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Enniatin B alters bovine polymorphonuclear leukocytes phagocytosis and extracellular reactive oxygen species production *in vitro*

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ABSTRACT

Enniatins (**ENNs**) affect human and animal health. Different ENN analogs have been identified, but Enniatin B (**ENN B**) is the most detected in foods and feeds. This study investigated the effect of ENN B on bovine polymorphonuclear leukocytes (**PMNs**) challenged with increasing ENN B concentrations (0.625, 1.25, 2.5, 5, and 10 μM). Bovine PMNs were isolated from the peripheral blood of dairy cows to evaluate the cell viability, chemotactic function, ability to phagocyte Gram+ and Gram– microorganisms, and extracellular Reactive Oxygens Species (**ROS**), with or without phorbol 12-myristate-13-acetate (**PMA**) as a pro-inflammatory challenge. Results demonstrated that ENN B did not affect bovine PMN viability and chemotactic activity at all concentrations ($p = 0.952$; $p = 0.218$, respectively). *E. coli* and *S. aureus* phagocytosis ability were reduced by ENN B at the highest concentrations (5 and 10 μM) compared to the negative control ($p \leq 0.001$; $p = 0.001$, respectively). Extracellular ROS production was increased by ENN B challenge under physiological and pro-inflammatory conditions ($p = 0.014$; $p < 0.001$, respectively). In conclusion, ENN B did not exert cytotoxic effects on bovine PMNs, while reduced phagocytic ability and increased the production of extracellular ROS, highlighting its potential role as an immunomodulator of the bovine innate immune response *in vitro*.

Implications: Emerging mycotoxin Enniatin B is a common grain contaminant worldwide that can exert cytotoxic and immunotoxic effects in animal cells. We hypothesized that Enniatin B could *in vitro* affect the bovine immune response. In our study, Enniatin B did not affect bovine polymorphonuclear cell viability and chemotaxis, while a reduction of phagocytosis and a modulation of extracellular reactive oxygen species were observed. The present study shows that Enniatin B *in vitro* exerts a potential role as an immunomodulator of the bovine innate immune response putting animals at an increased risk of infection diseases.

1. Introduction

Mycotoxins are known to impair the host's immune response (Korosteleva et al., 2009; Shandilya et al., 2023), and dairy ruminants can be chronically exposed to low mycotoxin levels in the diet since they can be present in a wide range of feedstuffs (*e.g.*, forages, grains, silages) (Cheli et al., 2013; Ogunade et al., 2018; Křížová et al., 2021). At present, the European Community is focusing on some emerging mycotoxins for their possible adverse effect on animals and humans and the lowest contamination levels have not been set so far (European Food Safety Authority, 2014).

Among the emerging mycotoxins, the presence of Enniatins (**ENNs**) in cereal grains and silages (Panasiuk et al., 2019; Reisinger et al., 2019; Křížová et al., 2021) is increasing worldwide (Cheli et al., 2013; Ogunade et al., 2018; Agriopoulou et al., 2020), continuously exposing dairy cows to their consumption and potentially impacting the innate immune system (Korosteleva et al., 2009). Enniatin B (**ENN B**) and Enniatin B1, produced by the *Fusarium* genus, are the two most prevalent analogs of ENNs and ENN B is currently the most detected in unprocessed and processed grains in Europe (Ivanova et al., 2006; Gauthier et al., 2013; Krug et al., 2018). So far, despite the recognition in cereal grains and silage contamination by ENNs, very few data are available on the effects of these emerging mycotoxins on dairy cow immunity.

Fusarium mycotoxins (*e.g.* deoxynivalenol, fumonisin B1) are reported to exert immunomodulatory effects on innate immune response by affecting the viability of phagocytic cells with a consequent reduction

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of host resistance to infectious diseases (Shandilya et al., 2023). Moreover, conditions of rumen dysbiosis or metabolic disorders, including subacute ruminal acidosis and impaired redox potential, can promote inadequate biodegradation of the emerging mycotoxins (Huang et al., 2018; Billenkamp et al., 2021), leading to a transfer of the mycotoxin itself or its metabolites to the blood and, potentially, to milk (Escrivá et al., 2017; Debevere et al., 2020; Xu et al., 2023).

The ENNs reported mechanism of action includes the lipophilic nature and the ionophoric properties of ENNs that promote their inclusion into cell membranes, interfering with cell physiology (Tonshin et al., 2010; Prosperini et al., 2017), and inhibiting acyl-CoA: cholesterol acyl transferase activity (Tomoda et al., 1992) with a consequent cytotoxic effect. ENN B can also enhance oxidative stress eliciting a higher lipid peroxidation and lysosomal functionality in *in vitro* models of Caco-2 cells (Ivanova et al., 2006b; Prosperini et al., 2013) inducing mitochondrial modifications and cell cycle disruption that result in increased apoptotic cell death; (Wätjen et al., 2009; Dornetshuber et al., 2007; Gammelsrud et al., 2012) and adrenal endocrine toxicity (Kalayou et al., 2015).

As the cow's first line of defense against external agents, bovine polymorphonuclear leukocytes (**PMNs**) are immune cells with a half-life of 7–12 h *in vivo* under physiological conditions protecting the body from infection by phagocyting and exposing pathogens to the destructive action of Reactive Oxygens Species (**ROS**) and hydrolytic granule proteins (Roos et al., 2003).

