



Original Research Article

Melatonin shapes bacterial clearance function of porcine macrophages during enterotoxigenic *Escherichia coli* infection

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ABSTRACT

Due to the immature gastrointestinal immune system, weaning piglets are highly susceptible to pathogens, e.g., enterotoxigenic *Escherichia coli* (ETEC). Generally, pathogens activate the immune cells (e.g., macrophages) and shape intracellular metabolism (including amino acid metabolism); nevertheless, the metabolic cues of tryptophan (especially melatonin pathway) in directing porcine macrophage function during ETEC infection remain unclear. Therefore, this study aimed to investigate the changes in the serotonin pathway of porcine macrophages during ETEC infection and the effect of melatonin on porcine macrophage functions. Porcine macrophages (3D4/21 cells) were infected with ETEC, and the change of serotonin pathway was analysed by reverse transcription PCR and metabolomic analysis. The effect of melatonin on porcine macrophage function was also studied with proteomic analysis. In order to investigate the effect of melatonin on bacterial clearance function of porcine macrophages during ETEC infection, methods such as bacterial counting, reverse transcription PCR and western blotting were used to detect the corresponding indicators. The results showed that ETEC infection blocked melatonin production in porcine macrophages ($P < 0.05$) which is largely associated with the heat-stable enterotoxin b (STb) of ETEC ($P < 0.05$). Interestingly, melatonin altered porcine macrophage functions, including bacteriostatic and bactericidal activities based on proteomic analysis. In addition, melatonin pre-treatment significantly reduced extracellular lactate dehydrogenase (LDH) activity ($P < 0.05$), indicating that melatonin also attenuated ETEC-triggered macrophage death. Moreover, melatonin pre-treatment resulted in the decrease of viable ETEC in 3D4/21 cells ($P < 0.05$), suggesting that melatonin enhances bacterial clearance of porcine macrophages. These results suggest that melatonin is particularly important in shaping porcine macrophage function during ETEC infection.

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1. Introduction

The high mortality rate of piglets has brought huge economic losses to the pig industry (Zhang et al., 2018). Due to the immature

gastrointestinal tract and immune system, weaning piglets are highly susceptible to pathogens, leading to diarrhea and even death (Lalles et al., 2007). It has been well documented that post weaning diarrhea is a multifactorial disease (Dierick et al., 2020), and the most common pathogen associated with diarrhea is enterotoxigenic *Escherichia coli* (ETEC) (Chen et al., 2020; Ren et al., 2017; Xia et al., 2019b). ETEC mainly produces 2 types of enterotoxins, heat-labile enterotoxins (LT) and heat-stable enterotoxins (ST, including STa and STb), both of which are involved in the occurrence and development of diarrhea (Dierick et al., 2020; Dubreuil et al., 2016;

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Ren et al., 2014b). Primarily, ETEC adheres to the ileum epithelium and then produces enterotoxins to penetrate the epithelium (Dubreuil et al., 2016; Ren et al., 2014b).

Macrophage is the first line of defence against pathogens (Sedlyarov et al., 2018). When the pathogen enters, macrophages detect and phagocytize it and present fragmented peptides to T helper cells (Th), then release pro-inflammatory cytokines and chemokines to destroy pathogens and protect the host (Patel et al., 2017). Generally, macrophage undergoes 2 distinct states in the context of various external stimuli (Patel et al., 2017). Resting macrophage (M0) polarizes into classically activated (M1) macrophage supporting bactericidal activity while polarizes into alternately activated (M2) macrophage to clear parasites and/or repair damaged tissues (Russell et al., 2019). The fine-tuning of macrophage polarization is essential for disease control and tissue homeostasis (Van den Bossche et al., 2017). Macrophage polarization is determined by various aspects, including metabolic pathways and metabolites (Kelly and O'Neill, 2015). Of note, pathogens affect immune cell activation and shape intracellular metabolism (e.g., amino acid metabolism) (Chapman et al., 2017). Therefore, investigating the regulation of amino acid metabolism on the fate decision of porcine immune cells is helpful to improve the resistance of piglets to pathogen invasion and reduce the mortality of piglets.

Tryptophan (Trp), as an essential amino acid, is mainly metabolized through 3 pathways: serotonin pathway, kynurenine pathway, and intestinal microbial pathway (Agus et al., 2018). Studies have shown that there are differences in host Trp metabolic remodeling caused by different types and characteristics of pathogens (e.g., extracellular or intracellular bacteria) (El-Zaatari et al., 2014; Zhang et al., 2013). When pigs have chronic pneumonia, their plasma Trp levels gradually decrease (Melchior et al., 2004). Similarly, mycobacterial infection causes a decrease in the level of Trp in peripheral blood mononuclear cells (Plain et al., 2011). In addition, Trp metabolism varies in the context of different intestinal inflammatory diseases (Manocha and Khan, 2012). For example, in Crohn's disease, the expression of tryptophan hydroxylase 1 (Tph1), a relevant enzyme in the serotonin pathway, is increased (Lamas et al., 2016; Manocha and Khan, 2012; Monteleone et al., 2011). As for inflammatory bowel disease, the activity of the key enzyme indoleamine 2,3-dioxygenase 1 in the kynurenine pathway is higher, while the production of indole and its derivatives via the gut microbial pathway is reduced (Lamas et al., 2016; Monteleone et al., 2011). It should also be noted that the downstream products of Trp metabolism (e.g., serotonin and kynurenic acid) have distinct effects on macrophage functions which are largely dependent on the active concentrations of specific metabolites as well as the expressions of related receptors (Herr et al., 2017; Moroni et al., 2012). At present, most studies on Trp metabolism focus on kynurenine pathway, but few studies on serotonin pathway. The alterations in metabolic cues of Trp metabolism (chiefly by serotonin pathway) in macrophages during pathogen infection are not clear. And studies have shown that melatonin can affect the polarization and function of macrophages (Xia et al., 2022, 2019a). However, whether melatonin has the same effect on porcine macrophages needs further investigation. More importantly, the key regulatory mechanism of intermediates from serotonin pathway in shaping macrophage functions during pathogen infection warrants further investigation.

Therefore, the purpose of this study is to explore the changes, physiological significance, and potential mechanism of intracellular serotonin pathway in guiding porcine macrophage functions during ETEC infection, which we hope to provide nutritional targets for tackling microbial diarrhea of weaning piglets.

2. Materials and methods

2.1. Animals and animal ethics

All animal studies were approved by and under the conduction of the Laboratory Animal Ethical Commission of the South China Agricultural University. All studies used 6- to 8-week-old mice and were the same age and gender for individual experiments.

