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FOOD MICROBIOLOGY

Transcriptome Analysis of *Listeria monocytogenes* Exposed to Beef Fat Reveals Antimicrobial and Pathogenicity Attenuation Mechanisms

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ABSTRACT *Listeria monocytogenes* is a deadly intracellular pathogen mostly associated with consumption of ready-to-eat foods. This study investigated the effectiveness of total beef fat (BF-T) from flaxseed-fed cattle and its fractions enriched with monounsaturated fatty acids (BF-MUFA) and polyunsaturated fatty acids (BF-PUFA), along with commercially available long-chain fatty acids (LC-FA), as natural antimicrobials against *L. monocytogenes*. BF-T was ineffective at concentrations up to 6 mg/ml, while *L. monocytogenes* was susceptible to BF-MUFA and BF-PUFA, with MICs at pH 7 of 0.33 ± 0.21 mg/ml and 0.06 ± 0.03 mg/ml, respectively. The MIC of C14:0 was significantly lower than those of C16:0 and C18:0 ($P < 0.05$). Fatty acids *c*9-C16:1, C18:2n-6, and C18:3n-3 showed stronger inhibitory activity than *c*9-C18:1 and conjugated C18:2, with MICs of <1 mg/ml. Furthermore, global transcriptional analysis by transcriptome sequencing (RNA-seq) was performed to characterize the response of *L. monocytogenes* to selected fatty acids. Functional analysis indicated that antimicrobial LC-UFA repressed the expression of genes associated with nutrient transmembrane transport, energy generation, and oxidative stress resistance. On the other hand, upregulation of ribosome assembly and translation process is possibly associated with adaptive and repair mechanisms activated in response to LC-UFA. Virulence genes and genes involved in bile, acid, and osmotic stresses were largely downregulated, and more so for *c*9-C16:1, C18:2n-6, and C18:3n-3, likely through interaction with the master virulence regulator PrfA and the alternative sigma factor σ^B.

IMPORTANCE *Listeria monocytogenes* is a bacterial pathogen known for its ability to survive and thrive under adverse environments and, as such, its control poses a significant challenge, especially with the trend of minimally processed and ready-to-eat foods. This work investigated the effectiveness of fatty acids from various sources as natural antimicrobials against *L. monocytogenes* and evaluated their potential role in *L. monocytogenes* pathogenicity modulation, using the strain ATCC 19111. The findings show that long-chain unsaturated fatty acids (LC-UFA), including unsaturated beef fat fractions from flaxseed-fed cattle, could have the potential to be used as effective antimicrobials for *L. monocytogenes* through controlling growth as well as virulence attenuation. This not only advances our understanding of the mode of action of LC-UFA against *L. monocytogenes* but also suggests the potential for use of beef fat or its fractions as natural antimicrobials for controlling foodborne pathogens.

KEYWORDS *L. monocytogenes*, beef fat, long-chain unsaturated fatty acids, antimicrobial activity, pathogenicity attenuation

Listeriosis, with sporadic cases and large outbreaks, is largely attributed to the consumption of food contaminated with *Listeria monocytogenes* (1). In healthy persons,

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it can be manifested as febrile gastroenteritis, often associated with consuming highly contaminated food (up to 10^9 CFU/g) (2). In general, listeriosis is mainly found among pregnant women, their fetuses, and immunocompromised persons, with symptoms of abortion, neonatal death, septicemia, and meningitis, and can result from consuming food with low levels of contamination (10^2 to 10^4 CFU/g) (3). The fatality rate of *Listeria* infection can range from 20 to 40% and, as such, is a huge concern for public health, despite its low infection rate (4, 5). The largest foodborne outbreak of listeriosis reported so far was in South Africa in 2017-2018, with 1,060 confirmed cases and 216 deaths, and was linked to consumption of ready-to-eat meat products (6).

L. monocytogenes employs various mechanisms to cope with acid, bile, and osmotic stresses encountered in the gastrointestinal (GI) tract (7). Glutamate decarboxylase (GAD), arginine deiminase (ADI), and agmatine deiminase (AgDI) systems are involved in maintaining internal pH against acid stress in the stomach (8–10). Bile salt hydrolase (Bsh) and the bile exclusion system (BilE) are involved in the response to bile stress (11, 12). In addition, *L. monocytogenes* can accumulate compatible solutes through transport systems (OpuC, BetL, and Gbu) to reduce osmotic pressure (10, 13). Successful survival in the GI tract contributes to the pathogenicity of *L. monocytogenes*. Once inside the gut, the infection cycle of *L. monocytogenes* is initiated by adhesion and subsequent entry into the intestinal epithelial cells via the use of internalins (InlA and InlB) (14). After cell entry, *L. monocytogenes* is entrapped into a vacuole and then escapes with the assistance of the pore-forming toxin listeriolysin O (LLO) and phosphatidylinositol phospholipase C (PI-PLC) (15). Within the cytosol, the hexose phosphate transporter (UhpT) mediates the direct import of phosphorylated sugars for intracellular bacterial growth and replication (16). Meanwhile, ActA promotes host cell actin assembly, allowing *L. monocytogenes* to move to the membrane and to form a membrane protrusion, with the assistance of InlC (17–19). After the invasion of neighboring cells, in addition to LLO, a zinc-dependent metalloprotease (Mpl) and phosphatidylcholine phospholipase C (PC-PLC) are required for efficient lysis of the double-membrane vacuole to continue the next infection cycle (14, 20). Many genes involved in responses to stress in the GI tract and pathogenesis of *L. monocytogenes* are regulated by the alternative sigma factor σ^B and/or the positive regulatory factor A (PrfA) (21) and are thus key targets for antimicrobial intervention.

The antimicrobial effects of medium-chain saturated fatty acids (MC-SFA) and long-chain unsaturated fatty acids (LC-UFA) have been tested against *L. monocytogenes* (22, 23). The potential use of fatty acids as alternatives to antibiotics has drawn more attention in recent years because it is difficult for bacteria to develop resistance to them, due to the multiple-target nature of the mode of action. Work on other Gram-positive bacteria, in particular *Staphylococcus aureus*, has suggested that the disruption of cell membrane structure and perturbation of membrane-associated biological functions (i.e., membrane phospholipid biosynthesis and electron transport) could be involved (23–28). Other detrimental effects on the bacterial cells induced by LC-UFA may include glucosyltransferase inhibition and interference with amino acid uptake, fatty acid synthesis, and peroxidation (29–32). It has been suggested that medium- and long-chain free fatty acids may influence the PrfA-dependent activation of virulence genes in *L. monocytogenes* (33). However, information is lacking on the effects of LC-UFA on the expression of genes involved in survival and adaptation of *L. monocytogenes* within the GI tract, subsequent cell invasion, and how *L. monocytogenes* responds to LC-UFA-related stress.

The objectives of this study were to determine the effectiveness of fatty acids from various sources as natural antimicrobials against *L. monocytogenes* and to evaluate their potential role in *L. monocytogenes* pathogenicity modulation using the MIC assay and transcriptome sequencing (RNA-seq). This included a number of pure commercially available saturated, monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA). In addition, because beef fat can have some of the most complex fatty acid compositions found in nature, due to microbial biohydrogenation of PUFA in the rumen, fatty acids from total beef fat and fractions enriched with PUFA and MUFA

TABLE 1 MICs of commercial fatty acids and beef fat at different pH values against *Listeria monocytogenes* ATCC 19111

Fatty acid		MIC (mg/ml)[b]	
Source	Name	pH 7	pH 5.5
Commercial fatty acids	C14:0	2.00 ± 0.87 $^{B, c}$	>6.00 $^{A, a}$
	C16:0	>6.00 ^{a}	>6.00 ^{a}
	C18:0	>6.00 ^{a}	>6.00 ^{a}
	*c*9-C16:1	0.38 ± 0.00 ^{cd}	0.88 ± 0.43 ^{c}
	*c*9-C18:1	5.00 ± 1.73 ^{b}	>6.00 ^{a}
	*c*9,*t*11/*t*10,*c*12-C18:2	5.00 ± 0.87 ^{b}	3.88 ± 2.50 ^{b}
	C18:2n-6	0.94 ± 0.50 ^{cd}	0.88 ± 0.57 ^{c}
	C18:3n-3	0.25 ± 0.11 ^{cd}	0.28 ± 0.09 ^{c}
Beef fat[a]	BF-MUFA	0.33 ± 0.21 $^{B, cd}$	>6.00 $^{A, a}$
	BF-PUFA	0.06 ± 0.03 $^{B, d}$	1.38 ± 0.22 $^{A, c}$
	BF-T	>6.00 ^{a}	>6.00 ^{a}

[a]Beef fat samples were total beef fatty acids (BF-T), monounsaturated fatty acids (BF-MUFA), and polyunsaturated fatty acids (BF-PUFA).

