after desiccation and rehydration.

242 Fluorescence probes used in bacteria to determine oxidative stress employed alkaline
243 phosphatase (Aertsen et al., 2005), redox-sensitive GFP2 (roGFP2) (Müller et al., 2017),
244 H_2DCFDA (2',7'–dichloro-dihydro-fluorescein diacetate) (Marcén et al., 2017), MitoSOX (Mols
245 et al., 2011), and C_{11}-BODIPY$^{581/591}$ (Johnson et al., 2012). These assays detect cellular oxidative
246 stress by direct interaction with ROS. C_{11}-BODIPY$^{581/591}$ is lipophilic and oxidised by peroxide
247 radicals but not by hydroperoxides (Drummen et al., 2002). The oxidation of C_{11}-BODIPY$^{581/591}$
248 is thus caused by lipid peroxide radicals that are formed after the challenge with H_2O_2 (Laguerre
249 et al., 2007). In the current study, C_{11}-BODIPY$^{581/591}$ was introduced after the removal of H_2O_2 to
250 ensure that H_2O_2 or HO• did not directly oxidize the fluorescent probe. The lipid chain of C_{11}-
251 BODIPY$^{581/591}$ is highly hydrophobic and directs the probe to the cytoplasmic membrane (Ball and
252 Vo, 2002). In prokaryotes, the determination of C_{11}-BODIPY$^{581/591}$ fluorescence with
253 spectrophotometry and fluorescence microscopy were used in *Pseudomonas* (Johnson et al., 2012)
254 and *Lactobacillus* (Carlsen et al., 2009). Flow cytometric quantification of C_{11}-BODIPY$^{581/591}$ was
255 previously applied only in eukaryotic cells and algae (Cheloni and Slaveykova, 2013;
256 Makrigiorgos et al., 1997), but not in bacteria. This study quantified C_{11}-BODIPY$^{581/591}$ by flow
257 cytometry to determine the level of lipid peroxidation in *E. coli*. Flow cytometry allows the
258 quantification of the C_{11}-BODIPY$^{581/591}$ stained and non-stained cells, which shows that C_{11}-
259 BODIPY$^{581/591}$ had a low penetration to the membrane of Gram-negative bacteria. The problem

was undetected by previous assays that employed spectrophotometry or fluorescence microscopy to detect lipid oxidation in bacterial membranes (Carlsen et al., 2009; Johnson et al., 2012).

C_{11}-BODIPY$^{581/591}$ is hydrophobic and mostly excluded from the cytoplasm and periplasm of Gram-negative bacteria (this study). The outer membrane has a low permeability to hydrophobic inhibitors because the outer leaflet consists of highly ordered and hydrophilic lipopolysaccharides (Vaara, 1992). Disruption of the outer membrane with EDTA and lysozyme (Vaara, 1992) allowed the staining with C_{11}-BODIPY$^{581/591}$ without affecting the oxidation of lipids in the cytoplasmic membrane (this study). Chelating agents, such as EDTA were used to enhance the membrane permeability of hydrophobic dye (Szivak et al., 2009). In this study, flow cytometry ensured that the C_{11}-BODIPY$^{581/591}$ was incorporated in the majority of the cells after treatment with EDTA and lysozyme.

The role of oxidative stress in desiccation tolerance was documented in *Lactobacillus* (Laguerre et al., 2007) and *E. coli* (this study). Trehalose increases the desiccation tolerance as it maintains the membrane structure (Leslie et al., 1995) and reduces the level of lipid peroxidation in yeast (De Jesus Pereira et al., 2003) and *E. coli* (this study). Trehalose was suggested to prevent the lipid from oxidation by forming a stable complex between hydroxyl groups of trehalose and double bond of unsaturated fatty acids (Oku et al., 2003).

CFA in membrane lipids are synthesized from unsaturated fatty acids and protect bacterial cells against several physical and chemical stressors (Chen and Gänzle, 2016) including desiccation (this study); CFA synthesis also improved survival of *Pseudomonas putida* after freeze-drying (Muñoz-Rojas et al., 2006). CFA and unsaturated fatty acids were oxidized during desiccated storage (Castro et al., 1995; Grogan and Cronan, 1986; Teixeira et al., 1996). The comparison of *E. coli* AW1.7 and AW1.7 Δ*cfa* suggests that conversion of unsaturated fatty acids

to CFA had differential effects in hydrated membranes and in dry membranes (this study). The relative oxidative stability of CFA and unsaturated fatty acids to oxidation in dehydrated membranes, however, remains unknown; moreover, the composition of membrane fatty acids may change during desiccation.