2.2. Cell culture

The porcine alveolar macrophages (3D4/21 cells) were cultured in RPMI 1640 (Gibco, New York, USA) supplemented with 10% FBS (Excell Bio, Taicang, China) and 1% penicillin/streptomycin (Gibco, New York, USA) at 37 °C with 5% CO₂. The 3D4/21 cells were provided by Professor Huahua Du, College of Animal Science, Zhejiang University, China.

2.3. Murine primary peritoneal exudate macrophages (PEMs)

Mice (Changsheng, Liaoning, China) were intraperitoneal injected with 4% thioglycolate for 2 to 4 d, then macrophages were collected from peritoneal cavity with PBS (Gibco, New York, USA) and cultured in complete DMEM (Gibco, New York, USA).

2.4. ETEC infection of 3D4/21 cells

The ETEC F4ac C83902, ETEC F4ac ΔLT and ETEC F4ac ΔSTb were used in this study, among which wild strains were *E. coli* W25K (O149: K91, K88ac; LT, STb, EAST) (Ren et al., 2014a), and the knockout strains were provided by Professor Guoqiang Zhu, College of Veterinary Medicine, Yangzhou University, China. The ETEC cells were cultured in 10 mL Luria–Bertani (LB) (Sangon Biotech, Shanghai, China) at 37 °C and harvested at the log phase. The 3D4/21 cells were infected with ETEC for 1 h (multiplicity of infection [MOI] = 10:1), and culture supernatant and cells were collected. In some experiments, 3D4/21 cells were pre-treated with 1 mM melatonin (Sigma–Aldrich, St Louis, USA) for 18 h followed by ETEC infection.

2.5. ETEC co-culture with cellular lysate

The 3D4/21 cells were pre-treated with or without melatonin (1 mM) for 18 h and the cellular lysate was collected to incubate with ETEC. From 0 to 32 h, OD values at 600 nm were measured every 4 h and the growth curve of ETEC was plotted. In some experiments, cellular lysate was collected in the same way and treated by different methods (including DNAase, RNAase, and/or boiled) before incubation with ETEC for 12 h. The OD_{600 nm} value of ETEC cultured with pre-treated cellular lysate for 12 h was measured.

2.6. Cellular viable ETEC counting

The 3D4/21 cells were pre-treated with or without melatonin (1 mM) for 18 h followed by ETEC infection (MOI = 10:1, for 1 h). Then, the cells were lysed to obtain bacterial suspension for plating on solid agar after a dilution series, and the number of viable cells was then determined by counting colony forming units (CFU).

2.7. Metabolites analysis

The contents of intracellular metabolites associated with serotonin pathway were determined by isotope dilution liquid chromatography–mass spectrometry (LC–MS) methods.

2.8. Quantitative reverse transcription-PCR (RT-PCR) analysis

The total RNA of the samples was extracted using EZ-Press RNA Purification Kit (EZBioscience, Roseville, USA) according to the manufacturer's instructions. RNA was reverse transcribed to complementary DNA by using the Color Reverse Transcription Kit (EZBioscience, Roseville, USA) following the manufacturer's recommendations. RT-PCR was conducted via $2 \times$ Color SYBR Green qPCR Master Mix (EZBioscience, Roseville, USA) on the Quant Studio 6 Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA). Fold change was assessed using the $2^{-\Delta\Delta Ct}$ method using β -actin as a reference gene. The primers used in this study are shown in Table 1.

2.9. Western blotting

Western blotting was performed as follows. Briefly, cells were washed with frozen cold PBS and dissolved in a radio-immunoprecipitate analysis buffer containing proteases and phosphatase inhibitors (Beyotime, Shanghai, China). The protein concentration was detected with the bicinchoninic acid protein detection kit (Beyotime, Shanghai, China), and the same amount of protein was detected by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to polyvinylidene fluoride membranes, then incubated with primary and secondary antibodies, and visualized using chemiluminescence reagents. The optical density of the signal on the film was quantified by Image J software.

2.10. Detection of cytokines and mediators

Culture supernatants from cell culture and cell samples were collected, and mediators such as reactive oxygen species (ROS) (Beyotime, Shanghai, China) and lactate dehydrogenase (LDH) (Njjcbio, Nanjing, China) were measured by commercial kits following the protocols.

2.11. Proteomics analysis

Melatonin treated-3D4/21 cells were collected for proteomics analysis in NovoGene (Beijing, China). Differentially expressed proteins (DEPs) were used for Volcanic map analysis, cluster heat map analysis, and enrichment analysis of Gene Ontology (GO), InterPro (IPR), and Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.12. Statistical analysis

All statistical analyses were performed using GraphPad Prism V.8.0. Software, and results were represented as means \pm SEM or SD. Non-paired *t*-test was used to analyse the data between the 2 groups. Non-paired *t*-test was used if the data obeyed normal distribution and the variance was equal, non-paired *t*-test with Welch's correction was used if the data obeyed Gaussian distribution but the variance was not equal, and non-parametric test was used if the data did not obey normal distribution. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. ETEC infection specially leads to metabolic remodeling of serotonin pathway in porcine macrophages

Almost 90% to 95% Trp is metabolized through kynurenine pathway (Wilson et al., 2012); nevertheless, few studies have focused on Trp metabolism via serotonin pathway in macrophages during pathogen infection. To determine Trp metabolism through serotonin pathway in macrophages during ETEC infection, we detected the contents of corresponding metabolites in 3D4/21 cells after ETEC infection. After ETEC infection, the serotonin pathway in 3D4/21 cells was blocked, as evidenced by the decreased levels of Trp, 5-hydroxytryptophan (5-HTP), serotonin, and melatonin (Fig. 1A). Moreover, the reduced mRNA expressions

Table 1
Primer used for mRNA expression analysis by RT-PCR.