[b]Within a row, values obtained at different pHs for the same treatment that do not share a capital letter are significantly different ($P < 0.05$). Within a column, values obtained from different treatments at the same pH that do not share a lowercase letter are significantly different ($P < 0.05$).

were utilized to study the effects on *L. monocytogenes*. Specifically, fat from flaxseed-fed cattle was chosen, as it would be relatively rich in α-linolenic acid (C18:3n-3) and its biohydrogenation intermediates, including conjugated linoleic acid, conjugated linolenic acid, *trans*-C18:1, as well as nonconjugated nonmethylene interrupted C18:2 (atypical C18:2) isomers (34, 35).

RESULTS

Antimicrobial activities of fatty acids against *L. monocytogenes*. BF-T, containing 31.69% *c*9-C18:1, 20.65% C16:0, and 7.48% C18:0 (see Table S1 in the supplemental material), did not show any inhibitory activity against *L. monocytogenes* at concentrations up to 6 mg/ml at either pH (Table 1). However, *L. monocytogenes* was susceptible to its fractions, BF-MUFA and BF-PUFA, with MICs of 0.33 ± 0.21 mg/ml, and 0.06 ± 0.03 mg/ml, at pH 7. The corresponding MICs at pH 5.5 were more than 20 times higher ($P < 0.05$) (Table 1). *L. monocytogenes* was not sensitive to the C16:0 or C18:0 saturated fatty acids at concentrations up to 6 mg/ml. However, the C14:0 saturated fatty acid showed an MIC of 2.00 ± 0.87 mg/ml at pH 7 ($P < 0.05$) (Table 1). Following the same trend as saturated FA, *c*9-C18:1 showed higher MIC values than *c*9-C16:1 at both pH values ($P < 0.05$), by more than 4 mg/ml (Table 1). For FA with equal chain length, the MICs of C18:2n-6 and C18:3n-3 were significantly lower than the MIC of *c*9-C18:1 at both pH values ($P < 0.05$) (Table 1). Remarkably, the MIC of the conjugated fatty acid cjC18:2 (*c*9,*t*11/*t*10,*c*12-C18:2) at pH 7 was similar to that of *c*9-C18:1, and the MICs at both pH values were higher than those for C18:2n-6 ($P < 0.05$) by more than 3 mg/ml.

Transcriptome profiling of *L. monocytogenes*. Total post quality control (QC) reads mapped to the sense strand in each sample ranged from approximately 4 to 20 million, with a mean of 9.53 (standard deviation [SD] 3.89). The reads for the antisense strand ranged from 0.05 to 0.24 million, with a mean of 0.14 (SD 0.06). Only the sense strand-specific read counts were used for further analysis. The genome of *L. monocytogenes* EGD-e contains 2,940 annotated genes, of which 2,799 were identified as expressed after filtering out genes with very low expression (Table S3). A principal-component analysis (PCA) plot based on the normalized read counts showed a clear separation between the transcriptome profiles of *L. monocytogenes* ATCC 19111 with different fatty acid treatments and that of the control group (Fig. 1), indicating that fatty acid treatments shifted the gene expression profile differently. The fatty acid treatments with stronger antimicrobial activities (*c*9-C16:1, C18:2n-6, and C18:3n-3) (MICs < 1.00 mg/ml at pH 7; Table 1) clustered together and separate from the control

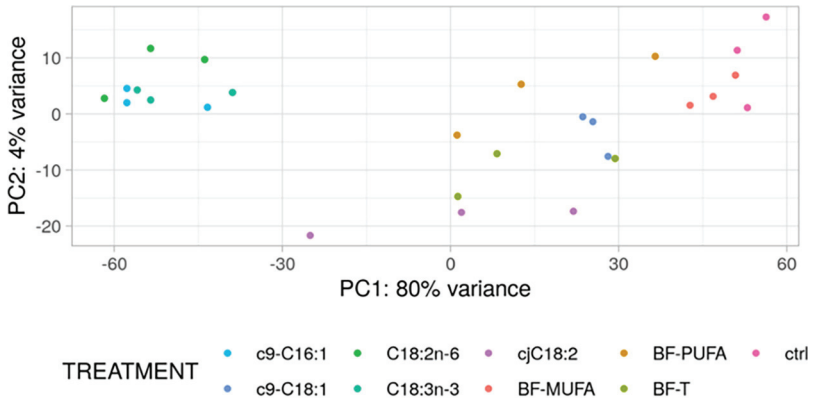


Fig 1 Principal-component analysis score map of global gene expression in *Listeria monocytogenes* ATCC 19111 with or without (ctrl) fatty acid treatments. Beef fat samples were total beef fatty acids (BF-T), monounsaturated fatty acids (BF-MUFA), and polyunsaturated fatty acids (BF-PUFA).

and other treatment groups. While the BF-MUFA samples clustered close to the control group, all remaining treatments formed a loose cluster, also separated from the control group along PC1. The numbers of differentially expressed (DE) genes for each treatment, identified using a fold change threshold of 2.5 and false-discovery rate (FDR)-adjusted *P* value of $\leq$0.05, are shown in Table S4. The numbers are correlated with the distance of separation of treatment groups from control as observed in the PCA plot (Fig. 1). Heatmaps depict the clear distinction in expression profiles of the DE genes in treatment versus control comparisons (Fig. S1). Among the tested fatty acids, *c*9-C16:1, C18:2n-6, and C18:3n-3 impacted the gene expression profile of *L. monocytogenes* to a much greater extent than did other fatty acids.

Gene ontology terms enriched in sets of differentially expressed genes. Fisher's exact tests for statistically overrepresented gene ontology (GO) terms in sets of DE genes using the whole genome of *L. monocytogenes* as reference revealed enriched terms in all three GO categories. The enriched terms in the GO category "molecular function" are depicted in Fig. 2 and those in categories "biological process" and "cellular component" are shown in Fig. S2 and Fig. S3. Because of the large number of DE genes in several of the treatment versus control contrasts, the terms that were found to be significantly enriched not only were many in number but also had terms in common with both the upregulated and the downregulated sets of DE genes. To identify the unique terms that differentiate the contrasts, dot plot overviews of the terms were created using the *dotplot* function of Bioconductor package enrichplot (36). While there were many enriched molecular function terms common to both the up- and downregulated sets of DE genes (Fig. 2), the dot plot also points to some unique differences. For example, the terms related to rRNA, tRNA, ribosome structure, and translation are only found in upregulated sets of genes, from some treatments like *c*9-C16:1, C18:2n-6, C18:3n-3, BF-PUFA, and BF-T, while the majority of transmembrane transporter activity-related terms, particular channel activity, are limited to the BF-PUFA treatment. In contrast, the terms related to certain transmembrane transport systems and phosphorus-dependent energy metabolism are specific to the downregulated sets of DE genes, mainly in treatments *c*9-C16:1, C18:2n-6, and C18:3n-3. Plots depicting enriched biological process terms indicated that ribosome, amino acid, peptide, and protein biosynthetic and metabolic processes were upregulated across treatments, while carbohydrate catabolic processes were downregulated in general (Fig. S2). Finally, plots depicting enriched cellular component terms showed ribosome-related terms in upregulated sets of genes from all treatments and "ATPase complex" terms only in the upregulated sets of genes from BF-PUFA treatment (Fig. S3). The reader is pointed to these plots for a more detailed overview and to the tabulated list of enriched GO terms in all treatments (Table S5).