The role of CFA on the oxidative stability of bacterial membranes may also depend on other biophysical properties of the membrane. The packing density of phospholipids is altered when the membrane transition from the liquid to the gel phase upon dehydration (Crowe and Hoekstra, 1992). CFA maintain the membrane integrity because the cyclopropane ring of CFA is rigid and resistant to packing into acyl chain array of phospholipids, which maintains the lamellar structure of the phospholipid membrane (Poger and Mark, 2015). In contrast, *cis*-unsaturated fatty acids are flexible and can bend and promote a compact bilayer (Poger and Mark, 2015). This suggests that physical and chemical properties, particularly the oxidative stability of unsaturated and CFA were different in the hydrated or dry membrane; however, the mechanisms that cause this differential effect remain unexplored.

Desiccation induces oxidative stress; however, the transcriptional response to oxidative stress is poorly documented in desiccated bacteria. During desiccation, the Fe-protein and Fe-superoxide dismutase (*sodF*) were overexpressed in desiccated *Salmonella* and *Cyanobacteria*, suggesting that Fe-proteins improve survival after desiccation (Gruzdev et al., 2012; Shirkey et al., 2000). In *E. coli* and related bacteria, SoxR and OxyR are cytoplasmatic transcription factors which control the transcription of proteins that prevent or repair oxidative damage. Oxidation of SoxR and OxyR reduces binding to the promoter region and activates transcription of genes that encode functions related to peroxide detoxification (Choi et al., 2001; Ding et al., 1996). An *oxyR* deficient derivative of *E. coli* also produced higher levels of λ prophages when compared to the

wildtype (Glinkowska et al., 2010), indicating that OxyR mediated gene expression also relates to induction of λ prophages. This study found that *soxR* and *oxyR* were not overexpressed in STEC after drying and rehydration. In *E. coli,* the protein level of OxyR was not different after H_2O_2 treatment (Zheng et al., 1998), suggesting that OxyR mediated gene expression is mediated by changes in protein conformation upon oxidation rather than changes in *oxyR* mRNA abundance. Oxidative stress also relates to the SOS response and the RpoS-regulated general stress response (Farr and Kogoma, 1991). RpoS regulated gene expression is related to the growth phase (Farr and Kogoma, 1991), which may limit the transcription of RpoS following a short re-hydration period as used in this study. RecA regulated by the SOS response was overexpressed in STEC after drying and rehydration, which indicates that oxidative stress in dry cells contributes to DNA damage.

The mechanism of desiccation tolerance of pathogenic *E. coli* matches the response of commensal and generic *E. coli* (Louis et al., 1994) and STEC have a similar resistance to desiccation as generic *E. coli*. In addition, STEC over-expressed genes encoded by the Stx prophage after desiccation and re-hydration (this study). Induction of the λprophage during desiccation was reported previously (Webb and Dumasia, 1967). Stx phages belong to lambdoid phages but the regulation of prophage induction is different from the λ phage (Monse et al., 2012). The induction of Stx prophage after desiccation corresponds to the overexpression of *recA* (this study), which mediates the expression of prophage late region by proteolysis of the prophage repressor CI (Waldor and Friedman, 2005). The induction of genes encoded by the Stx prophage after desiccation and rehydration may thus lead to the production of infectious phage particles that potentially transduce non-virulent *E. coli* before or after ingestion and thus amplifies the production of toxin (Bielaszewska et al., 2007). The outbreak in 2011 likely involved long-term survival of *E. coli* O104:H4 on dry fenugreek seeds, followed by rehydration during sprouting

(Beutin and Martin, 2012). Survival and persistence of STEC in dry foods is a concern of the food industry and consumer safety (Beuchat et al., 2013). The overexpression of GFP in *E. coli* O104:H4Δ*stx2a*:*gfp*:*amp*^{r} was highly correlated to expression of *stx*, and RecA mediated GFP overexpression also corresponded to the production of infectious phage particles (Fang et al. 2017). Whether or not dried cells of STEC produce Stx-phages after drying and re-hydration, however, remains to be confirmed.