Species	Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	
Pig	β -actin	GCACCGTGTGGCGTAGAGG	GGACTTCGAGCAGGAGATGG	
	TpH1	CCGTCTGTGGCTGTTACTTATC	GTCCGAACCGTGTCTCATACTG	
	AADC	GGAAGTGCCAGTGAAGCTACCTTG	AGGAGTGTGCCTGGTCGGATG	
	AANAT	GCCACCTACCATCCCAGAGTCC	TCAAACAGCCAGCAGCATCC	
	ASMTL	TGCCAGGGCACAAAGTGAACG	GCTGCTTCTGCCACCAGGATG	
	MAOa	TGACCTCCGAACCCACGAAAG	GCTTACTTGACCAGATCCGCCTAC	
	MAOb	AATTTGTGGGGGATCTGGTCAAG	ACACCTCGTGGTTAGGGTCTCC	
	IL-1 β	CATTGCACGTTTCAAGGATG	AGTGAGAAGCCGATGAAGA	
	TNF- α	GCTGTCCCTCGGCTTGAC	CCAGCTTCTGCTACTGCTG	
	IL-10	TCAAAGGGGCTCCCTAGTTT	CTGCCTCCCACTTCTCTTG	
	MARCO	TCAGGATTATCGGCTCCAGGAACC	GCGGCAGAAGACAGTGGCATC	
	CLEC7A	ATCAAGGCATGTGTCTTCCCAACC	GAGCCATCCTCCAAAGCCATG	
	Mouse	GAPDH	AGGTCGGTGTGAACGGATTG	TGTAGACCATGTAGTTGAGGTCA
		TpH1	GGACATGCCAAAGTCAAGCCCTTT	GTACTTCAGTCCAAACGGGGCGCTTC
		AADC	GCATTGAGGGTCTGTCAGTG	CTGGGTATGAGCTAGCCGCTG
AANAT		TCACACCTGAGGATGCCACCAG	CCACAGCGAGCCAATGATGAAGG	
ASMT		GCACCGAGGTACCCGTGTTT	GAAGAAGTCGCTGACAGGAAGC	
MAOa		CAACACAGTGGAGTGGCTACATGG	AGCAGACCAGGCACGGAAGG	
MAOb		TGTGAAGCAGTGTGGAGGTACAAC	CCGTTCACTCACTTGACCAGATCC	
ETEC	LT	AGCCGGTTTGTGTTCTCTC	AGGTTTATACCCAGAGGGCA	
	STb	TGCCACAGATACAACGGAT	GGAATGCCCTTCTGTGTT	

TpH1 = tryptophan hydroxylase 1; AADC = aromatic amino acid decarboxylase; AANAT = arylalkylamine N-acetyltransferase; ASMTL = acetylserotonin O-methyltransferase like; MAO = monoamine oxidase; IL-1 β = interleukin-1 β ; TNF- α = tumour necrosis factor- α ; IL-10 = interleukin-10; MARCO = macrophage receptor with collagenous structure; CLEC7A = C-type lectin domain containing 7A; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; ASMT = acetylserotonin O-methyltransferase; ETEC = enterotoxigenic *Escherichia coli*; LT = heat-labile enterotoxins; STb = heat-stable enterotoxin b.

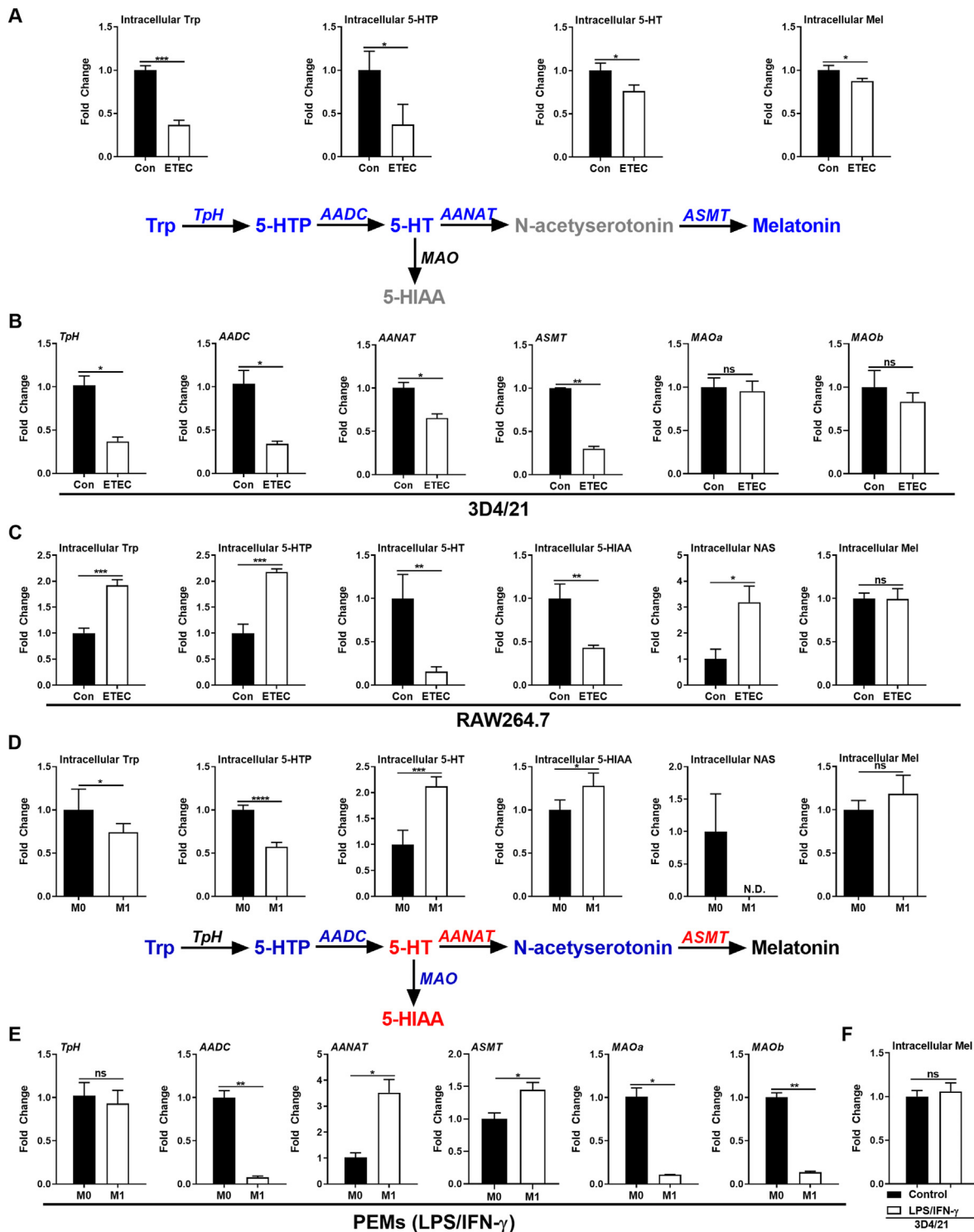


Fig. 1. ETEC infection specially leads to metabolic remodeling of serotonin pathway in porcine macrophages. (A and B) The changes of intracellular metabolites and genes in serotonin pathway of porcine macrophages (3D4/21 cells) with or without ETEC infection ($n = 3$). Results represent 2 independent experiments. (C) The changes of intracellular metabolites in serotonin pathway of RAW264.7 cells with or without ETEC infection ($n = 4$). Results represent 3 independent experiments. (D and E) The changes of intracellular metabolites and genes in serotonin pathway of PEMs stimulated with or without LPS/IFN- γ ($n = 3$ to 6). Results represent 2 independent experiments. (F) The changes of intracellular melatonin content in 3D4/21 cells stimulated with or without LPS/IFN- γ ($n = 4$). Results represent 3 independent experiments. Trp = tryptophan; 5-HTP = 5-hydroxytryptophan; 5-HT = serotonin; Mel = melatonin; ETEC = enterotoxigenic *Escherichia coli*; TpH = tryptophan hydroxylase; AADC = aromatic amino acid decarboxylase; AANAT = arylalkylamine N-acetyltransferase; ASMT = acetylserotonin O-methyltransferase; MAO = monoamine oxidase; 5-HIAA = 5-hydroxyindoleacetic acid; NAS = N-acetylserotonin; M0 = resting macrophage; M1 = classically activated macrophage; PEMs = peritoneal exudate macrophages; LPS/IFN- γ = lipopolysaccharides plus interferon gamma. Blue color indicates down-regulated, and red color represents up-regulated. ns for no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