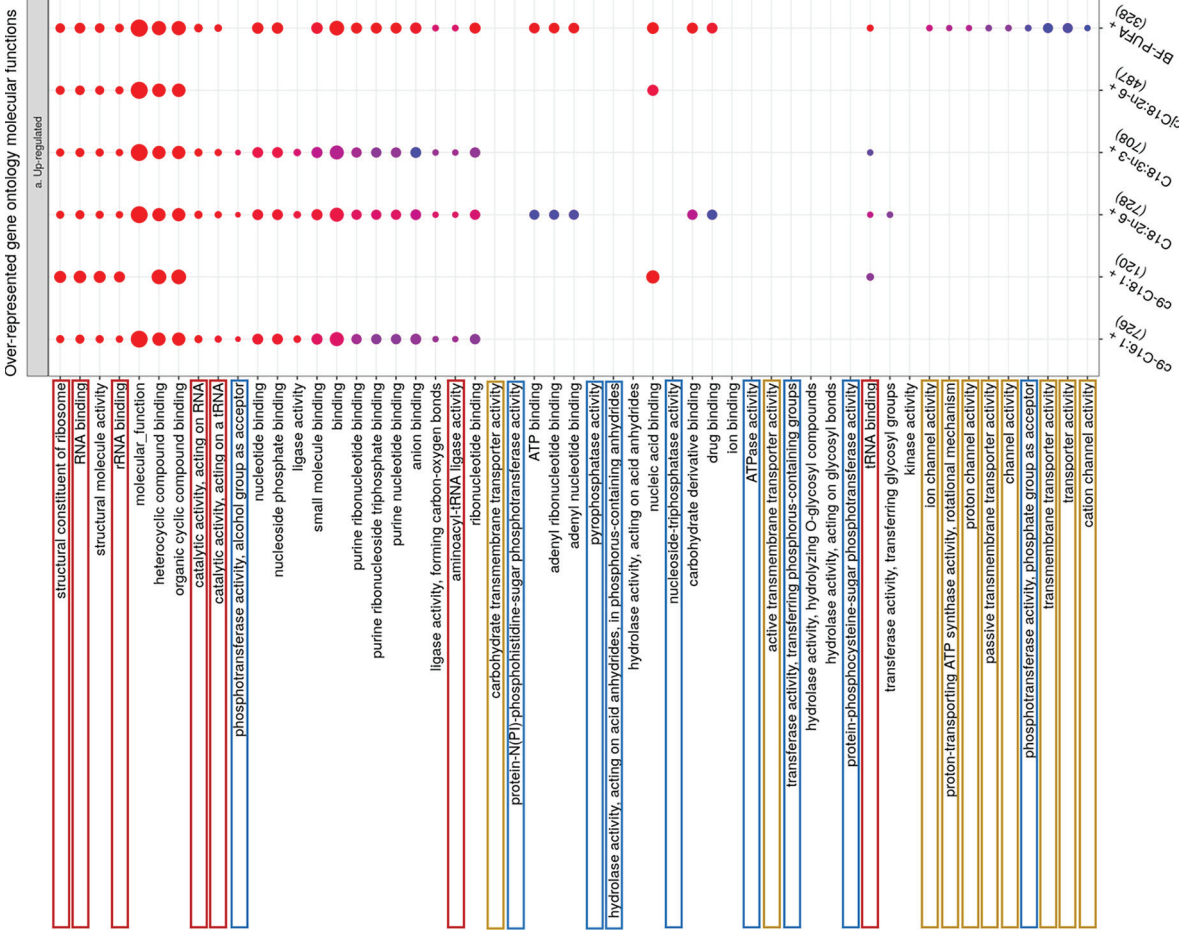
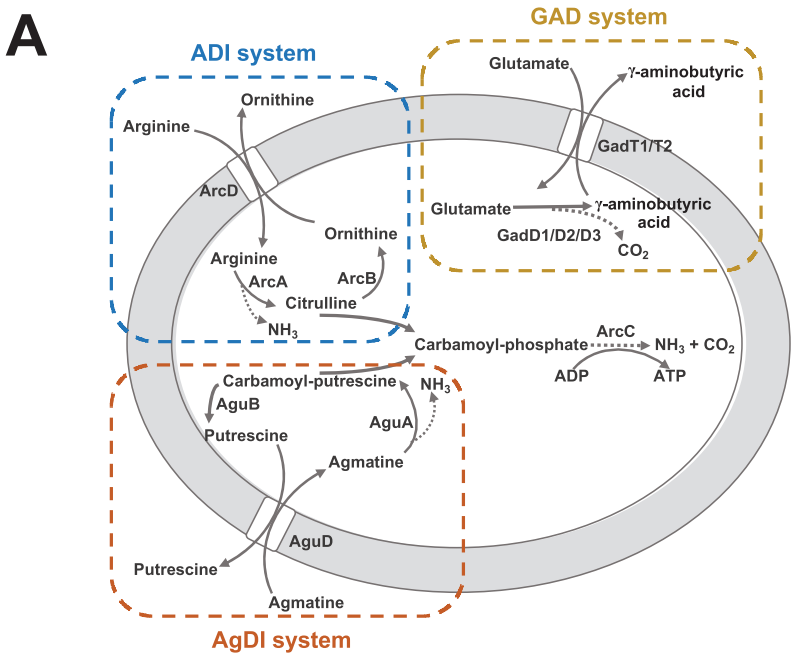
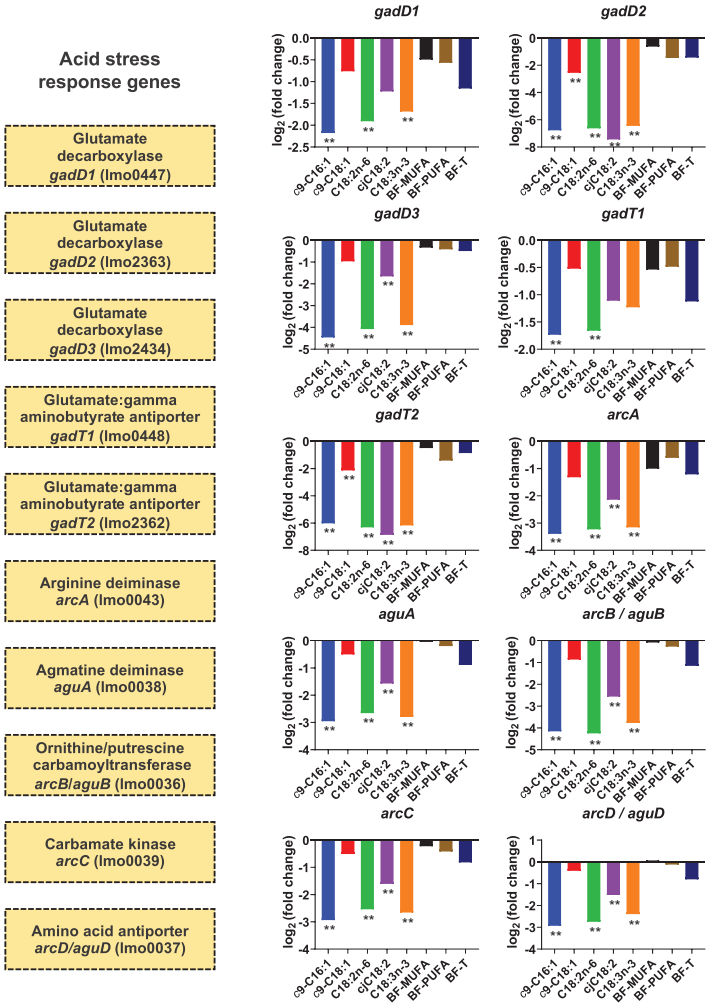


Fig 2 Dot plot summary of all overrepresented gene ontology molecular function terms in sets of differentially expressed genes in *Listeria monocytogenes* ATCC 19111 treated with fatty acids and contrasted with control. The size of the dot indicates the gene ratio, i.e., the ratio of the number of genes annotated to that term of the total identified as up- or downregulated for that comparison. The color of the dot indicates the adjusted *P* value (FDR) for that term based on the overrepresentation test used. Numbers of upregulated or downregulated genes for respective fatty acid treatment are shown in the brackets. Beef fat samples were total beef fatty acids (BF-T), monounsaturated fatty acids (BF-MUFA), and polyunsaturated fatty acids (BF-PUFA). The terms related to rRNA, tRNA, ribosome structure, and translation are in red frames, the terms related to transmembrane transporter activity are in yellow frames, and those related to phosphorus-dependent metabolism are in blue frames.