In conclusion, the current study developed a methodology using the fluorescence dye C_{11}-BODIPY$^{581/591}$ to probe lipid oxidation of desiccated bacteria; and used GFP-labeled STEC to indicate that drying induced *stx* expression. Oxidative damage is lethal to desiccated bacteria and could enhance the production of Stx. This study improved the knowledge on the impact of drying on oxidative stress and its effect on the regulation of genes for oxidative stress response and *stx* in STEC.

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Figure legends

Figure 1. Dot plot of red and green fluorescence of *E. coli* AW1.7 that were not stained with C_{11}-BODIPY$^{581/591}$ (blue symbols), or stained with C_{11}-BODIPY$^{581/591}$ (red and black clusters). Stationary phase culture was treated with H_2O_2 (50 mM) for 30 min, treated with EDTA and lysozyme and stained with C_{11}-BODIPY$^{581/591}$ (10 μM) for 30 min (black symbols). Untreated bacteria were treated with EDTA and lysozyme, and then stained with C_{11}-BODIPY$^{581/591}$ (red symbols). The population was divided into four sub-groups by reference lines for green and red fluorescence. The reference lines were set to include at least 99% of unstained cells; the threshold value was 100 for green and red fluorescence. The inset shows the % of cells in the three samples that were not oxidized (Q1); oxidized and non-oxidized (Q2); fully oxidized (Q3), or not stained (Q4).

Figure 2. Percentage of C_{11}-BODIPY$^{581/591}$ labeled cells of *E. coli* AW1.7 (white bars) and *E. coli* AW1.7 Δ*cfa* (grey bars). Stationary cultures were oxidized with 50 mM H_2O_2 and analysed by flow cytometry to differentiate between unstained cells, cells exhibiting only red fluorescence corresponding to the non-oxidized probe, and cell exhibiting green fluorescence corresponding to the oxidized probe. Untreated cultures after incubation in LB media for 30 min (solid colored bars) served as control. Significant differences between *E. coli* AW1.7 and *E. coli* AW1.7 Δ*cfa* are indicated by an asterisk (*) (P<0.05). Values for the same strain treated under different conditions that do not share a common superscript differ significantly (P<0.05). Data are means ± standard deviation for three independent experiments.

Figure 3. Percentage of C_{11}-BODIPY$^{581/591}$ labeled cells of *E. coli* AW1.7 (panel A) and *E. coli* AW1.7 Δ*cfa* (panel B) after drying. Treated cells were analysed by flow cytometry to differentiate between unstained cells, cells exhibiting only red fluorescence corresponding to the non-oxidized

probe, and cell exhibiting green fluorescence corresponding to the oxidized probe. *E. coli* cells were air-dried with 0.1% peptone (white), 1% trehalose (white-hatched), 10% trehalose (grey), and dried with 10% trehalose at anaerobic conditions (grey-hatched). C_{11}-BODIPY$^{581/591}$ fluorescence between *E. coli* AW1.7 and *E. coli* AW1.7 Δ*cfa* after treatment at the same conditions was not significantly different (P<0.05). Values for the same strain treated under different conditions that do not share a common superscript differ significantly (P<0.05). Data are means ± standard deviation of the means for three independent experiments.

Figure 4. Reduction of cell counts of *E. coli* AW1.7 (white bars) and *E. coli* AW1.7Δ*cfa* (grey bars) after drying in different matrices and aerobic or anaerobic atmospheres. Liquid cultures were suspended in 0.1% peptone or 10% trehalose before drying in a vacuum desiccator and equilibration to a_w 0.75. Significant differences between *E. coli* AW1.7 and *E. coli* AW1.7Δ*cfa* after the same treatment are indicated by an asterisk (P<0.05). Values for the same strain treated with different conditions that do not share a common superscript are significantly different (P<0.05). Data are means ± standard deviation for at least three independent experiments.