of serotonin pathway-related enzymes were observed (Fig. 1B), including *TpH*, aromatic amino acid decarboxylase (*AADC*), arylalkylamine N-acetyltransferase (*AANAT*), and acetylserotonin O-methyltransferase (*ASMT*). The change of serotonin pathway in RAW264.7 cells (murine macrophage cell line) infected with ETEC was also detected. The contents of Trp, 5-HTP, and N-acetylserotonin (NAS) were increased, while the contents of serotonin and 5-hydroxyindoleacetic acid (5-HIAA) were decreased (Fig. 1C). Notably, there was no difference in intracellular melatonin content (Fig. 1C). Therefore, the blockage of serotonin pathway caused by ETEC infection may be unique to pigs rather than mice.

Considering *E. coli* could polarize macrophage towards M1 phenotype through the lipopolysaccharides (LPS) (Benoit et al., 2008), thus, we detected the change of serotonin pathway in PEMs stimulated with LPS plus interferon gamma (IFN- γ) (LPS/IFN- γ). The concentrations of intracellular Trp and 5-HTP were dramatically decreased, while the concentrations of serotonin and 5-HIAA were increased (Fig. 1D). The content of melatonin had no difference in PEMs stimulated with LPS/IFN- γ (Fig. 1D). LPS/IFN- γ

stimulation promoted the expressions of *AANAT* and *ASMT*, but inhibited the expression of *AADC* (Fig. 1E). Moreover, there was also no difference in the content of melatonin in 3D4/21 cells with LPS/IFN- γ stimulation (Fig. 1F). Therefore, these data suggest that ETEC infection-induced the blockage of serotonin pathway in 3D4/21 cells may be largely associated with ETEC itself (e.g., enterotoxins).

3.2. Intracellular serotonin pathway remodeling in porcine macrophages induced by ETEC infection may depend on *STb*

Given that ETEC has virulence factors (e.g., LT and ST) (Dierick et al., 2020), we used various ETEC strains, including normal strain (F4ac C83902), ETEC lacking LT strain (F4ac Δ LT), and ETEC lacking *STb* strain (F4ac Δ STb), to infect 3D4/21 cells, respectively (Fig. 2A). Only F4ac Δ STb could restore the intracellular content of melatonin (Fig. 2B) and the expression of *AANAT* (Fig. 2C). These findings suggest that the *STb* of ETEC may inhibit the expression of *AANAT* and ultimately lower the intracellular melatonin. The