Expression profile of pathogenicity-associated genes. The expression levels of genes related to the acid, bile, and osmotic stress responses, as well as the virulence factors, were evaluated to investigate the role of the selected FA in the pathogenicity of *L. monocytogenes*. For acid resistance, treatments with *c*9-C16:1, C18:2n-6, and C18:3n-3 downregulated the expression levels of genes associated with the GAD system, including *gadD1*, *gadD2*, *gadD3* (encoding glutamate decarboxylase), and *gadT2* (encoding a glutamate:gamma aminobutyrate antiporter), as well as the ADI and AgDI system, including *arcA* (encoding arginine deiminase), *aguA* (encoding agmatine deiminase), *arcB*/*aguB* (encoding ornithine/putrescine carbamoyltransferase) (37), *arcC* (encoding carbamate kinase), and *arcD*/*aguD* (encoding an antiporter), by up to 174-fold (fold change $>$2.5, FDR $<$0.05) (Fig. 3A). *c*9-C18:1 inhibited the expression of *gadT2* and *gadD2* only, and cjC18:2 repressed all but *gadD1* and *gadT1* in these systems. BF-T and its fractions had no significant effect on the expression level of any of these genes. For genes related to the bile stress response, *bsh* (encoding bile salt hydrolase) was downregulated by all treatments except for BF-PUFA, which did not have any significant effect, but *btlA* (a bile tolerance locus) was upregulated by all treatments except for *c*9-C18:1 and BF-MUFA, for which no significant effects were noted (Fig. 3B). Genes of the bile tolerance locus *btlB*, ABC transporter ATP-binding protein-encoding *bilEA*, and ABC transporter permease-encoding *bilEB* were largely unaffected by any of the treatments except for *c*9-C16:1, which downregulated *bilEA*. For the osmotic stress response, carnitine transporter-associated genes *opuCA*, *opuCB*, *opuCC*, and *opuCD* were all downregulated by treatments *c*9-C16:1, C18:2n-6, and C18:3n-3. However, glycine betaine transporter-related genes *gbuA*, *gbuB*, and *gbuC* were upregulated by all the fatty acid treatments except for BF-MUFA (Fig. 3C). Glycine betaine transporter *betL* was not significantly affected by any of the treatments. In addition to the genes involved in the bile, acid, and osmotic stress responses, genes encoding σ^B, the key transcriptional regulator essential for responding to environmental stress conditions, and the stressosome (RsbR, RsbS, and RsbT) were also examined in terms of their response to fatty acid treatments. There was no significant effect on the expression level of any of these genes with any of the treatments (data not shown). In the genome of strain EDG-e, 240 protein-coding genes are likely under the regulation of σ^B (38), 142, 34, 134, 134, 96, 23, 18, and 41 of which were among the DE genes in *L. monocytogenes* treated with *c*9-C16:1, *c*9-C18:1, C18:2n-6, C18:3n-3, cjC18:2, BF-MUFA, BF-PUFA, and BF-T, respectively. The great majority of those DE genes (89 to 100%) were downregulated by different FA treatments, with the downregulated genes being 134, 33, 126, 126, 91, 23, 16, and 38, respectively. The expression levels of the 10 confirmed genes in the PrfA regulon, responsible for the key virulence determinants in *L. monocytogenes* (39), were examined. They included genes encoding internalins (*inlA*, *inlB*, and *inlC*), listeriolysin O (*hly*), phosphatidylinositol phospholipase C (*plcA*), zinc metalloproteinase (*mpl*), hexose phosphate transporter (*uhpT*), actin assembly-inducing protein (*actA*), and phosphatidylcholine phospholipase C (*plcB*), as well as positive regulatory factor A (*prfA*) (Fig. 3D). Treatments with *c*9-C16:1, C18:2n-6, and C18:3n-3 downregulated all genes except for *mpl*, for which no significant impact was observed with any of the treatments, while *c*9-C18:1 and BF-MUFA resulted in no significant changes in the expression level of any of these genes. On the other hand, cjC18:2, BF-T, and BF-PUFA inhibited the expression of certain key virulence genes, such as the genes encoding the master regulator *prfA* and listeriolysin O *hly*.

The expression levels of genes involved in the oxidative stress response in *L. monocytogenes* were assessed. Treatments with *c*9-C16:1, C18:2n-6, and C18:3n-3 significantly inhibited almost all genes associated with oxidative stress tolerance, including *trxA*, *trxB*, *gsr2*, *oxr*, *sod*, and *fri*, which encode thioredoxin, thioredoxin reductase, glutathione reductase, oxidoreductase, superoxide dismutase, and ferritin, respectively (Fig. 4). Downregulation of some of those genes was noted for cjC18:2 and BF-PUFA, but none was observed for BF-T and BF-MUFA. For the cell envelope stress response, the expression levels of two-component systems LisRK, CesRK, VirRS, and LiaSR (40) and





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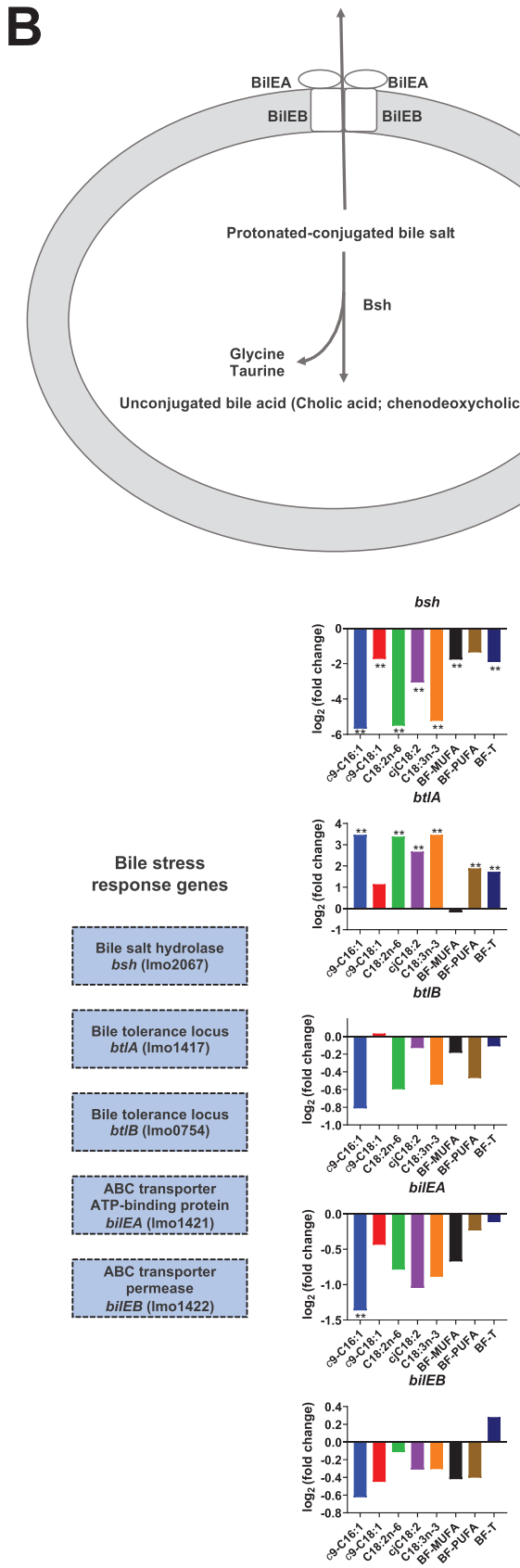

