Potential of oxygen and nitrogen reactive intermediates to disperse *Listeria monocytogenes* from biofilms

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Abstract

Studying biofilm dispersal is important to prevent *Listeria monocytogenes* persistence in food processing plants and to avoid finished product contamination. Reactive oxygen and nitrogen intermediates (ROI and RNI, respectively) may trigger cell detachment from many bacterial species biofilms, but their roles in *L. monocytogenes* biofilms have not been fully investigated. This study reports on ROI and RNI quantification in *Listeria monocytogenes* biofilms formed on stainless steel and glass surfaces; bacterial culture and microscopy combined with fluorescent staining were employed. Nitric oxide (NO) donor and inhibitor putative effects on *L. monocytogenes* dispersal from biofilms were evaluated, and transcription of genes (*prfA*, *lmo 0990*, *lmo 0807*, and *lmo 1485*) involved in ROI and RNI stress responses were quantified by real-time PCR (qPCR). Microscopy detected the reactive intermediates NO, peroxyxynitrite, H₂O₂, and superoxide in *L. monocytogenes* biofilms. Neither NO donor nor inhibitors interfered in *L. monocytogenes* growth and gene expression, except for *lmo0990*, which was downregulated. In conclusion, ROI and RNI did not exert dispersive effects on *L. monocytogenes* biofilms, indicating that this pathogen has a tight control for protection against oxidative and nitrosative stresses.

Keywords Dispersal · Reactive oxygen intermediate · ROI · Reactive nitrogen intermediate · RNI

Introduction

Listeria monocytogenes causes a rare, but life-threatening foodborne illness named listeriosis, which has been linked to ready-to-eat food consumption; food contamination usually occurs after heat treatment. Post-processing contamination is a concern for the food industry, especially if we consider that many bacteria can persist in the environment due to biofilm formation [1].

Biofilms are sessile microorganism communities attached to biotic or abiotic surfaces, embedded in a self-produced polymeric matrix. To eradicate this “city of microbes,” it is crucial to understand the steps that lead to its build up and maintenance [2, 3]. In this sense, dispersal is a critical stage in biofilm life cycle because it enables bacterial cells to escape from harsh conditions to reach less hostile environments [1]. This stage depends on numerous regulatory signals, and studying them is important to manage cell dispersal: converting sessile cells back to a planktonic phenotype makes bacterial cells more vulnerable to the action of antimicrobials [2, 4].

Stress caused by exposure to low nitric oxide (NO) concentrations enhances cell dispersal of various Gram-positive and Gram-negative bacteria from biofilms, without posing significant toxicity risks to humans or the environment [2, 4–8]. Despite these findings, NO influence on *L. monocytogenes* biofilms is not well known. This paper contributes to better understanding of cell dispersal regulation in biofilms formed by this foodborne pathogen.

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Material and methods

Bacterial strains

Listeria monocytogenes ATCC 19115 (serotype 4b) and *L. monocytogenes* IAL 633 (serotype 1/2a) were acquired from Adolfo Lutz Institute (São Paulo, Brazil) and kept as glycerol stocks in brain heart infusion broth (BHI, Oxoid, UK) at -80°C . Working cultures were grown in BHI broth (37°C , 24 h) as needed.

L. monocytogenes biofilm growth

Two different surfaces were used for *L. monocytogenes* biofilm growth: system A (stainless steel) and system B (special glass for microscopic observations). For system A, stainless steel coupons (AISI-304, 2.45 cm^2) were cleaned and sterilized according to Winkelströter et al. [9], and one coupon was placed *per* well of a polystyrene microtiter plate (24-wells, Costar®, USA). As described by Silva et al. [10], the wells were filled with 2 mL of overnight *L. monocytogenes* BHI broth culture (ca. 10^8 CFU/mL) and incubated without shaking (25°C , 3 h) to favor bacterial attachment [11]. After adhesion, the coupons were rinsed with phosphate-buffered saline (PBS), to remove non-adherent cells, and transferred to another plate. Then, fresh BHI broth was added to the wells for re-incubation (25°C , 120 rpm, 4 days). On the fourth day, the procedure was repeated, and the biofilms were re-incubated for up to 8 days.

For system B, aliquots of overnight *L. monocytogenes* BHI broth culture (0.5 mL) were transferred to a chambered coverglass (Nunc™ Lab-Tek™ II 8, Nalgel Nunc Int., USA) and statically incubated at 25°C . After initial adhesion (3 h), loose cells were removed by rinsing the glass surface with PBS, fresh BHI broth was added, and the culture was re-incubated for up to 4 days. The procedure for planktonic cell removal was repeated, and the biofilm culture was further incubated (for up to 8 days). No shaking was applied to this system because, although the glass chamber was appropriate for confocal laser scanning microscopy, it was very delicate and could break with agitation.