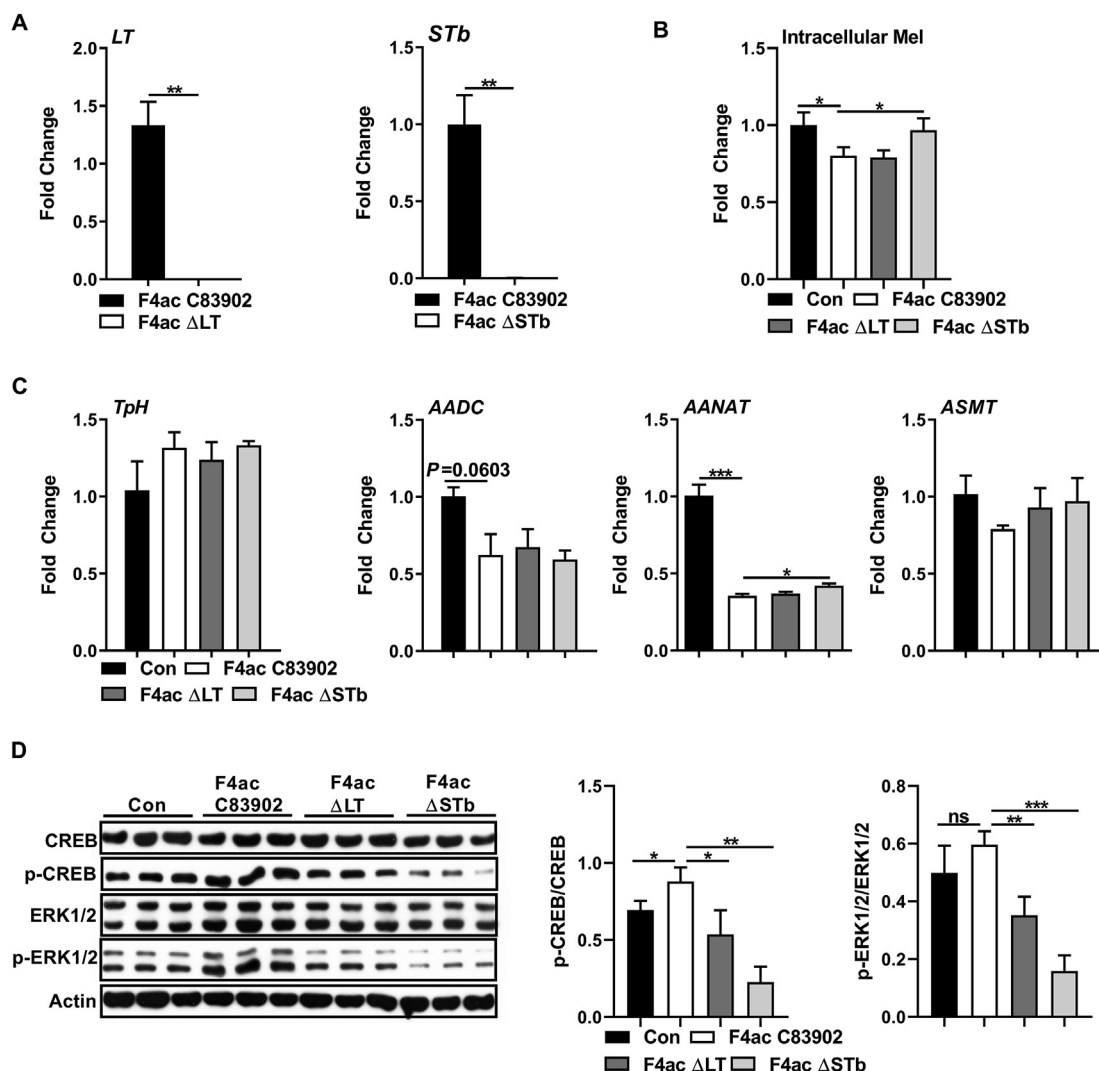


Fig. 2. Heat-stable enterotoxin b of ETEC transcriptionally inhibits *AANAT* expression and decreases melatonin in porcine macrophages. (A) The expressions of *LT* gene of F4ac Δ LT and *STb* gene of F4ac Δ STb ($n = 3$). (B–D) The changes of intracellular melatonin content, melatonin synthesis-related gene expression, and activation of CREB and ERK in 3D4/21 cells infected with F4ac C83902, F4ac Δ LT, and F4ac Δ STb, respectively ($n = 3$). Results represent 2 independent experiments. ETEC = enterotoxigenic *Escherichia coli*; LT = heat-labile enterotoxins; STb = heat-stable enterotoxin b; Mel = melatonin; *TpH* = tryptophan hydroxylase; *AADC* = aromatic amino acid decarboxylase; *AANAT* = arylalkylamine N-acetyltransferase; *ASMT* = acetylserotonin O-methyltransferase; CREB = cyclic adenosine monophosphate-responsive element binding protein; p-CREB = phosphorylated CREB; ERK1/2 = extracellular signal-regulated kinase 1/2; p-ERK1/2 = phosphorylated ERK1/2. ns for no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

expression of AANAT is mainly regulated by protein kinase A/cyclic adenosine monophosphate/cAMP-responsive element binding protein (PKA/cAMP/CREB) and Raf-1 proto-oncogene, serine/threonine kinase/mitogen-activated protein kinase/extracellular signal-regulated protein kinase (RAF1/MEK/ERK) signalling pathways (Qiu et al., 2019; Zheng et al., 2021). Thus, we detected the abundance of key proteins in these 2 signalling pathways and found that the wild strain F4ac C83902 did not inhibit the activation of these 2 pathways. More importantly, F4ac ΔSTb strain failed to activate these 2 pathways (Fig. 2D), suggesting that ETEC-mediated lower AANAT

expression is independent on these 2 signalling pathways. Thus, other potential mechanisms by which ETEC-mediated expression of AANAT need to be revealed.

3.3. Melatonin affects the bacteriostatic or bactericidal functions of porcine macrophages

ETEC infection specifically reduces melatonin content in porcine macrophages, which suggests that melatonin may be involved in regulating the functions of porcine macrophages (e.g.,

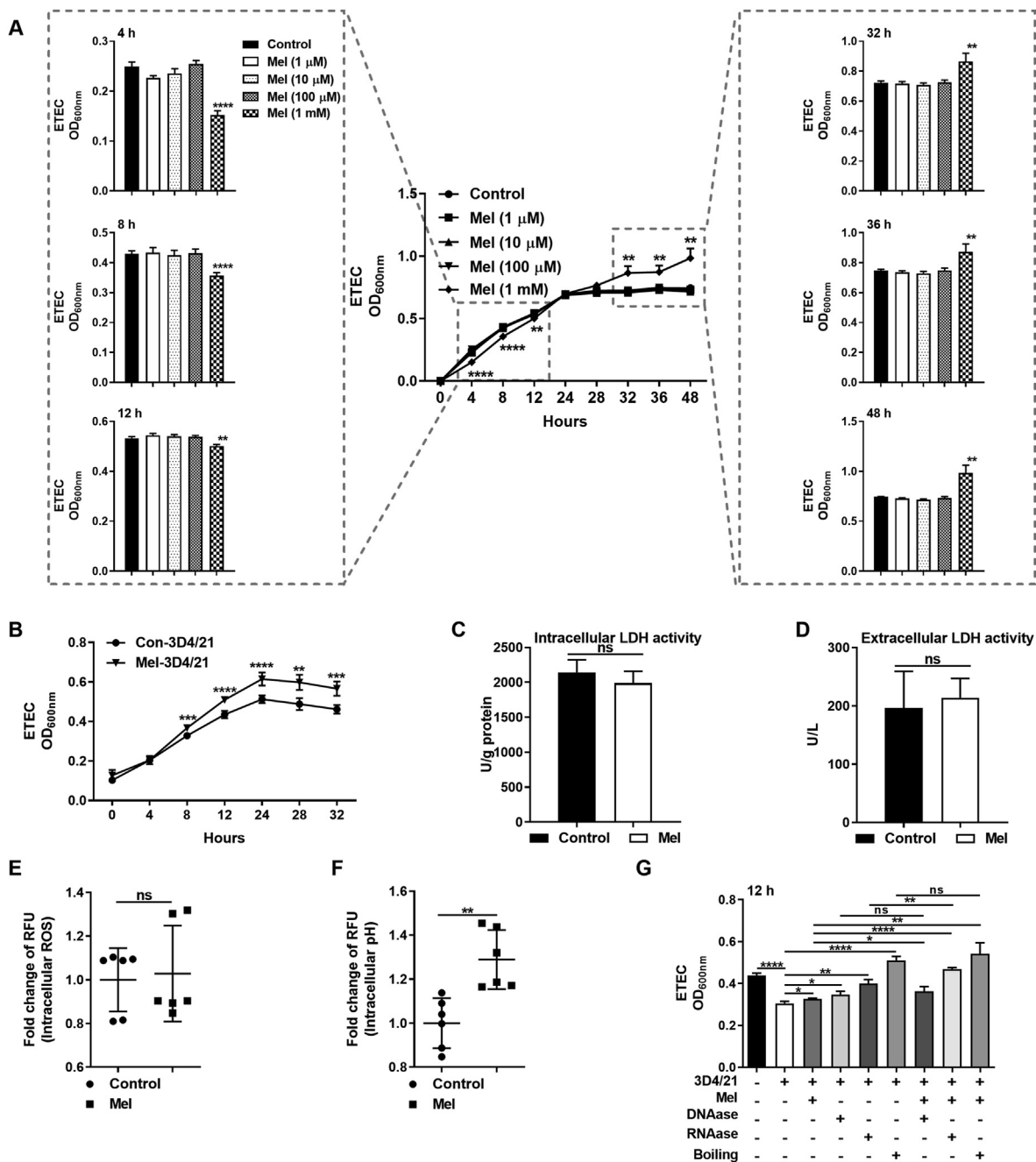


Fig. 3. Effect of melatonin on bactericidal function of porcine macrophages. (A) The effect of different concentrations of melatonin on the growth of ETEC at different time points ($n = 6$). Results represent 2 independent experiments. (B) The growth curve of ETEC incubated with lysates of 3D4/21 cells treated with or without melatonin (1 mM, for 18 h, the same as below unless indicated) ($n = 6$). (C–F) The changes of intracellular and extracellular LDH activity (C and D), intracellular ROS level (E), and intracellular pH value (F) of 3D4/21 cells with or without melatonin treatment ($n = 3$ to 6). Results represent 2 or 3 independent experiments. (G) The OD_{600 nm} value of ETEC cultured with cell lysates treated by different methods. ETEC = enterotoxigenic *Escherichia coli*; Mel = melatonin; LDH = lactate dehydrogenase; ROS = reactive oxygen species; RFU = relative fluorescence units. ns for no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