We hypothesize that ENN B, similarly to other *Fusarium* mycotoxins, could affect the function of bovine innate immune cells such as PMNs, finally leading to an impairment of the host defense. Thus, the present study investigates the *in vitro* effects of ENN B on bovine PMNs by evaluating the impact of different concentrations of ENN B on cell viability, chemotactic activity, phagocytosis of Gram + and Gram - bacteria, and extracellular ROS production.

2. Material and methods

2.1. Reagents and media

Cytochrome c, phorbol 12-myristate-13-acetate (**PMA**), Phosphate-buffered Saline without Ca^{2+} and Mg^{2+}, endotoxin-free water, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Red Blood Lysis Buffer Hybri-Max and Percoll were purchased from Sigma–Aldrich Corporation (St.Louis, MO, USA). RPMI-1640 cell culture media with phenol red were obtained from Invitrogen Life Technologies (Paisley, UK). Fetal Bovine Serum was provided by Biochrom AG (Berlin, Germany). *Escherichia coli* (K-12 strain cat. n° E2861) and *Staphylococcus aureus* (Wood strain without protein A; cat. n° S2851) fluorescein conjugate BioParticles™ were provided by Molecular Probes (Invitrogen, San Giuliano Milanese (Mi), Italy).

2.2. Preparation of Enniatin B

The stock solution of ENN B (Bioaustralis, cat. n° BIA-E1167) was prepared as suggested by the manufacturer's instructions. ENN B was reconstituted in sterile Dimethyl sulfoxide (**DMSO**) at a concentration of 15.6 mM and stored at −20 °C. Based on the assays, further working concentrations of ENN B were prepared using the complete medium consisting of RPMI-1640 with 20 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid, 1 % or 10 % heat-inactivated fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin or the Hank's Balance Salt Solution. The ENN B working concentrations (0, 0.625, 1.25, 2.5, 5, and 10 μM) were selected based on previously reported results (Gammelsrud et al., 2012; Ficheux et al., 2013; Küden et al., 2022). DMSO diluted at the same concentration of 10uM ENN B was used as vehicle control (vehicle).

2.3. Bovine polymorphonuclear leukocyte isolation

Bovine PMNs were isolated as previously reported (Lecchi et al., 2013). Peripheral blood was collected during slaughtering routine procedures at a local slaughterhouse from clinically healthy multiparous mid-lactating Holstein Friesian cows (BW 747.12 kg ± 22.62; parity 4.3 ± 1.06). Blood samples were collected using sterile tubes containing 1.8 mg K_2EDTA per ml of blood from the jugular vein during the slaughtering procedure. Blood samples were subsequently centrifuged at 1000 *g* for 30 min at 4 °C and the plasma, buffy coat, and top third of the red blood cell pellet were discharged. The remaining pellet of cells was diluted 1:3 in sterile cold Phosphate-buffered Saline, gently layered onto 10 mL 1.087 g/mL Percoll (Sigma–Aldrich), and centrifuged at 400 *g* for 40 min at room temperature. After removal of the Percoll layer, the remaining red blood cells were lysed in a hypotonic solution, then the remaining PMNs were washed four times with sterile cold Phosphate-buffered Saline.

2.4. Cells viability assay

Water-soluble tetrazolium salt (**WST-1**) Cell Proliferation Assay Kit (Cayman Chemical, cat.n° 10,008,883) was used to determine the cytotoxic effects of ENN B on bovine PMNs, following the manufacturer's instructions. PMNs (2×10^5/well) were challenged with increasing concentrations of ENN B (0.625, 1.25, 2.5, 5, and 10 μM) or DMSO (vehicle) diluted in the complete medium (10 % fetal bovine serum) for 1 h at 37 °C and 5 % CO_2 in a 96-well plate due to their short viability *ex vivo*. The effect of each concentration was assayed in duplicate. After 1 h, PMNs were incubated with 10 % (*v*/v) WST-1 reagent, and after 2 h of incubation, the absorbance was measured at a wavelength of 450 nm using a LabSystems Multiskan plate reader spectrophotometer (LabX, Midland, Canada).

2.5. Chemotaxis assay

PMN chemotaxis toward zymosan-activated serum (**ZAS**) was measured as previously reported (Cai et al., 2019; Ávila et al., 2020). Briefly, PMNs were incubated for 1 h with increasing concentrations (1.25, 2.5, 5) of ENN B or DMSO as vehicle control in the complete medium (1 % fetal bovine serum) in 24-well Transwell migration plates (Corning Inc.) equipped with a 5-μm pore size membrane. A total of 5×10^5 cells (100 μL final volume) were added in duplicate in the upper chamber, and migration medium (RPMI-1640 with 1 % fetal bovine serum and 0.15 mg/ml ZAS) was added into the lower chambers to a final volume of 600 μL. Cells were incubated for 1 h at 37 °C in a humified atmosphere of 5 % CO_2. For the negative control, cells were incubated without ENN B and ZAS. Finally, the upper chambers were removed, and the media under the filters were collected in tubes to count the migrated PMNs using the Mindray BriCyte E6 flow cytometer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd).

