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NOTE

Toxicology

Effects of exposure to the neonicotinoid pesticide clothianidin on α -defensin secretion and gut microbiota in mice

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ABSTRACT. The mechanism by which the neonicotinoid pesticide clothianidin (CLO) disrupts the intestinal microbiota of experimental animals is unknown. We focused on α -defensins, which are regulators of the intestinal microbiota. Subchronic exposure to CLO induced dysbiosis and reduced short-chain fatty acid-producing bacteria in the intestinal microbiota of mice. Levels of cryptdin-1 (Crp1, a major α -defensin in mice) in feces and cecal contents were lower in the CLO-exposed groups than in control. In Crp1 immunostaining, Paneth cells in the jejunum and ileum of the no-observed-adverse-effect-level CLO-exposed group showed a stronger positive signal than control, likely due to the suppression of Crp1 release. Our results showed that CLO exposure suppresses α -defensin secretion from Paneth cells as part of the mechanism underlying CLO-induced dysbiosis.

KEYWORDS: α -defensin, clothianidin, dysbiosis, microbiota, neonicotinoid

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Neonicotinoid pesticides (NNs) were registered and marketed in the 1990s for their selective toxicity to insects via nicotinic acetylcholine receptors (nAChRs). However, imidacloprid and acetamiprid, which are NNs, were found to have excitatory effects on mammalian cerebellar neurons via nAChRs [24]. Subsequently, questions arose about the reproductive toxicity of NNs [17, 21, 25, 47, 53], their neurotoxicity and involvement in cognitive–emotional alterations [14, 16–18, 20, 26, 27, 33, 44, 45, 55], and their safety, even at the no-observed-adverse-effect level (NOAEL). Recently, subchronic exposure to clothianidin (CLO), a type of NN, has been reported to disrupt the intestinal microbiota of rats [35] and mice [30, 56]. CLO may affect the host via dysbiosis, as reduced diversity of the gut microbiota (dysbiosis) is associated with immunomodulatory dysfunction [37] and neurological disorders [4]. However, the mechanism underlying CLO-induced dysbiosis is not clear.

α -Defensins, which are responsible for intestinal innate immunity, are antimicrobial peptides secreted by Paneth cells in the intestinal crypts of the small intestine. They are bactericidal to pathogenic bacteria but have little effect on commensal bacteria [28], and they regulate the intestinal microbiota appropriately by participating in “elimination” and “symbiosis” of the intestinal environment.

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Recently it has been shown that a decrease in α -defensin causes dysbiosis in depression model mice [43]. Similarly, dysbiosis in Crohn's disease model mice has been linked to abnormal α -defensins [40], highlighting a clearer understanding of the connection between α -defensins and dysbiosis. The secretory stimuli of α -defensins are microbial stimulation of Paneth cells [2] and cholinergic nerve stimulation [39]. The sympathetic, parasympathetic, and enteric nervous systems are suggested to be involved in the secretion of α -defensins. The significant role of nAChRs in these neurotransmissions has been highlighted [1, 11, 12]. Additionally, the expression of $\alpha 2\beta 4$ nAChR in Paneth cells within mouse intestinal organoids has been documented [46]. Consequently, it is plausible that CLO may exert an influence, either directly or indirectly, on Paneth cells, thereby impacting α -defensin secretion. In this study, we evaluated the effects of CLO on α -defensin secretion in mice to clarify the mechanism underlying NN-induced dysbiosis.

Male C57BL/6N mice (8 weeks old) were purchased from Japan SLC (Hamamatsu, Japan) and reared as described previously [18]. Briefly, the mice were grouped (6 mice per cage) in individually ventilated cages (Sealsafe Plus Mouse; Tecniplast, Buguggiate, Italy) measuring $40.5 \times 20.5 \times 18.5$ cm and were kept under controlled temperature ($23 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$) in a 14:10 hr light/dark cycle at the Kobe University Life-Science Laboratory with *ad libitum* access to a pellet diet (DC-8; Clea Japan, Tokyo, Japan) and filtered water. A 10-day acclimation period was conducted prior to CLO administration. This study was approved by the Institutional Animal Care and Use Committee (Permission #30-01-01) and conducted in accordance with the Kobe University Animal Experiment Regulations.