bacteriostatic or bactericidal functions). Firstly, different concentrations of melatonin (1, 10 100 μ M, and 1 mM) were supplemented to ETEC culture medium. We found ETEC growth was significantly inhibited during 0–12 h while it was significantly enhanced during 32 to 48 h (Fig. 3A). Then, 3D4/21 cells were pre-treated with melatonin (1 mM) for 18 h, and then the cellular lysate was collected to incubate with ETEC. The growth of ETEC was even enhanced (4 to 32 h), which suggested that melatonin might inhibit the bacteriostatic or bactericidal functions of porcine macrophages (Fig. 3B). Interestingly, the attenuation of bacteriostatic or bactericidal functions of porcine macrophages

was not associated with the cell viability and intracellular ROS level of 3D4/21 cells treated with melatonin, but melatonin increased intracellular pH value of 3D4/21 cells (Fig. 3C–F). Like melatonin treatment, pre-treatment with DNAase, RNAase or boiling blocked the bacteriostatic or bactericidal functions of 3D4/21 cells, however, only DNAase or boiling treatment blocked the melatonin-mediated attenuation of bacteriostatic or bactericidal functions of porcine macrophages (Fig. 3G). Collectively, melatonin may alter bacteriostatic or bactericidal functions of porcine macrophages by affecting factors associating with DNA and proteins, especially about proteins.

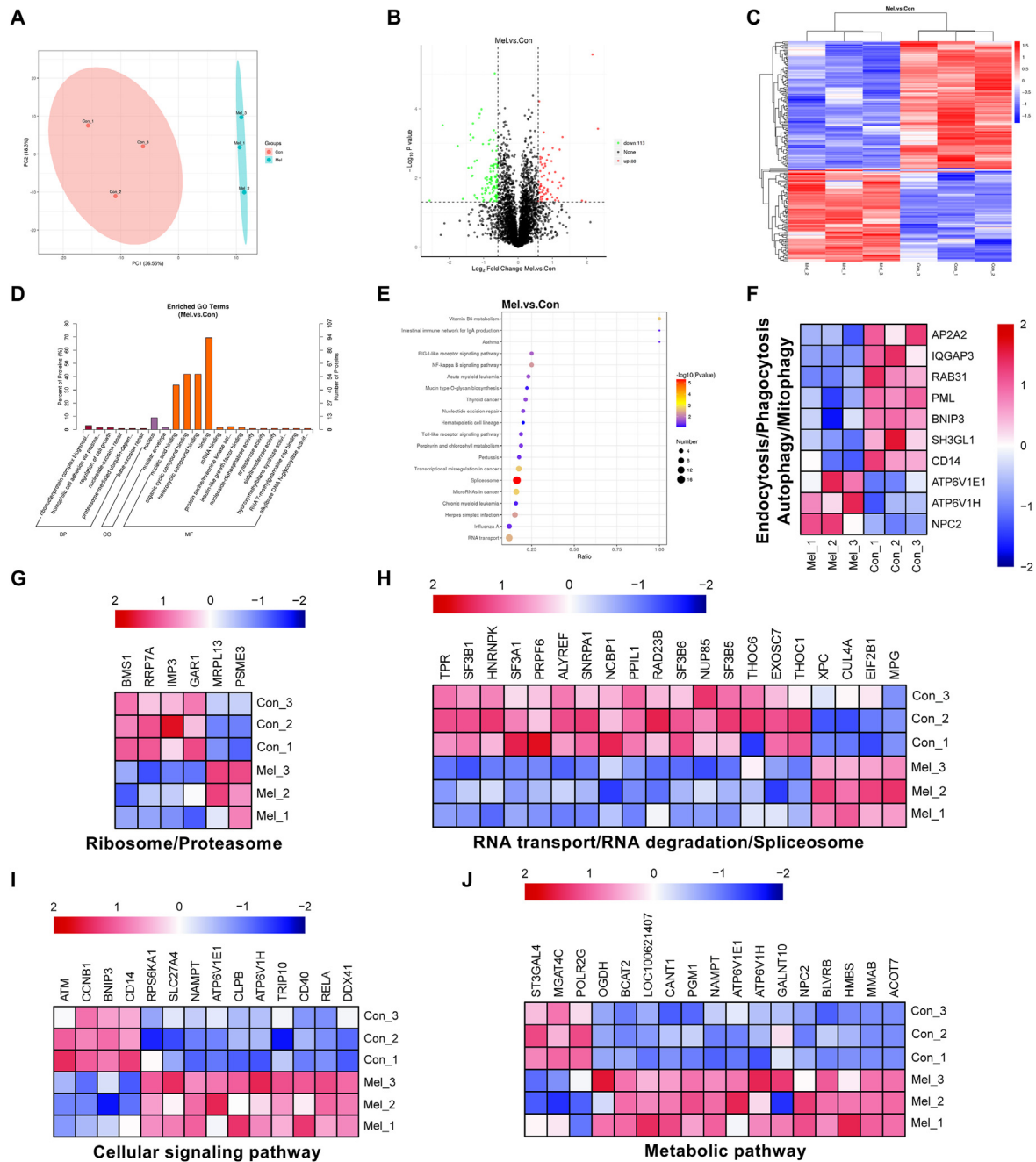


Fig. 4. The proteomic profile of 3D4/21 cells treated with or without melatonin ($n = 3$). (A) Principal component analysis. (B) Differential protein volcano map. (C) Differential protein clustering heatmap. (D) GO enrichment histogram. (E) KEGG enrichment bubble diagram. (F–J) The heatmap of differentially expressed proteins enriched in endocytosis/phagocytosis/autophagy/mitophagy, ribosome/proteasome, RNA transport/RNA degradation/Spliceosome, cellular signaling pathway, and metabolic pathway. Blue color indicates down-regulated, and red color represents up-regulated.