2.6. E. coli and S. aureus phagocytosis assays

Opsonization of fluorescein-labeled *Escherichia coli* (K-12 strain; cat. n° E2861) or *Staphylococcus aureus* (Wood strain without protein A; cat. n° S2851) bioparticles™ was performed by incubating the bacterial suspension (6×10^8) with 20 % autologous serum for 30 min at 37 °C, centrifuged at 800 ×*g* for 15 min and resuspended in sterile Phosphate-buffered Saline. Opsonized *E. coli* and *S. aureus* were stocked at −20 °C until use. Phagocytosis assay was carried out by incubating 5×10^4 PMNs/well in Hank's Balance Salt Solution for 1 h with increasing concentrations (0.625, 1.25, 2.5, 5, and 10 μM) of ENN B or DMSO (vehicle). Fluorescein-labeled *E. coli* or *S. aureus* bioparticles™ in a ratio of 45 particles/cell were added and incubated for 1 h. Cells were washed twice with Hank's Balance Salt Solution, incubated with 100 μl of 0.4 % trypan blue (2 min at room temperature) to quench the non-internalized

signal, washed twice with Hank's Balance Salt Solution, and then resuspended with 100 µl of Hank's Balance Salt Solution. The fluorescence (excitation, λ 494 nm; emission, λ 518 nm) intensity was detected using a fluorescence plate reader Fluoroscan Ascent (Thermo Electron Corporation, Vantaa, Finland) and was expressed as Relative Fluorescence Units (**RFU**).

2.7. Extracellular reactive oxygens species production

The modulation of oxidative burst activity of isolated bovine PMNs was studied by cytochrome *c* reduction assay, as previously reported (Pisani et al., 2009). PMNs (2×10^5/well) were challenged with increasing concentrations of ENN B (0.625, 1.25, 2.5, 5, and 10 µM) or DMSO (vehicle) and cytochrome *c* (1 mM) diluted in Hank's Balance Salt Solution and incubated at 37 °C and 5 % CO_2 in a 96-well plate for 150 min. To mimic a pro-inflammatory challenge, a second set of experiments was performed by adding PMA (2.5 µg/ml final concentration). The Optical density (**OD**) was measured at a wavelength of 550 nm using a LabSystems Multiskan plate reader spectrophotometer (LabX, Midland, Canada) every 30 min for 150 min.

2.8. Statistical analysis

Statistical analyses were performed in GraphPad Prism 8.0.2 (San Diego, CA). For the data normality assessment, the Shapiro–Wilk test was applied. Repeated measures of 1-way ANOVA for matched or paired data and Tukey's multiple comparison test were used to evaluate viability, chemotaxis, phagocytosis, and ROS production, under physiological and proinflammatory conditions. The applied model was:

$$y_{jk} = \mu + treat_j + time_k + (treat \times time)_{jk} + e_{jk}$$

Where y_{jk} is the i_{th} phenotypic record (viability, chemotaxis, phagocytosis, ROS production) from cow_n with treatment $_j$ (ENN B) at time $_k$ (hours on treatment); while e_{jk} is the residual. Treatment and time were considered fixed effects. Cows represented the experimental unit for all the parameters. Statistical differences were declared at $P \leq 0.05$.

3. Results

3.1. ENN B did not affect bovine PMN viability and chemotaxis

Bovine PMN viability was not affected by ENN B challenge at all the tested concentrations (0.625, 1.25, 2.5, 5, and 10 µM) when compared to the control (vehicle) ($p = 0.952$) (Fig. 1).

The influence of ENN B treatment on bovine PMN chemotactic activity is reported in Fig. 2. PMN chemotactic activity was not significantly influenced by ENN B treatment at all the tested concentrations

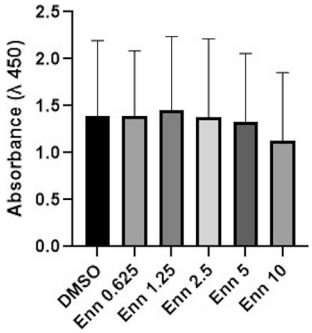

Fig. 1. Effects of different Enniatin B (ENN B) concentrations (0.625, 1.25, 2.5, 5 and 10 µM) on bovine polymorphonuclear leukocytes (PMNs) viability following Water-soluble tetrazolium salt (WST-1) assay. Data are means ± SEM of 8 independent experiments. Significance was declared for $P < 0.01$ (**) and $P < 0.05$ (*).

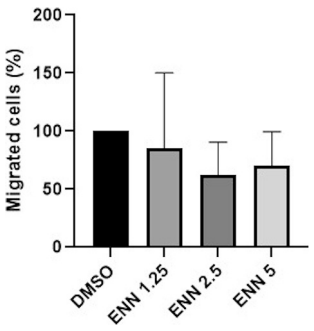

Fig. 2. Effects of different Enniatin B (ENN B) concentrations (1.25, 2.5, and 5 µM) on bovine polymorphonuclear leukocytes (PMNs) chemotaxis in response to exposure to zymosan-activated serum (ZAS). Data are means ± SEM of 8 independent experiments. Significance was declared for P < 0.01 (**) and P < 0.05 (*).

(1.25, 2.5, and 5 µM) when compared to the control (vehicle) although a slight reduction of migration was observed in cells challenged with ENN B 2.5 and 5 µM.