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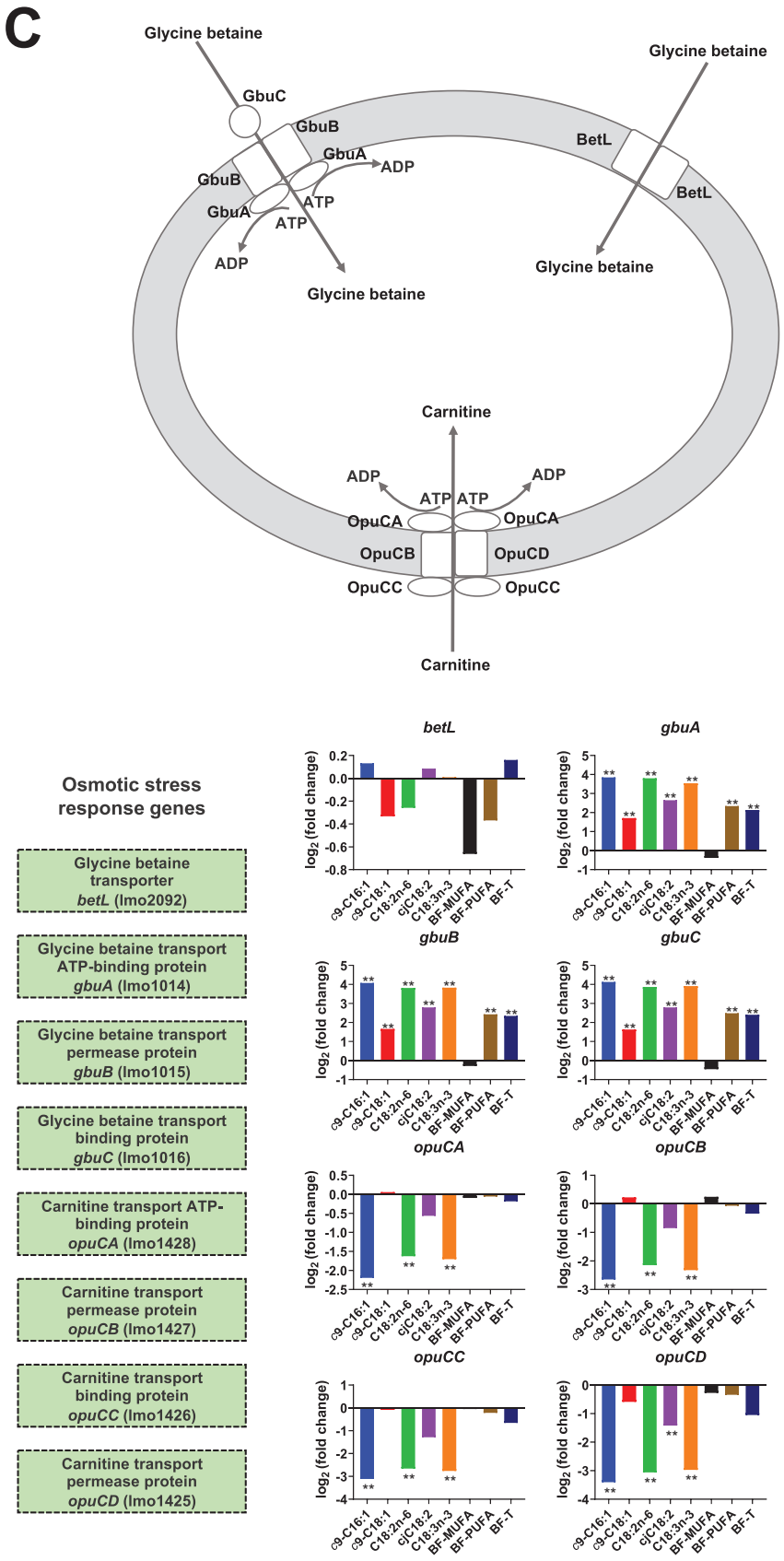

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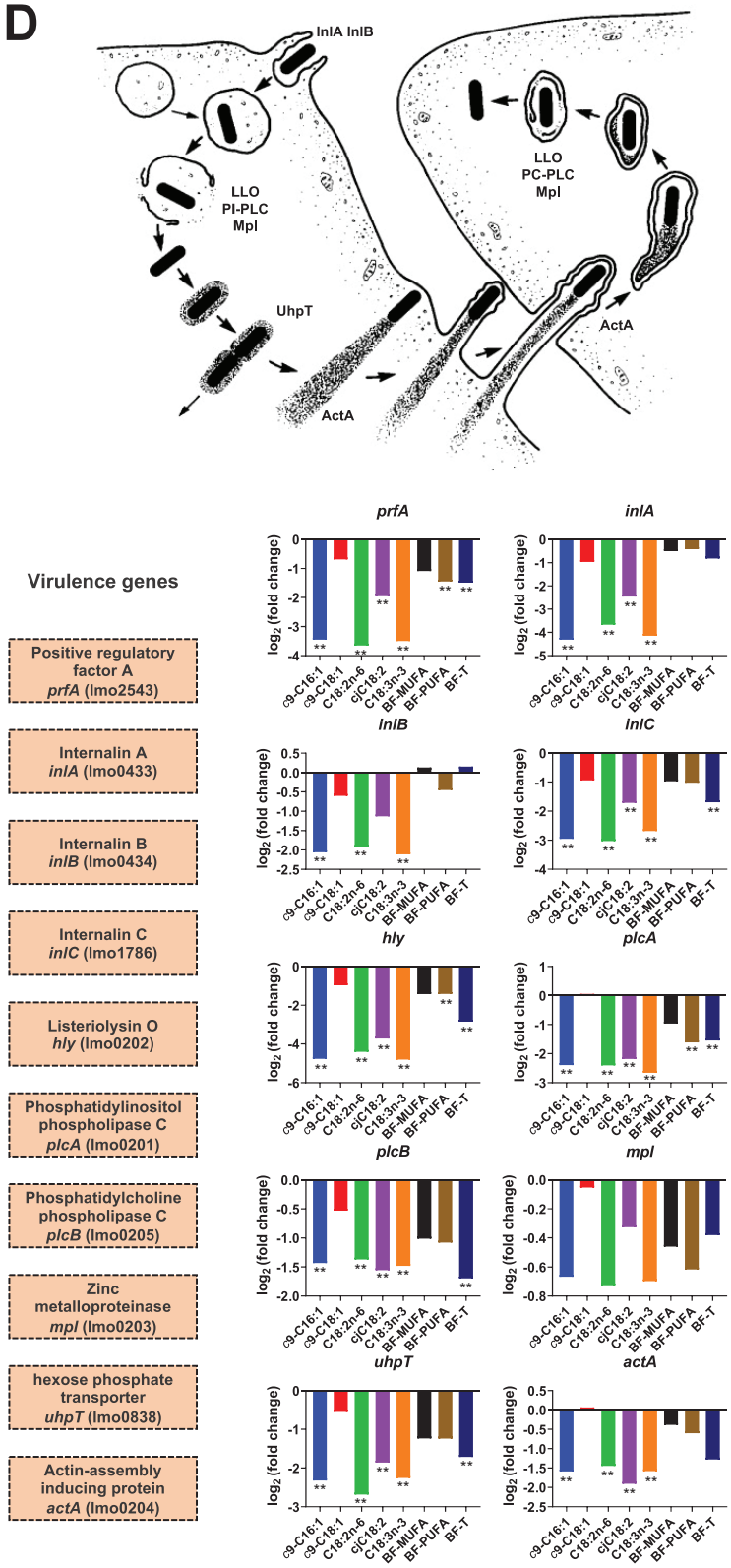

Fig 3 Expression levels of key genes involved in the pathogenesis of *Listeria monocytogenes* ATCC 19111 in response to fatty acid treatments. Shown are DE genes related to acid resistance (A), bile resistance (B), and osmotic stress response (C) and virulence (D). Schematic representation of the infectious process by *L. monocytogenes* was adapted from Tilney and Portnoy (92). Asterisks indicate genes with significant changes in expression (fold change >2.5, FDR <0.05) under various fatty acid treatments relative to control. Beef fat samples were total beef fatty acids (BF-T), monounsaturated fatty acids (BF-MUFA), and polyunsaturated fatty acids (BF-PUFA).

