**Helicobacter pullorum** induces nitric oxide release in murine macrophages that promotes phagocytosis and killing

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**INTRODUCTION**

*Helicobacter pullorum* is an avian enterohepatic species that, more recently, has also been found as a naturally acquired infection in mice and rats, and isolated from patients with gastrointestinal and hepatobiliary diseases. In this work, the interaction between *H. pullorum* and murine macrophages was examined. Firstly, the impact of nitric oxide, which is an antimicrobial produced by mammalian macrophages, on *H. pullorum* viability and morphology was studied by colony-forming assays and light microscopy, respectively. Exposure to nitric oxide lowered *H. pullorum* viability, in a growth-phase-dependent manner, and decreased the mean cell size. However, the number of coccoid forms remained low, contrasting with what has been observed for other *Helicobacter* species. Confocal microscopy showed that *H. pullorum* is internalized by murine macrophages, triggering nitric oxide production that promotes phagocytosis and killing of the pathogen. Interaction between *H. pullorum* and macrophages stimulated secretion of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and MIP-2. These results show that *H. pullorum* is able to infect mammalian murine cells triggering an inflammatory response.

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**Abbreviations:** DPTA, dipropylentriamine; GSNO, S-nitrosoglutathione; INF-γ, gamma interferon; iNOS, inducible nitric oxide synthase; L-NMMA, N°-monomethyl-L-arginine acetate salt.

One supplementary table and two supplementary figures are available with the online Supplementary Material.

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Fox et al., 1998; Laharie et al., 2009; Rocha et al., 2005; Steinbrueckner et al., 1997; Veijola et al., 2007.

*H. pullorum* adheres to and invades human intestinal Caco-2 cells, possesses a type VI secretion system (Sirianni et al., 2013) and expresses a cytolethal distending toxin (CDT) (Ceelen et al., 2006b; Varon et al., 2014; Young et al., 2000). The bacterium presents LPS with the highest biological activity within the genus *Helicobacter*, capable of inducing NF-κB activation (Hynes et al., 2004). In gastric and intestinal epithelial cell lines, *H. pullorum* triggers the production of IL-8 via NF-κB, probably due to its LPS involvement (Varon et al., 2009).

As sentinel cells of the human body, macrophages are activated by several signals, including cytokines (e.g. gamma interferon, INF-γ) and microbial compounds (e.g. LPS). In primed macrophages, a variety of pro-inflammatory cytokines, such as IL-1β and TNF-α mediate the expression of the inducible nitric oxide synthase (iNOS) (Pautz et al., 2010).

The mammalian iNOS enzyme produces nitric oxide (NO), a highly reactive molecule that combines with oxygen species, produced by activated macrophages and neutrophils, to generate species like nitrosonium (NO⁺), nitroxy (NO−), peroxynitrite (ONOO−) and nitrite (NO₂⁻).
Most of these reactive nitrogen species damage several bacterial components, such as DNA, lipids, protein metal centres and the amino acid residues of proteins, causing inactivation of key metabolic functions and ultimately leading to microbial clearance (Hughes, 2008; MacMicking et al., 1997; Qu et al., 2011).

In spite of the potential pathogenicity of this bacterium, the mechanisms of *H. pullorum* infection remain poorly understood. For example, there is still a gap in the knowledge on how *H. pullorum* behaves when facing antimicrobials produced by the host immune system and regarding the response of phagocytes to *H. pullorum*. In this work, we have evaluated the viability and morphological modifications of *H. pullorum* when exposed to nitrosative stress generated by different NO donors applied at different stages of bacterial growth. The interaction of *H. pullorum* with murine macrophages was studied by confocal microscopy and through the analysis of the cytokines stimulated upon *H. pullorum* infection.