3.2. ENN B reduced bovine PMN phagocytosis

The ability of PMNs to phagocyte Gram- ($p < 0.001$) and Gram+ ($p = 0.001$) bacteria was reduced after the ENN B challenge. Specifically, ENN B at 5 µM ($p < 0.001$) and 10 µM ($p = 0.003$) decreased *E. coli* bioparticle phagocytosis compared to the control (vehicle)(Fig. 3A). The same results were observed for the engulfment of *S. aureus* when PMNs were pre-incubated with ENN B at 5 ($p = 0.016$) and 10 ($p < 0.001$) µM (Fig. 3B).

3.3. ENN B modulated bovine PMN extracellular reactive oxygens species production in physiological and pro-inflammatory environment

PMN extracellular ROS production was significantly affected by ENN B treatment when tested under physiological conditions ($p = 0.014$) (Fig. 4A). Specifically, ENN 5 and 10 µm showed the highest values compared to the control (vehicle) ($p = 0.001$; $p < 0.001$, respectively). On the contrary, ENN B at the lowest concentrations (0.625, 1.25, and 2.5 µM) inhibited ROS production in PMA-stimulated PMNs compared to the vehicle (DMSO) ($p < 0.001$), and no variation was observed when the highest mycotoxin levels were tested (Fig. 4B).

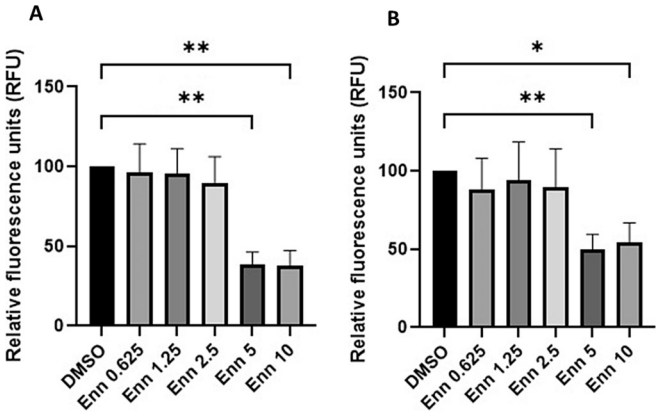

Fig. 3. Phagocytosis of fluorescein-labeled *Escherichia coli* (A) and *Staphylococcus aureus* (B) BioParticles™ by bovine polymorphonuclear leukocytes (PMNs) after incubation with different Enniatin B (ENN B) concentrations (0.625, 1.25, 2.5, 5 and 10 µM). Data are means ± SEM of 8 independent experiments. Significance was declared for P < 0.01 (**) and P < 0.05 (*).

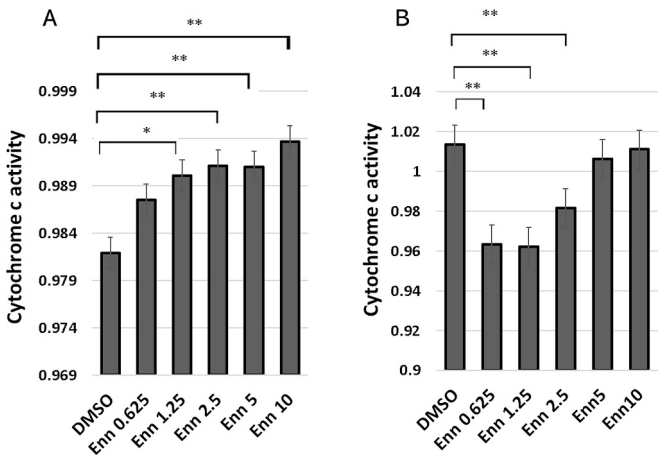

Fig. 4. Extracellular reactive oxygen specie (ROS) production by (A) not phorbol 12-myristate-13-acetate (PMA) and (B) PMA-stimulated bovine polymorphonuclear leukocytes (PMNs) exposed to increasing concentrations of Enniatin B (ENN B) (0.625, 1.25, 2.5, 5, and 10 μM) in the presence of cytochrome *c*. The graph refers to a time of 150′. Data are means ± SEM of 8 independent experimenti. Significance was declared for $P < 0.01$ (**) and $P < 0.05$ (*).

4. Discussion

The present study investigated the immunomodulatory activity of an emerging food and feed mycotoxin, ENN B, on dairy cow PMNs *in vitro*. Results demonstrated that ENN B did not affect the PMN viability and chemotaxis at the tested concentrations, reduced the phagocytosis of both Gram + and Gram – bacteria, and modulated the extracellular ROS production. Previous studies suggested that mycotoxins belonging to the class of ENNs may modulate the immune response both in animals and humans, affecting the function of the immune cells *in vitro* (Gammelsrud et al., 2012; Ficheux et al., 2012, 2013). ENNs are cytotoxic for different types of cells and the effect is directly related to the mycotoxin concentrations. Human immature and mature dendritic cells, macrophages, and lymphoblastoid Jurkat T-cells were negatively affected by ENN B at concentrations above 5 μM resulting in a drastic reduction of cell viability (Ficheux et al., 2013; Solhaug et al., 2016; Manyes et al., 2018). Similar results were reported in a murine macrophage model using RAW 267.4 cells, demonstrating that ENN B promotes the cell cycle arrest in G0/G1, the M2-like macrophage differentiation, apoptosis, and necrosis (Gammelsrud et al., 2012). ENN B exerts a cytotoxic effect also on a bovine mammary gland epithelial cells model, the MAC-T, reducing cell viability at concentrations greater than 5 μM (Xu et al., 2023). The present study pointed out that ENN B did not exert any effects on the viability of bovine PMNs even at 5 and 10 μM. To the best of the authors' knowledge, no data on the cytotoxic effect of ENNs have been investigated on bovine immune cells so far including PMNs. PMNs are produced in the bone marrow by committed progenitors, matured, and left the cell cycle entering the blood (Amulic et al., 2017; Hidalgo et al., 2019). We supposed that ENN B did not affect bovine PMN viability because circulating PMNs are mature cells without the capacity to divide since ENNs promote a cytotoxic effect by enhancing cell cycle arrest (Gammelsrud et al., 2012). Further study may investigate the effects of ENNs on myeloid progenitors.