The mice were divided into three groups ($n=6$ per group): CLO-0, CLO-50 (50 mg/kg/day), and CLO-150 (150 mg/kg/day). CLO was purified according to a previous report [17]. CLO or vehicle (0.5% carboxymethylcellulose, 10 mL/kg) was administered by oral gavage for 28 days at doses of 0, 50, and 150 mg/kg body weight, based on the lowest NOAEL of 47.2 mg/kg body weight obtained in previous male mouse toxicity studies [9, 48]. The oral gavage was performed under isoflurane anesthesia to avoid stress to the mice.

After 28 days of CLO administration, blood samples were collected from the heart while the mice were deeply anesthetized with isoflurane. The mice were then euthanized and various samples were obtained, including cecal contents, blood, and sections from the duodenum, jejunum, and ileum. Cecal contents were stored at -80°C until 16S rRNA analysis and cryptdin-1 (Crp1, a major α -defensin in mice) quantification. The 16S rRNA analysis was performed as previously reported [30]. Briefly, a nucleic acid purification system (Maxwell® RSC; Promega K.K., Tokyo, Japan) and a nucleic acid purification kit (Maxwell® RSC Fecal Microbiome DNA Kit [AS 1700]; Promega K.K.) were used to purify DNA from cecal contents following the manufacturer's instructions. The analysis of the intestinal microbiota by 16S rRNA sequencing of the collected samples was entrusted to Azenta Japan Corp. (Tokyo, Japan), which performed the analysis as previously described [35]. In short, sequencing was performed in a 2×300 paired-end (PE) configuration, and image analysis and base determination were performed by the MiSeq Control Software embedded in the MiSeq instrument. Valid sequences for cluster analysis were obtained from DNA samples, and taxonomic analysis was performed on representative sequences of each cluster. Clusters were formed by 97% sequence identity, and sequences were grouped into operational taxonomic units (OTUs) using the clustering program VSEARCH (1.9.6) [36].

On day 28 of CLO administration, each mouse was transferred to a new cage, and feces were collected from the cage 15 hr later. Feces and a portion of the cecal contents were used for α -defensin measurements. α -Defensin levels in feces and cecal contents were measured as previously reported [8, 31, 43]. Briefly, samples were air-dried and powdered using a bead beater-type homogenizer (Beads Crusher $\mu\text{T-12}$; TAITEC, Saitama, Japan). After mixing with phosphate-buffered saline (PBS) using a vortex mixer for 1 hr and subsequent centrifugation at $20,000 \times g$ for 20 min, extracts from feces and cecal contents were collected. The levels of Crp1 were then measured by sandwich ELISA. The Crp1 antibodies (77-R5 and 77-R20) detected Crp1-3 and 6 of the Crp1 family [8].

Blood was centrifuged ($800 \times g$, 10–15 min, 4°C) to separate the plasma. Plasma serotonin was analyzed by LC-ESI/MS/MS according to a previously reported method [23]. In short, 50 μL of plasma was thoroughly mixed with 20 μL of internal standard mix (1 $\mu\text{g/mL}$ in 70% methanol containing 0.1% formic acid). Then, 50 μL of ice-cold acetonitrile containing 0.05% formic acid was added, and the mixture was centrifuged ($10,000 \times g$, 10 min, 25°C). Subsequently, 10 μL of the supernatant was combined with 10 μL of the 2,4-diphenyl-pyranilium (DPP) mix, and the derivatization reaction was carried out in a thermal cycler (4 hr, 60°C). After completion of the reaction, 80 μL of distilled water was added, and the target substances were measured by LC-ESI/MS/MS. An Agilent 1290 Infinity UHPLC system (Agilent Technologies, Santa Clara, CA, USA) was used for LC, and an Agilent 6495B triple quadrupole mass spectrometer (Agilent Technologies) was used for MS as described elsewhere [15].