**METHODS**

**Bacterial strain manipulation.** *H. pullorum* 6350-92 (CCUG 33838), which was isolated from a stool sample of a patient with gastroenteritis and hepatitis (Melito et al., 2000), was used as the reference strain. The strain was routinely cultivated on blood agar (BA) plates, which are composed of solid medium blood agar base no. 2 (Oxoid) supplemented with 10 % (v/v) defibrinated horse blood (Probiofarmacéutica) and an antibiotic/antifungal mix containing 6.3 g vancomycin 1 g -cyclodextrin (Roth), 3.1 g trimethoprim 1 g amphotericin B 1 g (Roth). Cells were incubated in closed jars, at 37 °C, under a microaerobic atmosphere (7 % CO₂, 6 % O₂, 3.5 % H₂ and 83.5 % N₂) generated by an Anoxomat system (Mart Microbiología). Bacteria were taken as fully grown after culturing on BA plates for a total of 5 days, during which bacteria were twice transferred to fresh BA medium.

**Viability tests.** The viability of *H. pullorum* under nitrosative stress conditions was determined by exposing cells to the following nitrosative stress generators: spermine-NONOate (Sigma; t₁/₂ ~ 40 min; Thomas et al., 2002), dipropylentetramine (DPTA)-NONOate (Cayman; t₁/₂ ~ 3 h) and S-nitrosoglutathione (GSNO; t₁/₂ ~ 50 min; Yap et al., 2010). GSNO was freshly prepared by mixing equimolar amounts of sodium nitrite and reduced GSH under acidic conditions (0.05 M HCl) (Thomas et al., 2002). For these assays, fully grown bacteria were inoculated in 25 cm² cell culture flasks (Nunc) filled with 10 ml medium, at an initial optical density at 600 nm of 0.1–0.2, in brain heart infusion (BHI) broth (Oxoid) plus 10 % (v/v) defibrinated FCS (Gibco-Invitrogen) (BHI-FCS) and grown for 19 h at 150 r.p.m. These cells were used to inoculate BHI medium supplemented with 0.2 % (w/v) β-cyclodextrin (Sigma) (BHI-βCD) at an OD₆₀₀ of 0.05, and distributed into 24-well plates. The stress generators were added, at concentrations ranging from 50 to 500 µM, at different stages of *H. pullorum* growth: immediately after inoculation (OD₆₀₀ ~ 0.05, lag phase), after 12 h (OD₆₀₀ ~ 0.2, early exponential phase) and after 48 h (OD₆₀₀ ~ 1, stationary phase). The number of viable cells was monitored at 12 h intervals up to 48 h after the stress application. The number of c.f.u. ml⁻¹ was evaluated by serially diluting each sample in BHI medium and plating on antibiotic/antifungal cocktail containing BA plates, which were incubated for 48 h.

**Morphological studies.** *H. pullorum* morphology under nitrosative stress was studied in cells grown in BHI-FCS, as described above for the viability tests, and exposed to spermine-NONOate (10, 50 and 100 µM). Cells prepared similarly but not exposed to any stress were also visualized. After 24 h incubation, cell suspensions were pelleted by centrifugation (5 min at 2400 g), resuspended in 10–50 µl PBS and mounted onto 1.7 % (w/v) agarose-coated glass slides. Images were acquired with a iXonEMCCD + 885 cooled camera (Andor Technology) attached to a DMR600 Microscope (Leica), with ×64/1.6 NA magnification, and treated with ImageJ software (Schneider et al., 2012). The percentage of bacilli versus cocci forms and the cell size were evaluated by counting and measuring manually a total of 100 cells per condition using Metamorph software version 4 (Molecular Devices).