In situ detection of ROI and RNI in biofilms of *L. monocytogenes*

To detect ROI stress markers in biofilms, the chemicals hydroethidine (HET), and 5-(6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) were used to stain superoxide radicals and hydrogen peroxide, respectively. Regarding RNI stress markers, NO was detected with 4-amino-5-methylamino-2',7'-difluorescein diacetate (DAFFM-DA), and peroxynitrite was stained with dihydrorhodamine 1,2,3 (DHR). All fluorescent reagents were

acquired from Molecular Probes (USA), and the staining procedure was conducted according to Barraud et al. [5].

Stained *L. monocytogenes* biofilms (systems A and B) were observed under the microscope. Biofilms on stainless steel coupons (system A) were observed under an epifluorescence microscope (Nikon model 80i, Japan) equipped with filters B-2A (450–490 nm) and G-2A (510–560 nm), and images were treated with the software NIS-Elements AR 3.2. Biofilms on glass chambers (system B) were observed under a confocal laser scanning microscope (Leica TCS SP5-AOBS) equipped with argon (488 nm) and helium (543 nm) lasers, coupled to the image documentation software Leica LAS AF version 2.6.0 build 7266 (Germany).

NO donor and inhibitor putative effects on *L. monocytogenes* biofilms

To evaluate the possible impact of NO supply or limitation on biofilms of *L. monocytogenes*, the classical NO donor sodium nitroprusside (SNP) was chosen; the NO inhibitors N $^{\omega}$ -nitro-L-arginine methyl ester hydrochloride (L-NAME) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (c-PTIO) were also employed. All these reagents were all supplied by Sigma (USA), and the selected concentrations were based on minimum inhibitory concentration tests [12] and literature data available for other bacteria [13].

L. monocytogenes biofilms grown on coupons (system A) were harvested (days 4 and 8) and treated (at 25°C , for 1 h) with SNP ($100\text{ }\mu\text{g mL}^{-1}$ or $2000\text{ }\mu\text{g mL}^{-1}$), L-NAME ($100\text{ }\mu\text{M}$), or c-PTIO ($100\text{ }\mu\text{M}$) solution. *L. monocytogenes* sessile populations from the coupons were enumerated by drop plating [11] on BHI agar (CFU cm^{-2}) according to Leriche and Carpentier [14]. Free-floating *L. monocytogenes* cells remaining from system A were also treated with SNP ($2000\text{ }\mu\text{g mL}^{-1}$), L-NAME ($100\text{ }\mu\text{M}$), or c-PTIO ($100\text{ }\mu\text{M}$) solutions at 25°C for 1 h and used for quantitative gene transcription evaluation, as described in the next section.

Effect of NO donors and inhibitors on key gene transcription in *L. monocytogenes*

For system A, treated free-floating *L. monocytogenes* cell viability was checked by enumeration on BHI agar plates and the results were recorded as colony-forming units per milliliter (CFU mL^{-1}). Moreover, total RNA was extracted from these cells, to evaluate putative changes in gene transcription.

RNA was extracted with Tri-Reagent® (Zymo Research, USA), and extracts were treated with the kit DNase I, RNase-free (Thermo Scientific, UK) combined with phenol-chloroform extraction to eliminate DNA contamination [15,

16]. The RNA extracts were quantified, checked for purity (Nanodrop® 2000 spectrophotometer, Thermo Scientific, USA), and used as templates for cDNA synthesis (*High-Capacity cDNA Reverse Transcription* kit containing RNase inhibitor, Applied Biosystems, USA).

RNA transcripts were quantified by real-time PCR (qPCR) by using the primers listed in Table 1, all acquired from Invitrogen (USA). The qPCR reaction (10 µl) included 2 µL of cDNA extract, 5 µL of iQ™ SYBR® Green Supermix (Bio-Rad, USA), 2 µL of molecular biology-grade water, and 0.5 µL of each primer solution (0.5 µM). PCR was amplified and amplicons were detected with thermocycler Mini-Opticon® (Bio-Rad, EUA); the detection threshold was set at 0.014. For all the tested genes (Table 1), there was an initial denaturation step (95 °C, 15 min) followed by 40 denaturation (95 °C, 15 s), annealing (59 °C, 30 s), and extension (72 °C, 30 s) cycles. The exception was *lmo1485*, which required annealing at 56 °C for 30 s.

To calculate relative gene transcription levels, normalization was performed with *16S rRNA* as the reference gene, according to the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen [19]. Results are expressed as arbitrary units.