To explore the nature of how melatonin affects bacteriostatic or bactericidal functions of porcine macrophages through influencing intracellular proteins, we conducted the proteomics analysis. We found that melatonin significantly changed the protein profile of 3D4/21 cells, in which 113 proteins were down-regulated while 80 proteins were up-regulated (Fig. 4A–C; Supplementary File: the list of differentially expressed proteins between the melatonin-treated group and control group). GO analysis showed that the most differentially expressed proteins were enriched in molecular function, such as nuclear acid binding (Fig. 4D). KEGG analysis also showed that melatonin treatment affected various pathways, for instance, Spliceosome (Fig. 4E). Most interestingly, the most differentially expressed proteins related to bacteriostatic or bactericidal functions of porcine macrophages were significantly down-regulated, including endocytosis, phagocytosis, autophagy, and mitophagy (Fig. 4F–J).

3.4. Melatonin remodels the bacterial clearance function of porcine macrophages

Moreover, we also investigated the effects of melatonin on bacterial clearance function of porcine macrophages. Firstly, 3D4/21 cells were pre-treated with melatonin for 18 h followed by ETEC infection (MOI = 10:1, for 1 h). Melatonin alleviated the

death of porcine macrophages caused by ETEC infection (Fig. 5A). More importantly, the number of intracellular viable ETEC was markedly reduced with melatonin pre-treatment (Fig. 5B). We also found that melatonin pre-treatment promoted the mRNA expressions of pro-inflammatory cytokines, like interleukin-1 β (IL-1 β) and tumour necrosis factor α (TNF- α), and phagocytic receptors, like macrophage receptor with collagenous structure (MARCO) and C-type lectin domain containing 7A (CLEC7A) in porcine macrophages during ETEC infection (Fig. 5C and D). These results indicate that melatonin may enhance the bacterial scavenging function of porcine macrophages, protecting porcine macrophages from death during ETEC infection. The enhanced bacterial scavenging function of porcine macrophages with melatonin pre-treatment was not dependent on intracellular ROS level and pH value of 3D4/21 cells (Fig. 5E to F). However, we found that melatonin altered the abundance of autophagy-related proteins in porcine macrophages during ETEC infection. The abundance of p62 was significantly decreased while the abundance of microtubule associated protein 1 light chain 3B (LC3B) was highly increased (Fig. 5G). These results indicate that melatonin may enhance the bacterial scavenging function of porcine macrophages, which is largely involved in facilitating autophagy. However, the well-designed experiments still need to be conducted to validate the hypothesis.

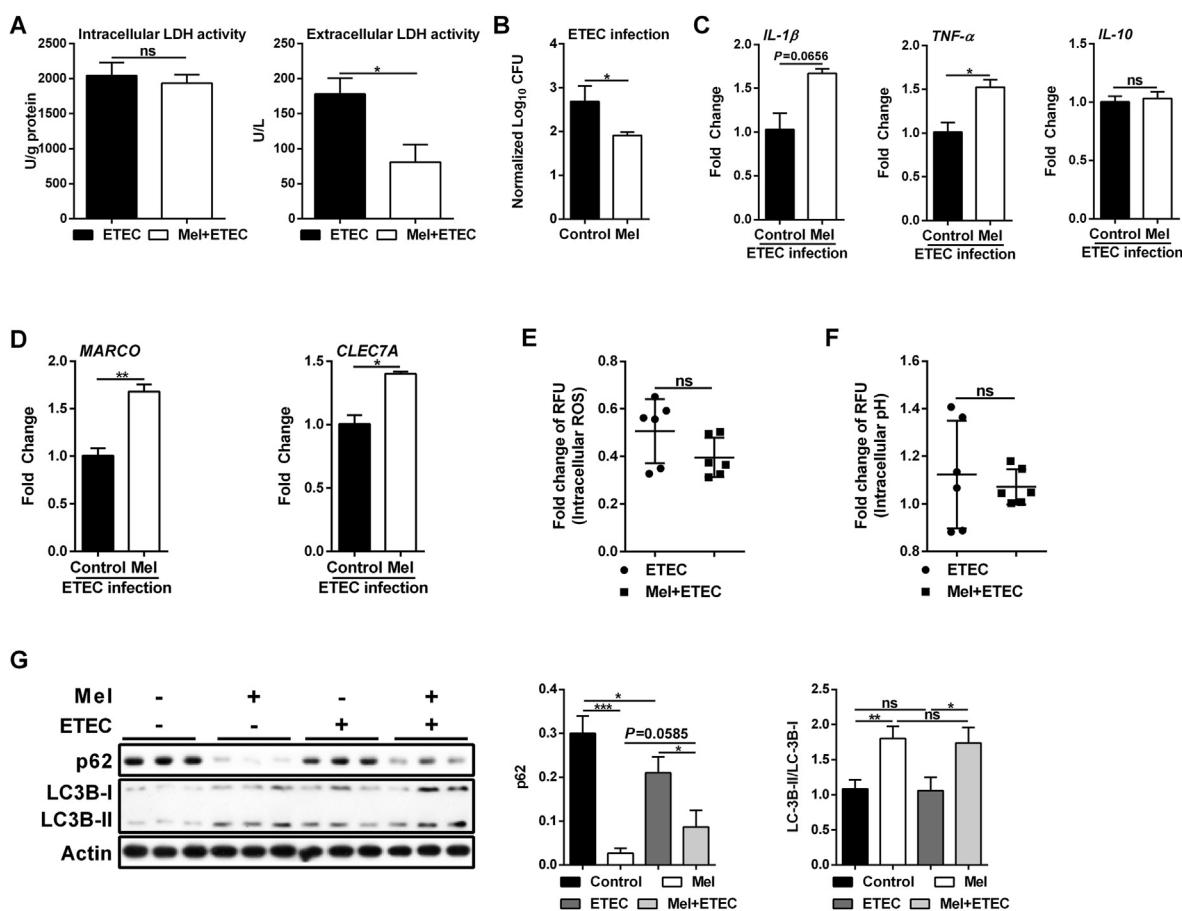


Fig. 5. Melatonin enhances the bacterial clearance functions of porcine macrophages. (A–F) The changes of intracellular (left panel) and extracellular LDH activity (right panel), number of viable bacteria, pro-inflammatory cytokine expression, MARCO and CLEC7A expression, intracellular ROS level, and intracellular pH value of ETEC-infected 3D4/21 cells with or without melatonin pretreatment ($n = 3$ to 6). Results represent 3 independent experiments. (G) The protein abundance of p62 and LC3B in 3D4/21 cells with various treatments as indicated ($n = 3$). LDH = lactate dehydrogenase; ETEC = enterotoxigenic *Escherichia coli*; Mel = melatonin; IL-1 β = interleukin-1 β ; TNF- α = tumour necrosis factor- α ; IL-10 = interleukin-10; MARCO = macrophage receptor with collagenous structure; CLEC7A = C-type lectin domain containing 7A; RFU = relative fluorescence units. ns=no significance ROS = reactive oxygen species; LC3B = microtubule associated protein 1 light chain 3B. ns for no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4. Discussion