Chemotaxis is an important component of wound healing, inflammation, and immunity and the migration of PMNs is necessary for the onset and success of immune response. The ability of ENNs mycotoxins to modulate the leukocyte chemotaxis has not yet been investigated. Cai et al. (2019) reported that zearalenone, a mycotoxin produced by several *Fusarium* species, inhibited T-cell chemotaxis at concentrations of 20 and 40 μM. The effect of deoxynivalenol, another mycotoxin produced by *Fusarium spp.*, has been investigated on swine PMNs (Gauthier et al., 2013b) demonstrating that deoxynivalenol at 10 and 50 μM reduced the chemotaxis, while did not exert any effect at the lowest concentration (2 μM). Our results demonstrated that ENN B did not affect the chemotactic activity of bovine PMNs suggesting that zearalenone and deoxynivalenol may impact different pathways compared to ENN B.

PMNs are the first line of defense against infection. ENNs are cyclic peptides exhibiting antibacterial activity against different bacteria species (Meca et al., 2011; Ola et al., 2013) and *Mycobacterium phlei* and *M. paratuberculosis* (Vesonder and Goliński, 1989; Supothina et al., 2004). To elucidate the role of ENNs in influencing the immune response of dairy cows, we investigated the ability of bovine PMNs to engulf Gram- (*E. coli*) and Gram+ (*S. aureus*) microorganisms, two of the main etiological agents of infection at the farms level. The results showed that PMNs, challenged with the highest concentrations of ENN B (5 and 10 μM), reduced their ability to engulf both Gram- and Gram+ bacteria. At concentrations lower than 10 μM, ENN B reduced ATP levels causing energy deprivation in Balb 3 T3 and HepG2 cells due to alteration of the mitochondrial electron transfer chain, leading to a cell activities decrease (Jonsson et al., 2016). The inflammatory microenvironment is ATP-rich (Bao et al., 2014; Borregaard and Herlin, 1982). ENN B influences mitochondrial activities through its ionophoric properties and the ability to transport cations (K+ and Ca2+) across the cell membranes, affecting ion transport and cell homeostasis (Hasuda and Bracarense, 2024). Moreover, ENNs may induce mitochondrial edema increasing the influx of K+ into the mitochondrial matrix and the outflow of Ca + from the mitochondria (Tonshin et al., 2010). In this context, since no data have been reported on the effect exerted by ENN B on PMN phagocytosis, we hypothesized that the impairment of mitochondrial activities caused by high concentrations of ENN B may reduce the ATP production affecting the effectiveness of bacteria phagocytosis. Since *S. aureus* and *E. coli* are two etiological agents of intramammary infections (Jamali et al., 2018; Rana et al., 2022), the reduction of PMN phagocytic capacity observed in this study, could reflect a decreased of *in vivo* ability to remove bacteria exposing the mammary gland to infection diseases (Overton and Waldron, 2004), further confirmed by the reduction of ROS in a PMA-induced pro-inflammatory environment. We speculated that the ENN B may modulate ROS production affecting mitochondria and that the effect may depend on the ability of cells to respond to environmental stimuli. However, our data differed from results reported on other cell models; the ROS production by Jurkat T-cells was not affected by ENN B (Manyes et al., 2018), while colorectal adenocarcinoma (Caco-2) increased ROS generation after a challenge with 3 μM ENN B (Prosperini et al., 2013). ROS production is central to the progression of many inflammatory diseases. At low concentrations, ROS serve complex signaling functions modulating cell growth, adhesion, differentiation, senescence, and apoptosis, while, at high concentrations, they are deleterious (Dröge, 2002). We speculate that ENN B at the lowest doses might exert a protective effect on PMN regulating the ROS production and promoting PMN activity, such as phagocytosis, while at the highest concentrations, ENN B might enhance the mitochondria impairment impacting phagocytosis and ROS production. To further investigate this hypothesis, the ENN B's ability to modulate the PMN antioxidant defense systems could be evaluated.

5. Conclusion

Results reported in our investigation showed that ENN B modulated the bovine PMN activities *in vitro*. In detail, ENN B at high concentrations reduced the PMNs phagocytosis of *E. coli* and *S. aureus*, while did not exert any effect at the lowest concentrations. At the same time, ENN B decreased the production of extracellular ROS in a pro-inflammatory condition thus modulating the antimicrobial activity of bovine PMNs. Altogether, our results supported the hypothesis that ENN B may negatively affect the bovine immune response *in vitro* potentially reflecting a more bovine susceptibility to infectious diseases *in vivo*.

Ethics approval

Not applicable.