Statistical analysis

Analyses of variance of the results obtained during culture and qPCR experiments were accomplished by one-way ANOVA, followed by Bonferroni post-test. For significant differences, a value of $P < 0.05$ was considered. The software GraphPad Prism 5 (USA) was used for statistics.

Results

Reactive oxygen intermediates were present in biofilms grown on stainless steel coupons—system A, according to results of fluorescence microscopy observations (data not shown). However, ROI were not detected in biofilms grown on glass—system B, according to CLSM results from Fig. 1,

which show there was no fluorescence for hydrogen peroxide staining (a and b), and the staining for superoxide radicals (e and f) was fading. Reactive nitrogen intermediates were naturally present in biofilm cultures of both *L. monocytogenes* strains tested on stainless steel coupons (system A), as indicated by specific staining combined with fluorescence microscopy observations (data not shown). CLSM results from system B (glass chamber) confirmed that NO and peroxynitrite were present in *L. monocytogenes* biofilms, as illustrated in Fig. 1c, d, g, h. In bright field, cell density was high. Also, there were green and red fluorescent cells, which indicated the presence of NO and peroxynitrite, respectively.

Experiments done in system A to investigate the putative effects of RNI on *Listeria monocytogenes* (8-day-old biofilms and free-floating cells) viability revealed similar bacterial counts (ca. 10^5 – 10^6 CFU/cm² for sessile population and ca. 10^9 CFU/mL for free-floating cells) irrespective of external NO donor (SNP) and NO inhibitor (L-NAME and C-PTIO) supply.

With regard to investigation into the transcription levels of genes previously reported to underlie bacterial tolerance to oxidative and nitrosative stresses, external NO donor and NO inhibitor supply did not affect any of the studied *L. monocytogenes* strains (Fig. 2). The exception was gene *lmo0990*, which was downregulated in all the tested conditions as compared to untreated control.

Discussion

To the best of our knowledge, possible ROI and RNI effects on *L. monocytogenes* biofilms have not been reported yet. However, these molecules have been detected in biofilms formed by other bacterial genera [5, 8]. Here, ROI was only present in *L. monocytogenes* biofilms grown on stainless steel (Fig. 1). This divergence could at least be partially explained by different aeration in systems A and B: for practical reasons, only the stainless steel coupons were agitated; the appropriate glass chamber for CLSM was too fragile for agitation.

Table 1 Primers used to quantify gene transcription in *Listeria monocytogenes* related to tolerance to oxidative and nitrosative stresses

Gene	Function	Primers	Reference
<i>16S-rRNA</i>	Endogenous control for PCR reactions	F: GATGCATAGCCGACCTGAGA R: TGCTCCGTCAGACTTTCGTC	Werbrouck et al. [17]
<i>PrfA</i>	Transcriptional activator factor	F: CAATGGGATCCACAAGAATATTGTAT R: GATGGTCCCGTTCTCTCTAA	Werbrouck et al. [17]
<i>lmo0807</i>	Tolerance to oxidative and nitrosative stresses	F: CACTATGAAGTAGGTTGTATCG R: ATCGAATTCTTCTCCTCGTTTC	Mraheil et al. [18]
<i>lmo0990</i>	Tolerance to oxidative and nitrosative stresses	F: TTATTGTGCTCTTGCTTTTCGAG R: CAGTCCTGTAATCCACTGAAA	Mraheil et al. [18]
<i>lmo1485</i>	Tolerance to oxidative and nitrosative stresses	F: CGCATTTAGAACCAATGG R: CGATGGAGTGTGGATGTT	Mraheil et al. [18]

Fig. 1 **a–h** Confocal laser scanning micrographs of 8-day-old biofilms of *L. monocytogenes* IAL633 and *L. monocytogenes* ATCC19115 grown on glass chamber—system B. Images were acquired with a Leica TCS SP5-AOBS microscope, equipped with argon (488 nm) and helium (543 nm) lasers. Results were documented with the software Leica LAS AF version 2.6.0 build 7266. Bright light micrographs were included to show that bacterial cells were present in the fields selected, regardless of specific

stainings. Staining for hydrogen peroxide was done with H₂DCF (red fluorescence would indicate positive results); nitric oxide was detected with DAFFM-DA (green fluorescence would indicate positive results); staining for superoxide radicals was done with HET (red fluorescence would indicate positive results); and peroxynitrite was detected with DHR (red fluorescence would indicate positive results). Scale bar = 10 µm

Nevertheless, mild oxidative stress might not have affected *L. monocytogenes* because catalase, a H₂O₂-degrading enzyme, can be produced by this bacterium [20].