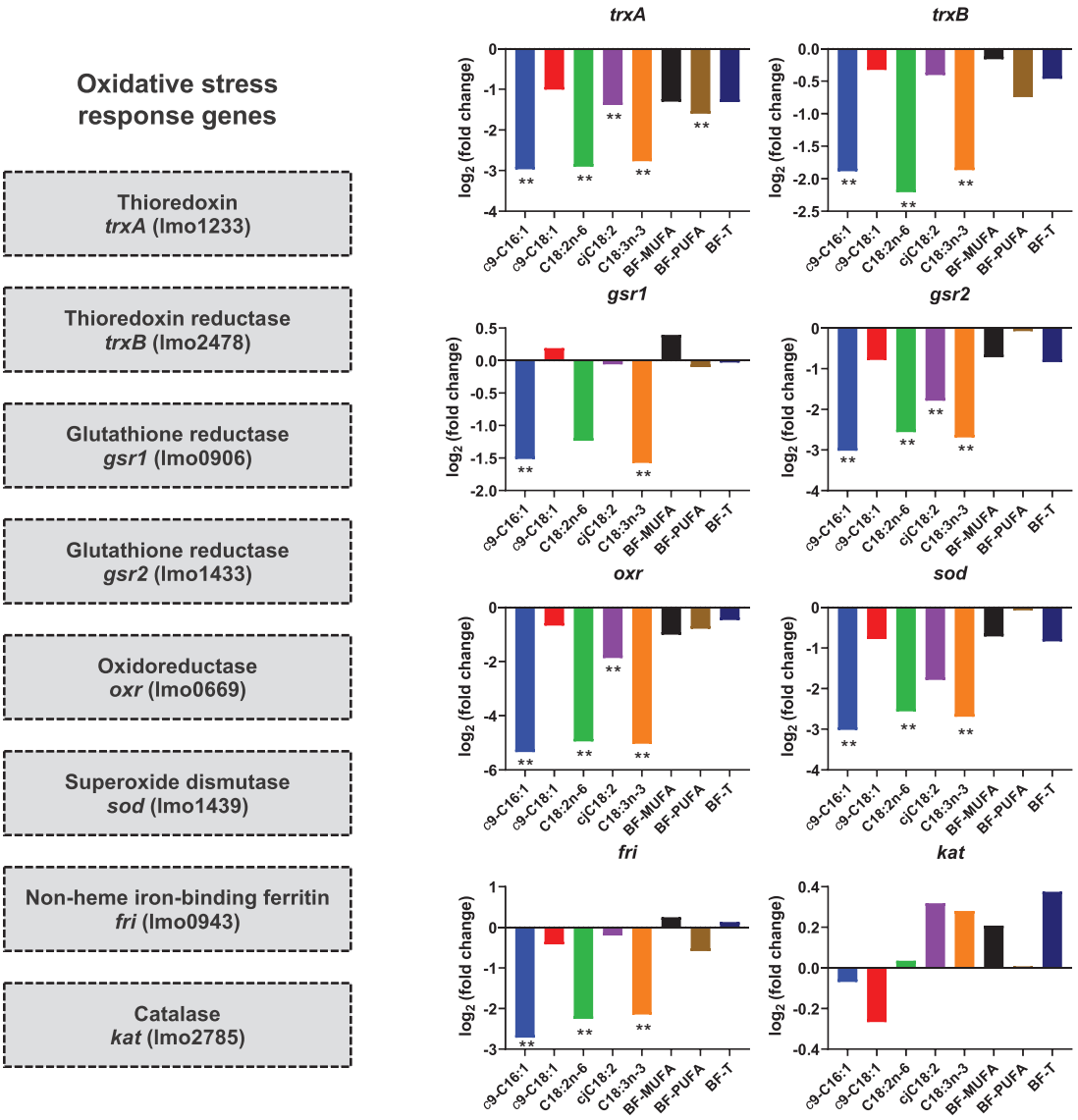

Fig 4 Expression levels of genes involved in the oxidative stress response of *Listeria monocytogenes* ATCC 19111 in response to fatty acid treatments. Asterisks indicate genes with significant changes in expression (fold change >2.5, FDR <0.05) under various fatty acid treatments relative to control. Beef fat samples were total beef fatty acids (BF-T), monounsaturated fatty acids (BF-MUFA), and polyunsaturated fatty acids (BF-PUFA).

genes under their regulation were evaluated. Of the genes encoding the four two-component systems, only *cesR* was significantly impacted; it was upregulated by treatments *c*9-C16:1, C18:2n-6, cjC18:2, C18:3n-3, and BF-T by up to 3-fold (Table S3). Of the genes under regulation by two-component systems, lmo1037 and lmo1215 were significantly upregulated (Table S3).

Validation of RNA-seq data by RT-qPCR. To validate the gene expression profiles obtained by RNA-seq analysis, virulence genes *prfA*, *hly,* and *actA* were selected and their expression levels were quantified by reverse transcription-quantitative PCR (RT-qPCR). The fold changes estimated from RNA-seq showed high correlation with those from the RT-qPCR analysis (Fig. S4).

DISCUSSION

Compared to Gram-negative bacteria, Gram-positive bacteria are more sensitive to medium- or long-chain free fatty acids (41). In this study, BF-MUFA and BF-PUFA

fractions exhibited very strong inhibitory activities against *L. monocytogenes*, with MICs of 0.33 and 0.06 mg/ml at neutral pH, respectively. Among the major constituents of BF-T as represented by the commercial fatty acids, C14:0 was more bacteriostatic than the longer-chain saturated fatty acids (SFA) C16:0 and C18:0. This is likely because C14:0 has a better balance between water solubility and sufficient lipophilicity to effectively interact with the hydrophobic cell surface of the bacterium in aqueous solution (42). However, the addition of double bonds affords fatty acids more solubility and lipophilic activity, more so in the *cis* rather than the *trans* configuration (43). Compared to the corresponding SFA, UFA of the same chain length exerted stronger antimicrobial activity, and the inhibitory effects increased with an increasing number of double bonds in fatty acids. Of all FA tested, BF-PUFA had the lowest MIC at pH 7, suggesting that some minor components or combinations of FA in this beef fat fraction have stronger antimicrobial efficacy than C18:2n-6 and C18:3n-3. These findings are in agreement with previous reports on the impact of chain length, degree of unsaturation, and configuration of double bonds on the antimicrobial activity of FA (44–49). The effect of pH on MICs was FA-dependent, i.e., ranging from no effect (*c*9-C16:1, C18:2n-6 and C18:3n-3, and cjC18:2) to lower efficacy at lower pH (C14:0, BF-MUFA, and BF-PUFA). No information on the effect of pH on the MICs of FA against *L. monocytogenes* appears to be available in the literature. The beneficial effect of lower pH on the minimum bactericidal concentrations (MBC) of medium- to long-chain FA has been reported for *L. monocytogenes* (22). For the Gram-negative bacterium *Escherichia coli*, however, a chain-length-dependent effect of pH was observed when tested against C2 to C18 fatty acids (50). Higher efficacy at lower pH was observed for C8 and C10 but not for C2 to C6 or C12 to C18 FA.

Most of the FA induced differential expression of a large number of genes, with *c*9-16:1, C18:2n-6, and C18:3n-3 inducing the highest number of changes and impacting approximately 50% of the coding genes in *L. monocytogenes*. This could be due to FA eliciting a general response in the bacterium, the multiple target nature of which makes the development of resistance in bacteria less likely, compared to antibiotic treatment. Even so, the FA tested in this study impacted gene expression differently, reflected not only in the number of genes but also in the ratio between up- and downregulated genes. As MIC was used for all FA, it is tempting to suggest that, in addition to affecting the structural component of cells, they may also impact regulatory genes differently. Even though BF-MUFA primarily contained C18:1 (56.5%), it impacted a much smaller number of genes than commercial *c*9-C18:1 (84 versus 180). This is likely due to the latter having a much higher MIC (0.33 versus 5.00 mg/ml) and, as such, the amount of *c*9-C18:1 in BF-MUFA was much lower in the samples treated with BF-MUFA. These findings also suggest that other components in BF-MUFA are likely stronger antimicrobials than commercial *c*9-C18:1.