Data and model availability statement

None of the data were deposited in an official repository.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

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CRediT authorship contribution statement

S. Sandrini: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **G. Salvi:** Writing – review & editing, Writing – original draft, Formal analysis. **D. Ravanelli:** Writing – review & editing, Writing – original draft, Formal analysis. **V. Perricone:** Writing – review & editing, Formal analysis. **L.G. De Matos:** Writing – review & editing, Formal analysis. **F. Ceciliani:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **C. Lecchi:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **A. Agazzi:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

None.

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None.

References

Agriopoulou, S., Stamatelopoulou, E., Varzakas, T., 2020. Advances in occurrence, importance, and mycotoxin control strategies: prevention and detoxification in foods. Foods 9, 137. https://doi.org/10.3390/foods9020137.

Amulic, B., Knackstedt, S.L., Abu Abed, U., Deigendesch, N., Harbort, C.J., Caffrey, B.E., Brinkmann, V., Heppner, F.L., Hinds, P.W., Zychlinsky, A., 2017. Cell-cycle proteins control production of neutrophil extracellular traps. Dev. Cell 43, 449–462.e5. https://doi.org/10.1016/j.devcel.2017.10.013.

Ávila, G., Catozzi, C., Pravettoni, D., Sala, G., Martino, P., Meroni, G., Lecchi, C., Ceciliani, F., 2020. In vitro effects of conjugated linoleic acid (CLA) on inflammatory functions of bovine monocytes. J. Dairy Sci. 103, 8554–8563. https://doi.org/10.3168/jds.2020-18659.

Bao, Y., Ledderose, C., Seier, T., Graf, A.F., Brix, B., Chong, E., Junger, W.G., 2014. Mitochondria regulate neutrophil activation by generating ATP for autocrine purinergic signaling. J. Biol. Chem. 289, 26794–26803. https://doi.org/10.1074/jbc.M114.572495.

Billenkamp, F., Schnabel, K., Hüther, L., Frahm, J., von Soosten, D., Meyer, U., Höper, D., Beer, M., Seyboldt, C., Neubauer, H., Dänicke, S., 2021. No hints at glyphosate-induced ruminal dysbiosis in cows. NPJ Biofilms Microbiomes 7, 30. https://doi.org/10.1038/s41522-021-00198-4.

Borregaard, N., Herlin, T., 1982. Energy metabolism of human neutrophils during phagocytosis. J. Clin. Invest. 70, 550–557. https://doi.org/10.1172/JCI110647.

Cai, G., Pan, S., Feng, N., Zou, H., Gu, J., Yuan, Y., Liu, X., Liu, Z., Bian, J., 2019. Zearalenone inhibits T cell chemotaxis by inhibiting cell adhesion and migration related proteins. Ecotoxicol. Environ. Saf. 175, 263–271. https://doi.org/10.1016/j.ecoenv.2019.03.045.

Cheli, F., Campagnoli, A., Dell'Orto, V., 2013. Fungal populations and mycotoxins in silages: from occurrence to analysis. Anim. Feed Sci. Technol. 183, 1–16. https://doi.org/10.1016/j.anifeedsci.2013.01.013.

Debevere, S., Cools, A., De Baere, S., Haesaert, G., Rychlik, M., Croubels, S., Fievez, V., 2020. In vitro rumen simulations show a reduced disappearance of Deoxynivalenol, Nivalenol and Enniatin B at conditions of rumen acidosis and lower microbial activity. Toxins 12, 101. https://doi.org/10.3390/toxins12020101.

Dornetshuber, R., Heffeter, P., Kamyar, M.-R., Peterbauer, T., Berger, W., Lemmens-Gruber, R., 2007. Enniatin exerts dependent cytostatic and independent cytotoxic activities against human cancer cells. Chem. Res. Toxicol. 20, 465–473. https://doi.org/10.1021/tx600259t.

Dröge, W., 2002. Free radicals in the physiological control of cell function. Physiol. Rev. 82, 47–95. https://doi.org/10.1152/physrev.00018.2001Escrivá, L., Font, G., Manyes, L., Berrada, H., 2017. Studies on the Presence of Mycotoxins in Biological Samples: An Overview. Toxins 9, 251. 10.3390/toxins9080251.

Ficheux, A.S., Sibiril, Y., Parent-Massin, D., 2012. Co-exposure of fusarium mycotoxins: in vitro myelotoxicity assessment on human hematopoietic progenitors. Toxicon 60, 1171–1179. https://doi.org/10.1016/j.toxicon.2012.08.001.

Ficheux, A.S., Sibiril, Y., Parent-Massin, D., 2013. Effects of beauvericin, enniatin b and moniliformin on human dendritic cells and macrophages: an in vitro study. Toxicon 71, 1–10. https://doi.org/10.1016/j.toxicon.2013.04.024.

Gammelsrud, A., Solhaug, A., Dendelé, B., Sandberg, W.J., Ivanova, L., Kocbach Bølling, A., Lagadic-Gossmann, D., Refsnes, M., Becher, R., Eriksen, G., Holme, J.A., 2012. Enniatin B-induced cell death and inflammatory responses in RAW 267.4 murine macrophages. Toxicol. Appl. Pharmacol. 261, 74–87. https://doi.org/10.1016/j.taap.2012.03.014.