**Infection assays with murine macrophages.** Murine macrophages J774.A.1 (ATCC TIB-67) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose, GlutaMAX supplement, pyruvate (GIBCO), 10 % (v/v) FCS and 100 µg penicillin/streptomycin ml⁻¹ (Sigma) (DMEMc medium), and incubated in a humidified 5 % CO₂-air-controlled atmosphere at 37 °C. For the infection assays, J774.A.1 macrophages were resuspended in Roswell Park Memorial Institute 1640 medium (RPMI), GlutaMAX supplement (Gibco) containing 10 % (v/v) FCS and 100 µg penicillin/streptomycin ml⁻¹ (RPMIc medium), and seeded for 3 h in 24-well plates at a density of 5 × 10⁵ cells per well. Macrophages were left untreated (non-activated macrophages), or activated overnight with 0.3 µg INF-γ ml⁻¹ (Sigma) and 1 µg LPS ml⁻¹ (Sigma) (activated macrophages). When required, murine iNOS activity was inhibited by N⁴-monomethyl-L-arginine acetate salt (L-NMMA; Sigma; 800 µM), which was added simultaneously to INF-γ and LPS. After 12 h of incubation, the macrophage medium was exchanged by infection medium that consisted of RPMI GlutaMAX plus 10 % FCS (RPMIf), and where indicated, supplemented with 800 µM L-NMMA.

For macrophage infection assays, fully grown bacteria were cultured on BA plates for another 24 h. These bacteria were used to inoculate, at an OD₆₀₀ ~ 0.1–0.2, Brucella broth liquid medium (BB; Gibco) containing 5 % FCS (BB-FCS), and the *H. pullorum* was grown for 15 h. Bacilli were then pelleted (10 min, 8740 × g, 4 °C) and resuspended in RPMIc at an OD₆₀₀ of 0.2, and the bacterial viability determined by c.f.u. counting prior to incubation within macrophages (time 0 of infection). Bacteria were used to infect macrophages, at a m.o.i. of 100, for 5 and 10 h. For each time point, the *H. pullorum*-infected macrophages were collected and bacteria present in the supernatant, adherent to the surface of the macrophages and localized inside the macrophages were plated together, and the c.f.u. count of viable *H. pullorum* bacilli was determined following incubation under microaerobic conditions. At the same time points of infection, the amount of NO produced by macrophages as the nitrite accumulated in the supernatants of the macrophage cell cultures was quantified. Nitrite production by macrophages that were not infected or infected with *H. pullorum*, and in the presence and in the absence of INF-γ/LPS/L-NMMA, was also analysed. The nitrite content was determined by microtitre plate colorimetric assay (Multiskan GO; Thermo Scientific), which consisted of readings of the absorbance at 540 nm of the 1:1 mixtures of standard (100 µM) and Griess reagent (1 %, w/v, sulfanilamide/0.1 % (w/v) naphthylethylene diamin dihydrochloride/2 % v/v, phosphoric acid). Sodium nitrite was used as standard.

**Confocal experiments.** For the confocal experiments, J774.A.1 macrophages (1 × 10⁶ cells per well) were seeded in 6-well plates containing submerged glass coverslips and cultivated in DMEMc for 12 h. Macrophages were left untreated (non-activated macrophages) or activated for 5 h with 0.15 µg INF-γ ml⁻¹ and 0.5 µg LPS ml⁻¹ (activated macrophages); where indicated, the inhibition of the NO production was achieved by the addition of 800 µM L-NMMA simultaneously with INF-γ/LPS.
H. pullorum bacilli grown in BB-FCS for 15 h, as described above for the macrophage assays, were washed with PBS, resuspended in DEMEM without antibiotics (DMEMi) and DMEMi plus 800 μM L-NMMA at an OD600 of ~0.2 and viability was determined by c.f.u. counting, and the cells were then used to infect macrophages at a m.o.i. of 100. After 30 min and 2 h of infection, each well was washed three times with PBS to remove non-adherent bacteria, the cells were fixed with 4% (w/v) formaldehyde, washed again (three times in PBS), stained with 2 μM HCS CellMask red (Molecular Probes) for 30 min at room temperature and PBS washed three times. The coverslips were mounted onto microscopy slides and confocal Z-stacks were acquired on a Leica SP5 confocal microscope, using a ×63/1.3 NA oil immersion objective, a 568 nm laser line and the spectral detection adjusted for the emission of the Alexa 568 fluorochrome. Stained macrophages that were activated by INF-γ and LPS, and incubated in DMEMi for 2 h, were also examined and served as internal controls. For quantitative assessment, adherent and intracellular bacteria of 100 random macrophages per condition were counted and images treated with ImageJ software. Two separate experiments were performed and cell counting was validated by two independent observers.