ATP generation by ATP synthase requires coupled electron transport and oxidative phosphorylation, which occurs in the membrane (51). It has been demonstrated that C18:2n-6 can uncouple oxidative phosphorylation, thus limiting oxygen uptake and interfering with ATP regeneration in *S. aureus* and *Bacillus subtilis* (25, 29, 52). In *S. aureus*, accumulation of *c*9-C16:1 triggered cell growth arrest, which was correlated with disruption of cytoplasmic membrane and leakage of solutes and low-molecular-weight proteins into the medium (24). In this study, only BF-PUFA upregulated genes in the proton transport two-sector ATPase complex, proton-transporting ATP synthase complex, membrane protein complex, and plasma membrane (Fig. S3). The reason for this difference is unclear. Numerous translation- and ribosome-associated genes were upregulated by most LC-UFA, but carbohydrate catabolic activity-associated genes were downregulated. Of the four two-component systems (CesRK, LiaSR, VirRS, and LisRK) involved in the cell envelope response, only *cesR* was significantly impacted (upregulated by *c*9-C16:1, C18:2n-6, cjC18:2, C18:3n-3, and BF-T). A signaling effect of LC-UFA on the two-component system in

Salmonella enterica has also been observed (53). These findings suggest *L. monocytogenes* may use increasing protein synthesis and two-component systems as compensatory responses and repair mechanisms to maintain cellular biophysical function in combating damage in membrane and energy production caused by LC-UFA. Taken together, these findings are in line with previously reported potential antimicrobial mechanisms of LC-UFA in cell membrane perturbation, dislocation of membrane-associated enzymes, and, consequently, disruption of downstream biochemical processes (54, 55). The bactericidal effects of polyunsaturated fatty acids have also been attributed to the peroxidation process (56, 57). The downregulation of most of the oxidative stress response genes, such as those encoding thioredoxin, thioredoxin reductase, glutathione reductase, oxidoreductase, superoxide dismutase, ferritin, and catalase (58–60), may have exacerbated the antimicrobial effects of *c*9-16:1, C18:2n-6, and C18:3n-3 on *L. monocytogenes*.

MC-SFA- and LC-UFA-mediated inhibition of virulence factor expression has been demonstrated in *Salmonella*, *Vibrio cholerae*, and *L. monocytogenes* (33, 61, 62). In both *Salmonella* and *V. cholerae*, the inhibition was through direct interaction of FA with regulatory proteins (53, 63). As for *L. monocytogenes*, specific FAs (eicosapentaenoic acid C20:5, linolenic acid C18:3, palmitoleic acid *c*9-16:1, and lauric acid C12:0, compared to palmitic acid C16:0 and stearic acid C18:0) prevented the PrfA-dependent activation of the *hly* promoter transcription. The findings of this study on the downregulation of *prfA* expression by LC-UFA, as well as genes under its regulation, are in line with these previous reports in that certain FAs attenuate virulence in *L. monocytogenes*, likely by direct inhibition of PrfA activity, making it unavailable for the activation of transcription of virulence genes under its direct regulation.

L. monocytogenes has multiple systems, which are essential for its survival and pathogenesis, to cope with stress encountered in the GI tract (58). To maintain pH homeostasis in gastric fluid, *L. monocytogenes* employs the GAD system to take up glutamate into the cell (via GadT1 or GadT2), which is converted to γ-aminobutyric acid (GABA; via GadD1, GadD2, or GadD3) with consumption of intracellular protons. Subsequently, GABA is transported out of the cell in exchange for another glutamate (8, 64). Also, ADI and AgDI systems are utilized by *L. monocytogenes* to accumulate intracellular arginine and agmatine (via ArcD and AguD), which yield ammonia as the by-product in subsequent reactions (via ArcA, ArcC, and AguA) (Fig. 3A) (9, 65). Bile salt hydrolase (Bsh) detoxifies bile through hydrolyzing and deconjugating glycine or taurine from the sterol core of primary bile acids (Fig. 3B) (66). Activity of the bile exclusion system dependent on both ATP-binding protein BilEA and permease BilEB, as well as the bile tolerance locus (Fig. 3B), remains unchanged upon exposure to LC-UFA (12, 67, 68). *L. monocytogenes* is able to accumulate compatible solutes using membrane transporters to maintain cell turgor (Fig. 3C). The uptake of glycine betaine is conducted via transporters BetL and Gbu, and the uptake of carnitine is by OpuC (10). Compared to BetL and Gbu, OpuC plays a greater role in listerial pathogenesis, and the uptake of glycine betaine by Gbu is preferred during growth in BHI broth (69). Key genes involved in the acid, bile, and osmotic stress responses were primarily downregulated by FA treatments, with *c*9-C16:1, C18:2n-6, and C18:3n-3 having similar and the most significant impact, followed by cjC18:2 and *c*9-C18:1. *L. monocytogenes* is known for its ability to survive and adapt in stressful environments, and the stress response genes are primarily regulated by the alternative sigma factor σ^B through the activation of their transcription; this subject has been reviewed extensively (38, 70, 71). σ^B also interplays with PrfA in regulating some virulence and stress response genes, for instance, transcription of *arcA*, *bsh*, *bilE*, and *opuC* and *inlA* and *inlB* (9, 12, 69, 72). In fact, transcription of *prfA*, in addition to its self-regulation, is also regulated by σ^B. These observations, together with the finding that expression of *sigB* was not affected by any of the treatments, suggest that specific FAs may block SigB directly or

indirectly, preventing it from activating genes under its regulation, including those involved in stress resistance and virulence.

In conclusion, BF-MUFA and BF-PUFA were potent antimicrobials against *L. monocytogenes*, with MIC values similar to or smaller than those of commercial, pure LC-UFA. Of the LC-UFAs, *c*9-16:1, C18:2n-6, and C18:3n-3 behaved similarly in both antimicrobial potency and impact on the gene expression of *L. monocytogenes*. The enhanced ribosome assembly and translation processes in response to LC-UFA are possibly associated with the repair mechanisms. LC-UFAs, especially *c*9-16:1, C18:2n-6, and C18:3n-3, downregulated many genes involved in virulence and GI tract survival of *L. monocytogenes*, likely by modulating the activity of the virulence regulator PrfA and the alternative sigma factor σ^B. This study demonstrates that LC-UFAs, including beef fat fractions from flaxseed-fed cattle, have the potential to be used as effective antimicrobials for *L. monocytogenes* through controlling growth as well as virulence attenuation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Listeria monocytogenes* (ATCC 19111) was obtained from the American Type Culture Collection and maintained as stock cultures at −80°C in brain heart infusion (BHI) broth (Oxoid, Mississauga, ON, Canada) supplemented with 15% glycerol (Fisher Scientific, Edmonton, AB, Canada). The stock culture was streaked on BHI agar which was incubated at 35°C for 24 h. A single colony from BHI agar was then inoculated into 10 ml of BHI broth, followed by a 24-h aerobic incubation at 35°C to obtain stationary-phase cultures.

Fatty acids. Commercial free fatty acids C14:0 (myristic acid), C16:0 (palmitic acid), C18:0 (stearic acid), *cis*(*c*)9-C16:1 (palmitoleic acid), *c*9-C18:1 (oleic acid), *c*9,*t*11/*t*10,*c*12-C18:2 (conjugated linoleic acid; cjC18:2), C18:2n-6 (linoleic acid), and C18:3n-3 (α-linolenic acid) with >99% purities were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). Beef fat from banked backfat samples collected from Angus cross steers that were fed a hay-based diet containing 12.5% flaxseed (34) was saponified to free fatty acids. Total beef fatty acids (BF-T) were then further fractionated to monounsaturated (BF-MUFA) and polyunsaturated (BF-PUFA) rich fractions using a combination of solid-phase extraction (Ag^+-SPE) and silver-ion high-performance liquid chromatography (Ag^+-HPLC) using procedures described by Turner et al. (73). The fatty acid compositions of BF-T and its fractions are listed in Table S1.