Figure 5. Relative gene expression of *oxyR, soxR, rpos, gfp and recA* in *E. coli* O104:H4 Δ*stx2*:*amp*:*gfp* after drying and rehydration. Relative gene expression was quantified by RT-qPCR with *gapA* as a housekeeping gene and stationary cultures in LB broth as reference conditions. Significant differences between target genes are labeled with different superscript (P<0.05). The single asterisk indicated the relative gene expression of the target genes is significantly different from one. Data are means ± standard deviation of three independent experiments with duplicate RNA isolations from each culture.

580 **Table 1.** Primers used for relative gene expression

Primers (forward, F; Reverse, R)	Direction: Sequence (5'-3')	Size (base pair)
Primer used for quantification of gene expression		
stx2	F: TATCCTATTCCCGGGAGTTT	200
	R: TGCTCAATAATCAGACGAAGAT	
gfp	F: TTCTTCAAGTCCGCCATG	200
	R: TGAAACGGCCTTGTGTAGTATC	
gapA	F: GTTGACCTGACCGTTCGTCT	116
	R: TGAAACGGCCTTGTGTAGTATC	
recA	F: ATTGGTGTGATGTTCGGTAA	200
	R: GCCGTAGAGGATCTGAAATT	
oxyR	F: CGGTCCAACTGTGGGAATCA	195
	R: CTGGAAGATGAGTTGGGCGT	
soxR	F: GTACCCTGTGATGAGCCGTT	203
	R: ATCAGGAACCCGCCAATACC	
rpos	F: TGCTGGGATAGAGACAGGCA	209
	R: TATCGCGATGCCACGAATGA	

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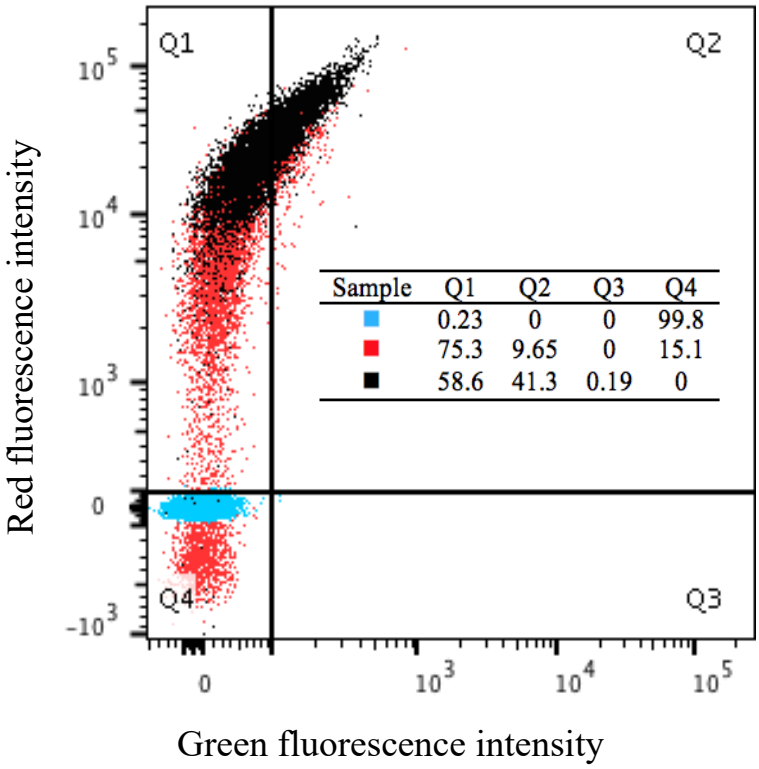
583 **Table 2.** Flow cytometric quantification of the expression of GFP in *E. coli* 104: H4
584 Δ*stx2*::*gfp*:: *amp* after drying with different matrices. Shown is the reduction of cell
585 counts as log(N_0/N), the proportion of cells (%) expressing GFP, and the proportion of
586 cells (%) not expressing the GFP. Data represent means ± standard deviation of three or
587 four independent replicates.