In parallel experiments, the viability of the adherent and intracellular H. pullorum bacilli was determined upon infection of macrophages for 30 min and 2 h. Each well was washed three times with PBS to remove bacteria present in supernatants, macrophages lysed with 2% (w/v) saponin and their bacterial content evaluated by c.f.u. counting.

### Analysis of cytokine gene expression

Expression of TNF-α, IL-1β, IL-6 and macrophage inflammatory protein 2 (MIP-2), which is the mouse homologue of the mammalian IL-8 (Tchkamp- Olson et al., 1990), was quantified in mRNA extracted from J774A.1 macrophages seeded, for 3 h, in 24-well plates (5 × 10^5 cells per well) containing RPMi medium. H. pullorum bacilli prepared in RPMi, as described above for the macrophage assays, were incubated with macrophages at a m.o.i. of 100, for 6 h. For comparison purposes, expression of cytokines in macrophages non-infected, non-activated and activated only with INF-γ (0.15 μg ml^-1) and LPS (0.5 μg ml^-1) was also measured. After incubation, the wells were washed with cold PBS and the total RNA was isolated with a High Pure RNA isolation kit (Roche). Residual DNA was digested using the enzymes of the Turbo DNA-free kit (Ambion). For each sample, 200 ng RNA was converted to cDNA using the anchored-oligo-dT primers and the Transcriptor High Fidelity cDNA synthesis kit (Roche). Semiquantitative reverse transcriptase PCR reactions contained 40 ng cDNA μl^-1, 2.5 U Taq DNA polymerase (Biolabs), 200 μM dNTPs (Nzytech) and 0.5 μM of each primer (Table S1, available in the online Supplementary Material), in 50 μl final volume. The reverse transcriptase PCR conditions were: 1 cycle of denaturation at 95 °C (30 s–2 min), followed by 30 cycles at 95 °C for 45 s, 50–55 °C for 30–45 s and 68 °C for 1 min, followed by a final cycle at 68 °C for 5 min. Bio-Rad Quantity One software was used in order to perform three steps that allowed the determination of the relative quantification of the cytokine expression: (i) image background subtraction; (ii) estimation and integration of all the pixels present in the DNA bands to an estimated area; and (iii) the area obtained for the gene band of interest, under the different conditions tested, was divided by the area estimated for the constitutive gene band, the murine glycerol-aldehyde 3-phosphate dehydrogenase (gapdh) gene, in the respective condition. These estimated relative quantifications enabled the establishment of comparisons of differences in cytokine expression upon macrophage infection with H. pullorum. Two independent biological samples were analysed in quadruplicate. Statistical analyses were performed by Student’s unpaired t-test using GraphPad Prism software.

### RESULTS

#### Effect of nitrosative stress agents on H. pullorum viability

The viability of H. pullorum 6350-92 under NO stress was analysed by exposing cells to different sources of NO. Two NONOates, spermine-NONOate and DPTA-NONOate, which differ in NO releasing rates, and GSNO, which typifies S-nitrosothiols and donates a nitroso functional group to another thiol via a transnitration reaction (Broniowska et al., 2013), were used. Hence, the bacterial phenotype responses to NONOates and GSNO are expected to be different.

H. pullorum grown to lag phase (OD600 ~0.05), early exponential phase (OD600 ~0.2) and stationary phase (OD600 ~1) (Fig. S1) was treated with nitrosative stress-generating agents in concentrations that ranged between 50 and 500 μM, and viability was evaluated at several intervals of time up to 84 h (Fig. 1).