Determination of the antimicrobial activity of fatty acids. Antimicrobial activities of the fatty acids were assessed by determination of the MICs, using a microplate assay (74). The assay was carried out at both pH 7.0 and pH 5.5, as low pH may enhance the antimicrobial activities of some fatty acids (75). A stock solution for each fatty acid or fatty acid fraction (24 mg/ml) was prepared in BHI containing 10% (vol/vol) dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Oakville, ON, Canada). The potential impact of DMSO on the growth of *L. monocytogenes* in BHI was examined and no inhibitory effect was noted at concentrations up to 3.13%. As such, the maximum concentration of DMSO used throughout this study was 2.5%. Fatty acid stocks (100 μl) were each mixed with BHI (100 μl) and then 2-fold serially diluted in 96-well microplates (Nunc MicroWell 96-well plates; Fisher Scientific). Stationary-phase cultures of *L. monocytogenes* were diluted to approximately 10^5 CFU/ml and aliquots (100 μl) of the cell suspensions were added to the fatty acid dilutions. For positive controls, fatty acid dilutions were replaced by BHI. For negative controls, bacterial suspensions were replaced by BHI. The plates with BHI at pH 7 were incubated at 35°C for 24 h, while those at pH 5.5 were incubated for 48 h. After incubation, a solution (80 μl) of 0.2 mg/ml *p*-iodonitrotetrazolium violet (*p*-INT) (Sigma-Aldrich) was added to each well and the plates were incubated at 35°C for 2 h to detect bacterial growth (76). The colorless tetrazolium salt acts as an electron acceptor and is reduced to a red-colored formazan product by biologically active organisms. The MIC for a fatty acid was defined as the lowest concentration at which no red color was observed (76). MIC values were determined by three independent experiments, each with two technical replicates ($n = 6$). Significant differences in MICs obtained from different fatty acid treatments at the same pH were determined by one-way analysis of variance (ANOVA); significant differences in MICs obtained from the same fatty acid treatment at different pH values were determined using Student's *t* test.

RNA isolation and sequencing. BF-T, BF-MUFA, and BF-PUFA and all commercial fatty acids except for C16:0 and C18:0, which did not show any antimicrobial effect at either pH at the maximum concentration of 6 mg/ml tested, were examined for their effects on the global gene expression of *L. monocytogenes*. *L. monocytogenes* was grown in BHI (pH 7) at 35°C to an optical density at 600 nm (OD_{600}) of 0.35 (33). Fatty acid solutions prepared in BHI supplemented with DMSO were then added to the *L. monocytogenes* BHI cultures to final concentrations of their respective MICs or 6 mg/ml if the MICs were ≥6 mg/ml, followed by incubation at 35°C with shaking at 80 rpm. Cultures with BHI supplemented only with 2.5% DMSO were included as positive controls. After 3 h of FA exposure, two volumes of RNAprotect (Qiagen, Mississauga, ON, Canada) were added to the cultures, and cells were harvested by centrifugation at 5,000 × *g* for 10 min and stored at −80°C. Total RNA was extracted from the cell pellets using an RNeasy Plus universal minikit (Qiagen) following the manufacturer's instructions. Each fatty acid

treatment was compared with control samples in three independent experiments. One treatment, C14:0, failed the RNA quality check and was then excluded from sequencing.

A strand-specific sequencing of the mRNA in total RNA was carried out by Genome Quebec (McGill University and Genome Quebec Innovation Centre, Montreal, QC, Canada). Briefly, RNA was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.) and its integrity was determined with a 2100 Bioanalyzer (Agilent Technologies). RNA samples with RNA integrity number (RIN) scores higher than 7.0 were used for the library construction and RNA-seq analysis. Bacterial rRNA was initially depleted using a Ribo-Zero rRNA removal kit specific for bacteria rRNA (Illumina), and then the remaining RNA was cleaned up using an Agencourt RNAClean XP kit (Beckman Coulter). Double-stranded cDNA was generated with NEBNext RNA first-strand synthesis and NEBNext Ultra Directional RNA second-strand synthesis modules (New England BioLabs) and converted to libraries using a NEBNext Ultra II DNA library prep kit (New England BioLabs). Libraries were quantified using a Quant-iT PicoGreen dsDNA assay kit (Life Technologies) and a Kapa Illumina GA with revised primers-SYBR Fast universal kit (Kapa Biosystems). The average fragment size was determined with a LabChip GX (PerkinElmer) instrument. The libraries were sequenced on an Illumina HiSeq4000 platform to generate 100-bp paired-end reads.

Bioinformatics and statistical analysis of RNA-seq data. The quality of sequence data was assessed with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (77) prior to and after performing quality control (QC) steps that included quality-based read trimming and adapter removal using the Trimmomatic (78) program (version 0.39) ILLUMINACLIP:/adaptors.fa:2:30:10 LEADING:20 TRAILING:20 SLIDINGWINDOW:4:15 MINLEN:75, where adaptors.fa is a fasta file containing the oligonucleotide sequences of the Illumina adapters used in library preparation kits. Reads that passed QC were mapped to the reference genome of *Listeria monocytogenes* EGD-e (ASM19603v1 assembly retrieved from release 43 of Ensembl Bacteria), which shares the same serotype (1/2a) with *L. monocytogenes* ATCC 19111. The read mapping software used was Bowtie2 (79) (version 2.3.5) with the preset option '-very-sensitive-local' selected. Read counts per gene were summarized using featureCounts (version 1.6.4) (80) in strand-specific mode for all genes in the gene annotation file corresponding to the ASM19603v1 assembly (from release 43 of Ensembl Bacteria).

Differential expression (DE) analyses were conducted using the Bioconductor package "edgeR" 3.28.1 (81) in R statistical programming language (version 3.6.0) on read counts from the sense strand. Genes with very low expression were filtered out, keeping only those that were expressed at counts per million (CPM) values that corresponded to a read count over 10 in at least 3 samples. Expression data were normalized with the trimmed mean of M-values (TMM) method to adjust compositional differences between the libraries (82). To visualize the general effects of fatty acid treatments on global gene expression in *L. monocytogenes*, a principal-component analysis (PCA) was performed based on normalized CPMs of expressed genes using the *plotPCA* function of the Bioconductor package DESeq2 1.26.0 (83). DE was evaluated between each of the eight treatment groups versus the control group by fitting a negative binomial generalized linear model (84), and genes were defined as differentially expressed if their absolute fold change was above 2.5 and false discovery rate (FDR)-adjusted *P* value using the Benjamini-Hochberg procedure was below 0.05. A power analysis was conducted using the Bioconductor package RNASeqPower 1.26.0 (85), which suggested that a fold change of 2.5 could be reliably detected at a power of 0.9 with the sample size of 3 per group and accounting for the average depth of coverage and biological variation in the groups. Finally, sets of DE genes were tested for enriched gene ontology terms using Fisher's exact test through the statistical overrepresentation tool in Panther (version 15.0) (86). Those terms with FDR-adjusted *P* values below 0.05 and supported by at least 5 genes were considered significant. Dot plot overviews of the enriched GO terms were created using the dotplot function of Bioconductor package enrichplot v1.6.1.

Validation of RNA-seq data by reverse transcription-quantitative PCR. The virulence genes *prfA*, *hly*, and *actA* detected in the RNA-seq data set were selected for validation using reverse transcription-quantitative PCR (RT-qPCR), with *gyrA* as a housekeeping reference gene (87). Primers used to quantify these genes are listed in Table S2. Total RNA was reverse transcribed to cDNA using a QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions. Real-time PCR was performed with the cDNA as the template using Brilliant II SYBR green PCR master mix (Agilent Technologies, Santa Clara, CA, USA) on a Stratagene M × 3005P PCR machine (Agilent Technologies, Waldbronn, Baden-Württemberg, Germany), with conditions described previously (88–90). The expression levels of virulence genes in response to fatty acids relative to the DMSO control were calculated according to the threshold cycle ($2^{-\Delta\Delta CT}$) method, with *gyrA* as a reference gene (91). DNase-treated RNA samples were also included in the amplification as negative controls. This experiment was carried out in three independent replicates, each with two technical replicates ($n = 6$). Correlations between the $\log_2$ fold change of gene expression levels estimated by RNA-seq and RT-qPCR were calculated using Spearman's rank correlation.

Data availability. All sequences were deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under BioProject PRJNA701752.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.7 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.9 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.

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