Gauthier, T., Waché, Y., Laffitte, J., Taranu, I., Saeedikouzehkonani, N., Mori, Y., Oswald, I.P., 2013. Deoxynivalenol impairs the immune functions of neutrophils. Mol. Nutr. Food Res. 57, 1026–1036. https://doi.org/10.1002/mnfr.201200755.

Hasuda, A.L., Bracarense, A.P.F.R.L., 2024. Toxicity of the emerging mycotoxins beauvericin and enniatins: a mini-review. Toxicon 239, 107534. https://doi.org/10.1016/j.toxicon.2023.107534.

Hidalgo, A., Chilvers, E.R., Summers, C., Koenderman, L., 2019. The neutrophil life cycle. Trends Immunol. 40, 584–597. https://doi.org/10.1016/j.it.2019.04.013.

Huang, Y., Marden, J.P., Julien, C., Bayourthe, C., 2018. Redox potential: an intrinsic parameter of the rumen environment. J. Anim. Physiol. Anim. Nutr. 102, 393–402. https://doi.org/10.1111/jpn.12855.

Ivanova, L., Skjerve, E., Eriksen, G.S., Uhlig, S., 2006. Cytotoxicity of enniatins a, A1, B, B1, B2 and B3 from fusarium avenaceum. Toxicon 47, 868–876. https://doi.org/10.1016/j.toxicon.2006.02.012.

Jamali, H., Krylova, K., Aïder, M., 2018. Identification and frequency of the associated genes with virulence and antibiotic resistance of *Escherichia coli* isolated from cow's milk presenting mastitis pathology. Anim. Sci. J. 89, 1701–1706. https://doi.org/10.1111/asj.13093.

Jonsson, M., Jestoi, M., Anthoni, M., Welling, A., Loivamaa, I., Hallikainen, V., Kankainen, M., Lysøe, E., Koivisto, P., Peltonen, K., 2016. Fusarium mycotoxin enniatin B: cytotoxic effects and changes in gene expression profile. Toxicol. in Vitro 34, 309–320. https://doi.org/10.1016/j.tiv.2016.04.017.

Kalayou, S., Ndossi, D., Frizzell, C., Groseth, P.K., Connolly, L., Sørlie, M., Verhaegen, S., Ropstad, E., 2015. An investigation of the endocrine disrupting potential of enniatin B using in vitro bioassays. Toxicol. Lett. 233, 84–94. https://doi.org/10.1016/j.toxlet.2015.01.014.

Korosteleva, S.N., Smith, T.K., Boermans, H.J., 2009. Effects of feed naturally contaminated with fusarium mycotoxins on metabolism and immunity of dairy cows. J. Dairy Sci. 92, 1585–1593. https://doi.org/10.3168/jds.2008-1267.

Křížová, L., Dadáková, K., Dvořáčková, M., Kašparovský, T., 2021. Feedborne mycotoxins beauvericin and enniatins and livestock animals. Toxins. https://doi.org/10.3390/TOXINS13010032.

Krug, I., Behrens, M., Esselen, M., Humpf, H.-U., 2018. Transport of enniatin B and enniatin B1 across the blood-brain barrier and hints for neurotoxic effects in cerebral cells. PLoS One 13, e0197406. https://doi.org/10.1371/journal.pone.0197406.

Küden, C., Karakaş, S.N., Batmaz, S.G., 2022. Comparative chemical properties, bioactivity, and cytotoxicity of resin-modified calcium silicate–based pulp capping materials on human dental pulp stem cells. Clin. Oral Investig. 26, 6839–6853. https://doi.org/10.1007/s00784-022-04713-5.

Lecchi, C., Scarafoni, A., Bronzo, V., Martino, P.A., Cavallini, A., Sartorelli, P., Ceciliani, F., 2013. α1-acid glycoprotein modulates phagocytosis and killing of *Escherichia coli* by bovine polymorphonuclear leucocytes and monocytes. Vet. J. 196, 47–51. https://doi.org/10.1016/j.tvjl.2012.07.022.

Manyes, L., Escrivá, L., Ruiz, M.J., Juan-García, A., 2018. Beauvericin and enniatin B effects on a human lymphoblastoid Jurkat T-cell model. Food Chem. Toxicol. 115, 127–135. https://doi.org/10.1016/j.fct.2018.03.008.

Meca, G., Sospedra, I., Valero, M.A., Mañes, J., Font, G., Ruiz, M.J., 2011. Antibacterial activity of the enniatin B, produced by *fusarium tricinctum* in liquid culture, and cytotoxic effects on Caco-2 cells. Toxicol. Mech. Methods 21, 503–512. https://doi.org/10.3109/15376516.2011.556202.

Ogunade, I.M., Martinez-Tuppia, C., Queiroz, O.C.M., Jiang, Y., Drouin, P., Wu, F., Vyas, D., Adesogan, A.T., 2018. Silage review: mycotoxins in silage: occurrence, effects, prevention, and mitigation. J. Dairy Sci. https://doi.org/10.3168/jds.2017-13788.

Ola, A.R.B., Thomy, D., Lai, D., Brötz-Oesterhelt, H., Proksch, P., 2013. Inducing secondary metabolite production by the endophytic fungus *fusarium tricinctum* through Coculture with *Bacillus subtilis*. J. Nat. Prod. 76, 2094–2099. https://doi.org/10.1021/np400589h.