Previous studies have revealed that Trp is mainly metabolized via kynurenine pathway in cells; however, little is known about metabolic cues of Trp through serotonin pathway in macrophages (especially in the context of bacterial infection). Indeed, the use of porcine intestinal macrophages could better illustrate the results and elucidate the mechanisms involved. But due to porcine primary macrophages are difficult to isolate, and the lacking of specific cytokines to induce porcine macrophage maturation and activation, we used porcine alveolar macrophage cell lines currently available for the study. Despite the heterogeneity of tissue-resident macrophages, their core functions are similar, especially for their ability to clear pathogens (Davies and Taylor, 2015; Jenkins and Allen, 2021). Furthermore, we demonstrate that exogenous melatonin can reduce the number of inflammatory macrophages and the level of inflammatory factors in jejunum of piglets (unpublished) in the context of bacterial infection. Combined these findings, we believe that the usage of 3D4/21 cells is fine and enough to reveal the anti-inflammatory effect of melatonin during bacterial infection.

Our study found that ETEC infection blocks the serotonin pathway of 3D4/21 cells, as evidenced by lower melatonin in particular. This phenomenon may be unique to pigs, which might be due to the existence of species specificity. The pathogenesis of ETEC infection strongly depends on the production of adhesive and enterotoxin (Noel et al., 2018). Its pathogenesis involves host factors, among which are adhesin and/or enterotoxin receptors (Dubreuil et al., 2016). Species specificity is a general feature of ETEC infection, mainly because specific receptors exist only in one or a limited range of animal species (Nagy and Fekete 2005; Sinha et al., 2019). In the case of diarrhea after weaning, the 2 main fimbriae F4 (K88) and F18 have different receptors specificity (Nagy and Fekete, 2005). The adhesion phenotype of F4ab and F4ac is highly correlated with the existence of the same intestinal mucin-type sialo glycoprotein receptor (Nagy and Fekete, 2005). The receptor is encoded by the related loci on porcine chromosome 13 (Nagy and Fekete, 2005). However, F18ab fimbriae, F18ac and their subvariants are closely related to the α (1,2) fucosyltransferase genes (*FUT1* and *FUT2*) on porcine chromosome 6 (Nagy and Fekete, 2005). Therefore, the blockage of intracellular serotonin pathway that is specially caused by ETEC infection might be closely related to the presence of specific receptors in pigs.

Notably, STb-mediated lower *AANAT* expression is not dependent on PKA/cAMP/CREB and/or RAF1/MEK/ERK signalling pathways. Some studies have shown that miRNAs regulate the expression of *AANAT*. For example, miR-483, miR-325-3p, and miR-7 inhibit the expression of *AANAT* (Clokic et al., 2012; Qiu et al., 2019; Yang et al., 2017). Therefore, miRNA is the probable target for STb to regulate the expression of *AANAT*.

Our findings have shown that ETEC infection causes a lower melatonin in porcine macrophages, indicating that melatonin could affect macrophage functions, especially during pathogen infection. At present, studies have shown that melatonin regulates macrophage functions (Xia et al., 2019a). For example, melatonin enhances the phagocytosis of rat testicular macrophages and peritoneal macrophages by increasing intracellular levels of free calcium (Esteban et al., 2004; Pawlak et al., 2005). This is consistent with our results that melatonin enhances the phagocytosis of 3D4/21 cells. However, our study found that melatonin might inhibit the bactericidal function of 3D4/21 cells. Although the function of macrophages was initially simply described as phagocytosis and killing microorganisms (Van den Bossche et al., 2017), with the deepening of the research, macrophages are not only an important

part of maintaining self-stability, but also widely involved in a series of physiological processes such as defence, cell metabolism, self-repair, and inflammation (Kotwal and Chien, 2017; Liu et al., 2017; O'Neill and Artyomov, 2019; Van den Bossche et al., 2017). Therefore, we cannot simply define whether melatonin is beneficial or harmful to macrophages according to the effects of melatonin on the phagocytosis and bactericidal functions of 3D4/21 cells.

Based on the current results, we speculate that melatonin may enhance the bacterial clearance function of porcine macrophages by promoting autophagy. After the activation of autophagy, light chain 3B (LC3B)–I is transformed into LC3B-II that could be integrated into the membrane of autophagosome (Mao et al., 2019). The conversion of LC3B–I to LC3B-II is a common indicator of autophagy, and the expression of LC3B-II is directly related to the number of autophagosomes (Mao et al., 2019). Moreover, the abundance of p62 (also known as sequestosome (SQSTM) 1) is negatively correlated with autophagy (Chang et al., 2018). Studies have shown that HeLa cells can clear *Streptococcus pyogenes* through autophagy (Nakagawa et al., 2004). And autophagy can inhibit mycobacterial survival in macrophages (Gutierrez et al., 2004). In addition, melatonin alleviates oxidative stress-induced cell death in retinal pigment epithelial cell line (ARPE-19) cells by promoting autophagy (Chang et al., 2018). In this study, during ETEC infection, melatonin down-regulated protein abundance of p62, while LC3B-II/LC3B–I ratio increased significantly. As a result, melatonin may enhance autophagy by regulating the expression of these 2 autophagy-related proteins, thus enhancing the bacterial clearance function of porcine macrophages.

In vivo experiments regarding the effects of melatonin on porcine macrophage function are ongoing. Our current results have shown that exogenous melatonin reduces the number of CD68⁺ macrophages in jejunum of piglets during ETEC infection (unpublished). In addition, other studies showed that exogenous melatonin can improve neural development, barrier integrity, intestinal absorption function, and microbiota in sucking piglets to promote intestinal development (Xia et al., 2022).

Collectively, ETEC infection specificity leads to metabolic remodeling of serotonin pathway and especially reduces melatonin level in porcine macrophages. Intriguingly, melatonin may inhibit the bactericidal function of porcine macrophages by affecting effectors associating with cellular proteins. Also, melatonin alleviates the macrophage death caused by ETEC infection and enhances the bacterial scavenging function of porcine macrophages. These results suggest that melatonin is specially required for shaping porcine macrophage function during ETEC infection, which provides a potential intervention strategy for resolving microbial infection-induced diarrhea and even death of piglets.

Author contributions

Lingfei Du: Validation, Writing - Original Draft; **Bingnan Liu:** Validation; **Ziyi Han:** Validation; **Yaoyao Xia:** Validation, Writing - Original Draft, Data Curation; **Miaomiao Wu:** Writing - Review & Editing; **Shaojuan Liu:** Writing - Review & Editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

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