Addition of NONOates to cells at the lag phase caused a viability decrease for all tested concentrations (Fig. 1a). When cells were treated with the same concentration of NONOate, the inhibitory effect of DPTA-NONOate was higher than that of spermine-NONOate, which is most probably related to the longer half-life of the former. Nevertheless, in both cases recovery of viability occurred 48 h after NO exposure, except for the highest NONOate concentration used (500 μM). Different growth behaviour was observed when the stress was applied to H. pullorum at the early exponential phase; for the same concentration, the addition of NONOates to cells at the early exponential phase caused a smaller decrease in viability when compared to cells treated at the lag phase. Moreover, growth recovery to levels similar to untreated cells was observed in a shorter interval of time (Fig. 1b). The results also showed that no viability impairment occurred when NONOates are applied to H. pullorum cells that had reached the stationary phase (Fig. 1c).

Exposure of lag phase-grown cells of H. pullorum to GSNO led to a decrease of viability, but only for high concentrations of GSNO (250 and 500 μM) (Fig. 1a). The inhibitory effect persisted over time as after 24 h treatment the cells exposed to 250 and 500 μM GSNO still displayed very low viability. However, H. pullorum did not suffer substantial viability impairment when GSNO was supplied to cells at the early exponential and stationary phases (Fig. 1b, c). Altogether, it was concluded that NO donors have an antimicrobial action against H. pullorum only when cells are at the initial growth phase.

#### H. pullorum cell size decreases under NO stress

To investigate the morphological alterations caused by nitrosative stress on H. pullorum, cells were treated with spermine-NONOate (10–100 μM) and examined by light microscopy. The majority of the H. pullorum NO-exposed...
cells exhibited a bacillary form similar to that observed for untreated cells. Cells with U, V, C and S shapes were also observed, mainly in samples exposed to 50 and 100 μM of spermine-NONOate (Fig. 2a), as these usually occur when bacteria are changing from bacilli to coccoid form (Benaissa et al., 1996; Kusters et al., 1997; Zeng et al., 2008). Interestingly, exposure of H. pullorum to NO stress caused alterations in the cell size, as judged by a decrease in the mean cell size of ~25% upon exposure to 50 and 100 μM spermine-NONOate. Moreover, the cell size shortening was accompanied by a decrease in length heterogeneity (Fig. 2a, b).

**H. pullorum viability is lower in NO-producing murine macrophages**

*H. pullorum* viability upon infection of J774A.1 murine macrophages was evaluated. When incubated with non-activated macrophages, *H. pullorum* suffered a decrease in survival of less than one log after 5 h, and approximately one log after 10 h. When in contact with IFN-γ/LPS-activated macrophages, *H. pullorum* viability was significantly reduced (Fig. 1).
macrophages, *H. pullorum* viability dropped significantly by approximately three logs after 5 h, and more than four logs after 10 h. Addition to macrophages of the mammalian iNOS enzyme inhibitor L-NMMA (Olken & Marletta, 1993; Olken et al., 1991) rescued *H. pullorum* viability to levels comparable to those observed within non-activated macrophages (Fig. 3a). Since the release of NO by macrophages killed *H. pullorum*, we next determined the amount of NO produced by macrophages upon infection. The results show that *H. pullorum* activates the mammalian iNOS as the nitrite content declined to zero when infection occurred in the presence of the iNOS inhibitor L-NMMA (Fig. 3b). Furthermore, *H. pullorum* viability was inversely related to the amount of NO generated by macrophages as a higher number of *H. pullorum* colonies was observed when lower nitrite contents were present in the supernatants (Fig. 3). Hence, *H. pullorum* triggers the mammalian innate immune system inducing the iNOS enzyme and the NO generated acts as a bactericidal agent against this pathogen.

**H. pullorum** is phagocytized by murine macrophages

*H. pullorum* loads in J774A.1 macrophages were quantified by analysis of fluorescent images obtained with confocal microscopy. IFN-γ/LPS-activated macrophages that were not infected with bacteria confirmed that stained macrophage cells could be distinguished from stained bacterial cells (Fig. S2a, b). Bacteria were considered as either internalized or adherent to macrophages based on the simultaneous inspection of three orthogonal slices (Fig. S2c).