Overton, T.R., Waldron, M.R., 2004. Nutritional Management of Transition Dairy Cows: strategies to optimize metabolic health. J. Dairy Sci. 87, E105–E119. https://doi.org/10.3168/jds.S0022-0302(04)70066-1.

Panasiuk, L., Jedziniak, P., Pietruszka, K., Piatkowska, M., Bocian, L., 2019. Frequency and levels of regulated and emerging mycotoxins in silage in Poland. Mycotoxin Res. 35, 17–25. https://doi.org/10.1007/s12550-018-0327-0.

Pisani, L.F., Lecchi, C., Invernizzi, G., Sartorelli, P., Savoini, G., Ceciliani, F., 2009. In vitro modulatory effect of ω-3 polyunsaturated fatty acid (EPA and DHA) on phagocytosis and ROS production of goat neutrophils. Vet. Immunol. Immunopathol. 131, 79–85. https://doi.org/10.1016/j.vetimm.2009.03.018.

Prosperini, A., Juan-García, A., Font, G., Ruiz, M.J., 2013. Reactive oxygen species involvement in apoptosis and mitochondrial damage in Caco-2 cells induced by enniatins A, A1, B and B1. Toxicol. Lett. 222, 36–44. https://doi.org/10.1016/j.toxlet.2013.07.009.

Prosperini, A., Berrada, H., Ruiz, M.J., Caloni, F., Coccini, T., Spicer, L.J., Perego, M.C., Lafranconi, A., 2017. A review of the mycotoxin enniatin B. Front. Public Health. https://doi.org/10.3389/fpubh.2017.00304.

Rana, E.A., Fazal, M.A., Alim, M.A., 2022. Frequently used therapeutic antimicrobials and their resistance patterns on *Staphylococcus aureus* and *Escherichia coli* in mastitis affected lactating cows. Intern. J. Veterin. Sci. Med. 10, 1–10. https://doi.org/10.1080/23144599.2022.2038494.

Reisinger, N., Schürer-Waldheim, S., Mayer, E., Debevere, S., Antonissen, G., Sulyok, M., Nagl, V., 2019. Mycotoxin occurrence in maize silage—a neglected risk for bovine gut health? Toxins 11, 577. https://doi.org/10.3390/toxins11100577.

Roos, D., van Bruggen, R., Meischl, C., 2003. Oxidative killing of microbes by neutrophils. Microbes Infect. 5, 1307–1315. https://doi.org/10.1016/j.micinf.2003.09.009.

Shandilya, U.K., Sharma, A., Xu, R., Muniz, M.M.M., Karrow, N.A., 2023. Evaluation of immunomodulatory effects of fusarium mycotoxins using bacterial endotoxin-stimulated bovine epithelial cells and macrophages in co-culture. Genes 14, 2014. https://doi.org/10.3390/genes14112014.

Solhaug, A., Karlsøen, L.M., Holme, J.A., Kristoffersen, A.B., Eriksen, G.S., 2016. Immunomodulatory effects of individual and combined mycotoxins in the THP-1 cell line. Toxicol. in Vitro 36, 120–132. https://doi.org/10.1016/j.tiv.2016.07.012.

Supothina, S., Isaka, M., Kirtikara, K., Tanticharoen, M., Thebtaranonth, Y., 2004. Enniatin production by the Entomopathogenic fungus Verticillium hemipterigenum BCC 1449. J. Antibiot. 57, 732–738. https://doi.org/10.7164/antibiotics.57.732.

Tomoda, H., Huang, X.-H., Caoa, J., Nishida, H., Nagao, R., Okuda, S., Tanaka, H., Omura, S., Arai, H., Inoue, K., 1992. Inhibition of acyl-CoA: cholesterol acyltransferase activity by cyclodepsiptide antibiotics. J. Antibiot. 45, 1626–1632. https://doi.org/10.7164/antibiotics.45.1626.

Tonshin, A.A., Teplova, V.V., Andersson, M.A., Salkinoja-Salonen, M.S., 2010. The fusarium mycotoxins enniatins and beauvericin cause mitochondrial dysfunction by affecting the mitochondrial volume regulation, oxidative phosphorylation and ion homeostasis. Toxicology 276, 49–57. https://doi.org/10.1016/j.tox.2010.07.001.

Vesonder, R.F., Goliński, P., 1989. Metabolites of fusarium. In: Fusarium. Elsevier, pp. 1–39. https://doi.org/10.1016/B978-0-444-87468-9.50006-7.

Wätjen, W., Debbab, A., Hohlfeld, A., Chovolou, Y., Kampkötter, A., Edrada, R.A., Ebel, R., Hakiki, A., Mosaddak, M., Totzke, F., Kubbutat, M.H.G., Proksch, P., 2009. Enniatins A1, B and B1 from an endophytic strain of *fusarium tricinctum* induce apoptotic cell death in H4IIE hepatoma cells accompanied by inhibition of ERK phosphorylation. Mol. Nutr. Food Res. 53, 431–440. https://doi.org/10.1002/mnfr.200700428.

Xu, R., Shandilya, U.K., Yiannikouris, A., Karrow, N.A., 2023. Traditional and emerging fusarium mycotoxins disrupt homeostasis of bovine mammary cells by altering cell permeability and innate immune function. Animal Nutrit. 12, 388–397. https://doi.org/10.1016/j.aninu.2022.10.007.