On the other hand, RNI were detected in *L. monocytogenes* biofilms formed under all the tested conditions, as demonstrated by microscopy observations with specific staining for NO

Fig. 2 Relative gene transcription levels normalized with reference to *16S rRNA* (arbitrary units) for free-floating *L. monocytogenes* cells detached from biofilms (system A—stainless steel coupon, brain heart infusion broth, 8 days of incubation, 25 °C). Treatments were done for 1 h at 25 °C: NO donor (SNP at 2000 $\mu\text{g ml}^{-1}$) or NO inhibitors (L-NAME

and c-PTIO, each one at 100 μM). Dotted lines indicate the expression rate of 1 (no change in comparison with the untreated control) and $P < 0.05$ was considered for evaluation of statistically significant difference

and peroxynitrite (Fig. 1). By comparing the images obtained under different light sources, it was possible to conclude that NO and peroxynitrite were not evenly distributed in the biofilms, which presented honeycomb-like structures with strongly fluorescent cells at the hollow border. This indicated the potential for cell dispersal from these microstructures. This kind of structural biofilm arrangement has been related to improved mechanical stability and nutrient flow, conferring fitness advantages to cells [1, 21].

Nitric oxide is generally produced by bacterial metabolism through the enzyme NO synthase (NOS), which occurs in several Gram-positive bacteria such as *Bacillus anthracis*, *Bacillus subtilis*, and *Staphylococcus aureus*. For these bacteria, NOS activity has been shown to influence antibiotic resistance patterns and virulence factor expression and to favor escape from host immune system oxidative burst. [13, 22]

Notwithstanding the NOS importance for bacterial survival and virulence, there are no literature data on the presence of NO synthase or NO production in *L. monocytogenes*. However, even in the absence of NOS activity, bacterial cells have been demonstrated to produce NO by alternative pathways by using arginine as precursor [23, 24]. Although this was not investigated in the present study, it is plausible to suppose that *L. monocytogenes* has NO synthase-independent ability to generate NO. In fact, Joseph and Goebel [25] reported that *L. monocytogenes* contains the enzyme arginine deiminase, which catalyzes arginine degradation to the possible NO precursors ammonia and citrulline.

Moreover, to corroborate the absence of NO inhibitory effect on *L. monocytogenes*, Cole et al. [26] studied intracellular *L. monocytogenes* survival in vivo, to find that the NO produced by macrophages increased pathogenic bacterium escape from phagolysosomes, thereby promoting systemic infection.

With regard to peroxynitrite, no data was found on its production by *L. monocytogenes* but it has been reported as a bacterial metabolite synthesized via NO oxidation in the

presence of ROI [7]. Our present findings regarding peroxynitrite detection in bacterial biofilms are also supported by the paper of Barraud et al. [5], who detected peroxynitrite in *Pseudomonas aeruginosa* biofilms.

In this research, experiments supplying NO donor and inhibitors were carried out to elucidate whether RNI interfered in *L. monocytogenes* growth and biofilm formation. The classical NO donor SNP was used. Two NO inhibitors that act through distinct mechanisms were chosen: L-NAME, a NO synthase inhibitor, and c-PTIO, a NO scavenger [22, 27].

The NO donor (SNP) did not influence *L. monocytogenes* populations or biofilm structure. This contradicted literature reports on *Pseudomonas aeruginosa* and *Clostridium sporogenes* biofilms, which are likely inhibited by the nitrosyl agent action on the bacterial cell wall. [27, 28] Additionally, many authors examined NO effects on other bacterial biofilms, to find that different NO donors increased cell dispersal and/or reduced biofilm formation even after exposure for a short time (1 h) [2, 5–7, 22]. Here, *L. monocytogenes* treatment with L-NAME or c-PTIO, which are NO inhibitors, did not affect bacterial cell viability.

Overall, neither NO donor nor NO inhibitors impacted transcription of selected genes in any of the studied *L. monocytogenes* strains. The exception was *lmo0990*, which was downregulated in *L. monocytogenes* IAL 633 (Fig. 2). The evaluated genes are considered key to counterbalancing oxidative and nitrosative stresses: *lmo0990* codifies a product similar to a multidrug efflux pump; *lmo0807* is homologous to the ABC complex transporter; and *lmo1485* participates in DNA methylation [24]. Taken together, our results are intriguing because they suggest that NO concentration does not directly impact *L. monocytogenes* biofilm formation or dispersal, which indicates that this foodborne pathogen has fine metabolic tuning to tolerate nitrosative and oxidative stress conditions.

Authors' contributions Study concept and design: FBRT, NC, and LPS. Data analysis and interpretation: ECPDM. Manuscript drafting: FBRT,

VFA, and ECPDM. Critical revision of the manuscript for important intellectual content: VFA and ECPDM.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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