Phagocytosis of *H. pullorum* by non-activated, activated and iNOS-inhibited macrophages was visualized after incubation periods of 30 min and 2 h (Fig. 4). *H. pullorum* cells were found to adhere to macrophages and to be internalized. The percentage of macrophages with phagocytized bacteria was 70 % after 30 min and 86 % after 2 h infection. The highest number of bacteria phagocytized per macrophage was observed for activated macrophages, i.e. when macrophages were producing significant quantities of NO (Fig. 4a, c). Concomitantly, suppression of NO production led to a decrease in the number of bacteria per macrophage. In parallel experiments, the viability of *H. pullorum* within macrophages was determined under conditions that replicated those used for confocal microscopy experiments. These results also showed that *H. pullorum* survival was lower in activated macrophages when compared to non-activated and iNOS-inhibited macrophages (Fig. 4c).

Prior to infection in macrophages, *H. pullorum* cells presented a bacillary shape (Fig. 4b, before). Upon incubation in macrophages, internalized bacteria exhibited coccoid and shorter bacillary forms (Fig. 4b, 30 and 120 min). Altogether these results indicate that the production of NO by macrophages increases the phagocytosis of *H. pullorum* cells, which upon internalization acquire a coccoid or shorter bacilli shape.

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**Fig. 2.** Morphology of *H. pullorum* NO-treated cells. (a) Phase-contrast microscopy images, and percentage of coccoid and bacillary forms, of *H. pullorum* cells exposed to the indicated concentrations of spermine-NONOate. Images are representative of the most common morphological phenotype observed for each condition. Morphological forms are indicated as follows: cocci (full arrows) and U/C shape (dashed arrows). Bar, 5 μm. (b) Mean cell length of *H. pullorum* untreated (white bar), and exposed to 10 μM (black bar), 50 μM (light grey bar) and 100 μM (dark grey bar) of spermine-NONOate (SP) for 24 h. Data are presented as the mean ± SEM. **, *P* <0.01; ***, *P* <0.0001 (t-test). The percentage of bacillary/coccoid forms and the mean length were determined by manually analysing 100 cells per condition in two independent biological samples.
**H. pullorum** induces the expression of cytokines in macrophages

J774A.1 macrophages were incubated with *H. pullorum* and the level of gene expression of TNF-α, IL-1β, IL-6 and MIP-2, which has been reported to be linked to iNOS activation (Bishayi et al., 2015; Srisuwan et al., 2014), was determined. Infection of macrophages with *H. pullorum* for 6 h resulted in the production of IL-1β, TNF-α, IL-6 and MIP-2 cytokines, in relation to non-infected and non-activated macrophages. *H. pullorum* induced the expression of IL-1β, TNF-α, IL-6 and MIP-2 at levels similar to those stimulated by INF-γ/LPS (Fig. 5).

**DISCUSSION**

*H. pullorum* is an emerging human pathogen with zoonotic potential (Borges et al., 2015), whose mechanisms of pathogenesis and resistance to immunity remain largely unknown. In this work, we have analysed the behaviour of *H. pullorum* when exposed to NO, which is an important antimicrobial chemical weapon of the mammalian immune system. *H. pullorum* is shown to be killed by several NO donors, namely spermine-NONOate, DPTA-NONOate and GSNO, in a growth phase-dependent way. The nitrosative stress strongly impaired *H. pullorum* viability when NONOates were added at the initial growth phase. On the contrary, the nitrosative stress toxicity was negligible for cells that were already at the early exponential and stationary phases. This behaviour was observed even when using very high concentrations of DPTA and spermine-NONOate, i.e. at concentrations that completely impair growth when added at the initial growth phase. GSNO is a less efficient bactericide in comparison with NONOates. When *H. pullorum* is at the initial growth phase, GSNO decreased viability only when applied at the highest concentrations. Hence, *H. pullorum* exhibits a higher resistance to NO stress than *Helicobacter pylori* since it was previously reported that GSNO and DPTA-NONOate significantly impair the growth of *H. pylori* at much lower concentrations (100 and 150 µM, respectively) (Justino et al., 2012).