	Reduction of cell counts $\log_{10}(N_0/N)$	**Proportion of the population (%) expressing GFP**	**Proportion of the population (%) not expressing GFP**
Control	0	0.61 ± 0.75^{b}	99 ± 0.76^{a}
Peptone (0.1%)	0.87 ± 0.05^{a}	4.7 ± 2.0^{a}	95 ± 1.96^{b}
Trehalose (10%)	0.28 ± 0.15^{b}	4.8 ± 1.2^{a}	95 ± 1.2^{b}

588 Values in the same column that do not share a common superscript are significantly
589 different (P<0.05). Data represent means ± standard deviation of four independent
590 experiments of treatments with peptone (0.1%) and trehalose (10%) and three
591 independent experiments for the control.
592

593 **Figure 1**



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595

596 **Figure 2**

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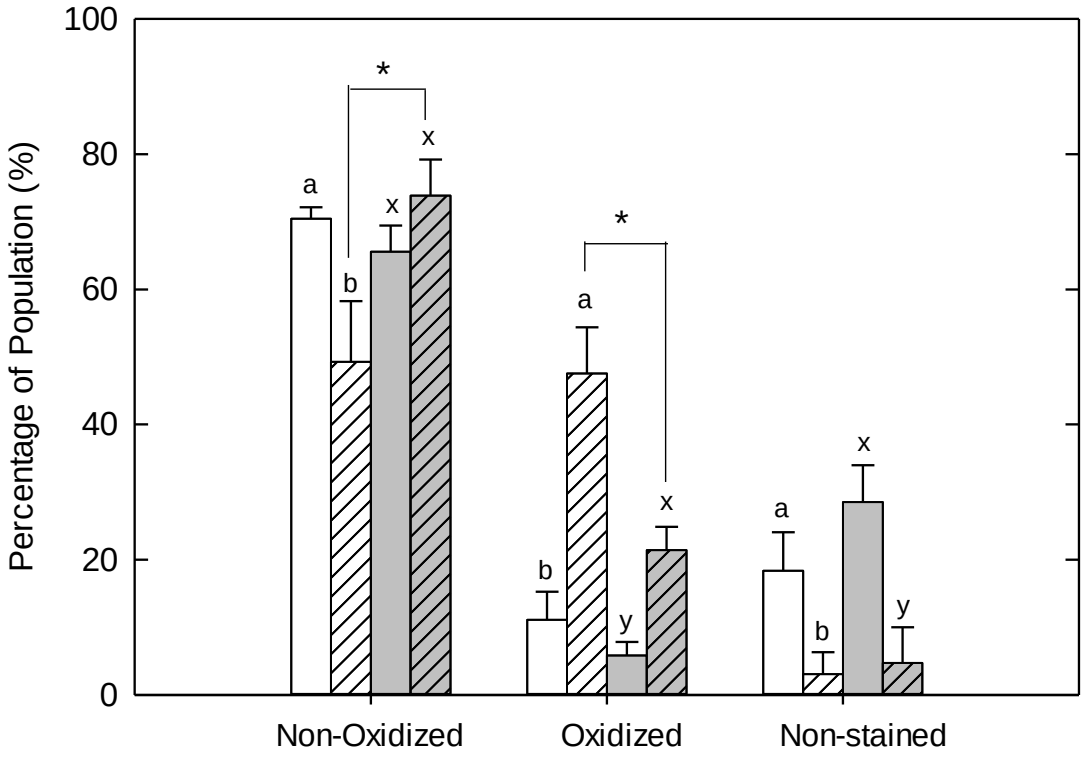
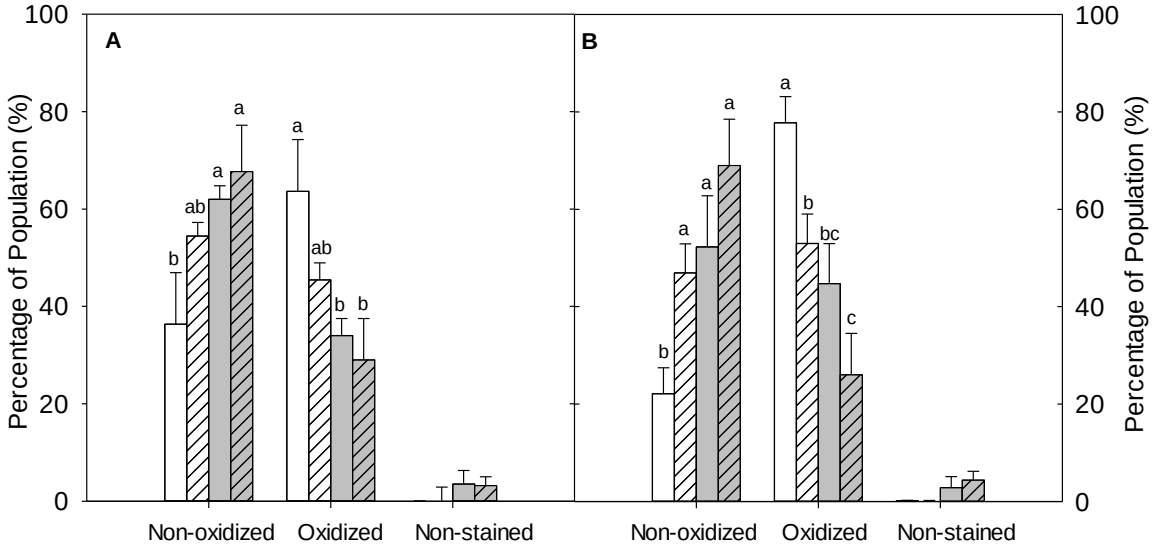
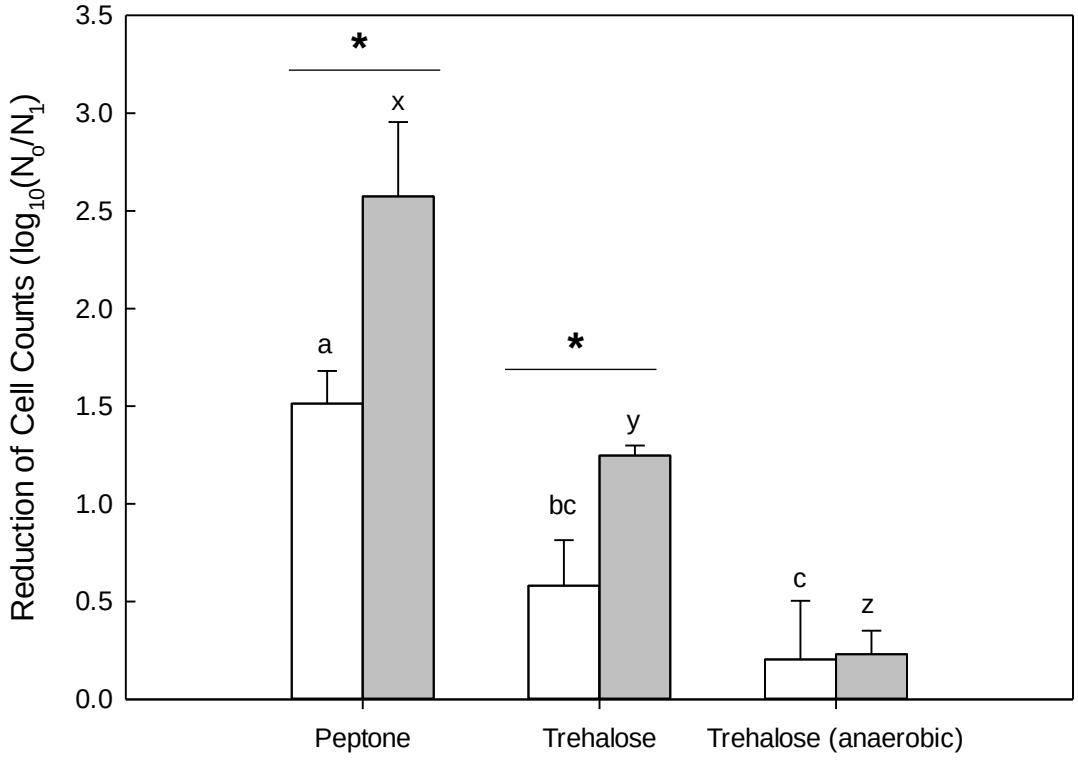


598 **Figure 3**



599

600 **Figure 4**



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603 **Figure 5**

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