Our morphological studies indicated that non-stressed cells of *H. pullorum* grown for 24 h retained the bacillary form, with only approximately 1% of the cells presenting a coccoid form. Therefore, coccoid forms of *H. pullorum* seem to occur at latter stages, a result which agrees with previous data showing that after 2 days growth in BHI liquid broth, only 20% of the *H. pullorum* cells exhibited a coccoid form, and that conversion of all cells to the coccoid form occurred after 4 days (Taneera et al., 2002).

The bacillar form of *H. pullorum* cells was not significantly altered upon exposure to nitrosative stress generated by spermine-NONOate at concentrations up to 100 µM. This behaviour of *H. pullorum* apparently contrasts with that of *H. pylori*, as the latter was reported to undergo a rapid conversion from bacillary to the coccoid form when exposed to NO donors (Benaissa et al., 1996; Cole et al., 1999).
Although NO did not induce the appearance of coccoid forms in *H. pullorum*, the stress led to a decrease in the mean length of the bacteria. A decrease in bacterial size due to environmental factors is not a very common phenomenon, but it was reported for *Escherichia coli* when entering the stationary phase and for marine bacteria during starvation. For marine bacteria, cell size shortening has been proposed to represent a survival mechanism that facilitates nutrient acquisition (Siegele & Kolter, 1992).

Incubation of *H. pullorum* with NO-generating murine macrophages lowered the viability of the bacterium, and...
the macrophage-killing ability was dependent on the NO produced as treatment of macrophages with an iNOS inhibitor increased the survival of internalized *H. pullorum* cells. The NO-producing murine macrophages phagocytized a higher number of *H. pullorum* cells that rapidly became non-viable. Interestingly, *H. pylori* was reported to increase the expression of iNOS by stimulating the number of macrophages and lymphocytes in the gastric mucosa, and nitrosative stress killed the bacterium (Cherdantseva et al., 2014; Justino et al., 2014). However, to the best of our knowledge, the increase of phagocytized cells by NO-producing murine macrophages has not yet been reported for any *Helicobacter* spp.

Our work indicates that *H. pullorum* adheres to murine macrophages, as also noted by Lutay et al. (2011). Furthermore, we report, for what is believed to be the first time, that *H. pullorum* is internalized in murine macrophages and induces the secretion of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and MIP-2. Similarly, *H. pylori* and *Helicobacter hepaticus* infections have been reported to induce IL-1β, TNF-α and IL-6 (Kleine et al., 2014; Rizwan et al., 2008; Wroblewski et al., 2010). Moreover, our observation that in murine macrophages *H. pullorum* increases the expression of MIP-2, the IL-8 homologue gene, is consistent with previous studies of *H. pullorum* done in human gastric and intestinal epithelial infected cell lines showing IL-8 induction (Varon et al., 2009).

The induction of NO and pro-inflammatory cytokines in macrophages by *H. pullorum* observed in this study may be associated with the interaction of macrophage receptors with *H. pullorum* LPS, possibly through activation of NF-κβ, similarly to the activation of IL-8 by *H. pullorum* previously observed in epithelial cells (Varon et al., 2009). Furthermore, the ability here shown to elicit inflammatory responses in a mammalian host suggests that *H. pullorum* can be pathogenic for humans.

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**Fig. 5.** Induction of host cytokines by *H. pullorum*. The expression of TNF-α, IL-1β, IL-6 and MIP-2 was quantified, by semiquantitative PCR, in INF-γ/LPS-activated and *H. pullorum*-infected J774A.1 macrophages. For each condition, expression values were quantified by band pixel intensity obtained for the cytokine and normalized to the correspondent band pixel intensity of the constitutive gapdh gene. Fold change was determined by dividing values by the normalized cytokine expression in non-infected and non-activated macrophages. Values are presented as the mean ± SEM of two independent biological samples with at least four technical replicas.


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