The duodenum, jejunum, and ileum were immersion-fixed in 0.1 M PBS containing 4% paraformaldehyde (PFA) at 4°C for 6 hr. Paraffin-embedded blocks were then prepared according to the standard method. Paraffin-embedded blocks were thinned to a 5 μm thickness using a sliding microtome (SM2000R; Leica Microsystems, Wetzlar, Germany), mounted on glass slides, and subjected to immunohistochemical analysis using Crp1 in duodenal, jejunal, and ileal Paneth cells. After deparaffinization, the sections were autoclaved in Histofine Antigen Activation Solution pH 9 (Nichirei Bioscience, Tokyo, Japan) at 105°C for 20 min to activate the antigen. The sections were brought to room temperature, washed with distilled water and 10 mM PBS with 0.05% Tween-20 (T-PBS), and then reacted with Blocking One Histo (Nacalai Tesque, Kyoto, Japan) for 1 hr at room temperature to prevent adhesion of nonspecific proteins. After the reaction, sections were incubated with rat monoclonal anti-Crp1 (77-R63) [32] overnight at 4°C . After washing with T-PBS and distilled water, endogenous peroxidase activity was removed by immersing the sections in 100% methanol and 0.5% hydrogen peroxide for 30 min each. After washing with distilled water and T-PBS, sections were reacted with Horseradish peroxidase labeling Goat anti-Rat IgG (H+L) Secondary Antibody (Thermo Fisher Scientific, Waltham, MA, USA) for 1 hr at room temperature. After the reaction, the sections were washed with T-PBS, and positive reactions were detected by DAB (3,3'-diaminobenzidine-tetrachloride) solution (DAKO EnVision®+ Kit/HRP [DAB]; Dako, Glostrup, Denmark). The contrast staining was performed with Meyer's hematoxylin solution. After dehydration and permeabilization, the sections were coverslipped with Eukitt mounting medium (O. Kindler, Freiburg, Germany) and examined under an optical microscope.

Statistical analyses were performed with BellCurve for Excel (Version 4.04; SSRI, Tokyo, Japan). All data were analyzed by the Kruskal–Wallis test followed by the Steel–Dwass test. The results were considered significant when the P -value was less than 0.05.

CLO administration had no significant effect on the body weight gain of mice from before to after the administration period (Supplementary Fig. 1). After 28 days of CLO administration, a tendency toward soft stool was observed in the CLO-exposed group, although it was not pronounced; this finding was consistent with a previous report [35]. Feces appeared to become looser in a CLO concentration-dependent manner.

Intestinal microbiota analysis showed that the relative abundance of Firmicutes, Bacteroidota, and Verrucomicrobiota among the seven phyla detected in the cecal contents showed differences ($P < 0.1$ or < 0.05) between groups (Supplementary Fig. 2). The relative abundance of Firmicutes tended to be lower in CLO-150 than in CLO-0 ($P = 0.094$) and significantly lower than in CLO-50 ($P < 0.05$) (Fig. 1A). The relative abundance of Bacteroidota was significantly higher in CLO-150 than in CLO-50 ($P < 0.05$) (Fig. 1B). The relative abundance of Verrucomicrobiota tended to be lower in CLO-50 than in CLO-0 ($P = 0.055$) (Fig. 1C). On the other hand, there were significant differences ($P < 0.1$ or < 0.05) in the relative abundance of 14 of the top 30 genera at the genus level within the intestinal microbiota between groups (Supplementary Figs. 3 and 4). The relative abundance of *Lachnoclostridium*, a butyrate-producing bacterium [50], was significantly lower in CLO-50 than in CLO-0 ($P < 0.05$) and tended to be lower in CLO-150 than in CLO-0 ($P = 0.094$) (Fig. 2A). Furthermore, the relative abundance of *[Eubacterium]_xylanophilum_group*, another butyrate-producing bacterium [49], was significantly lower in CLO-50 and CLO-150 than in CLO-0 ($P < 0.05$) (Fig. 2B). Butyrate is a major energy source for colonic epithelial cells [7] and has anti-inflammatory effects via induction of colon regulatory T-cell differentiation [10]. We previously reported that subchronic administration of CLO to rats induced dysbiosis, leading to changes in the relative abundance of short-chain fatty acid-producing bacteria [35]. Furthermore, exposure of maternal mice to CLO resulted in alterations in the relative abundance of such bacteria within the gut microbiota of their offspring [30]. Additionally, combined exposure of mice to CLO and environmental stress altered the relative abundance of short-chain fatty acid-producing bacteria [56]. In the present study, CLO exposure altered the relative abundance of short-chain fatty acid-producing bacteria in the intestinal microbiota of mice, which is consistent with these reports. This study confirms that CLO induces dysbiosis with a decrease in such bacteria and underscores the

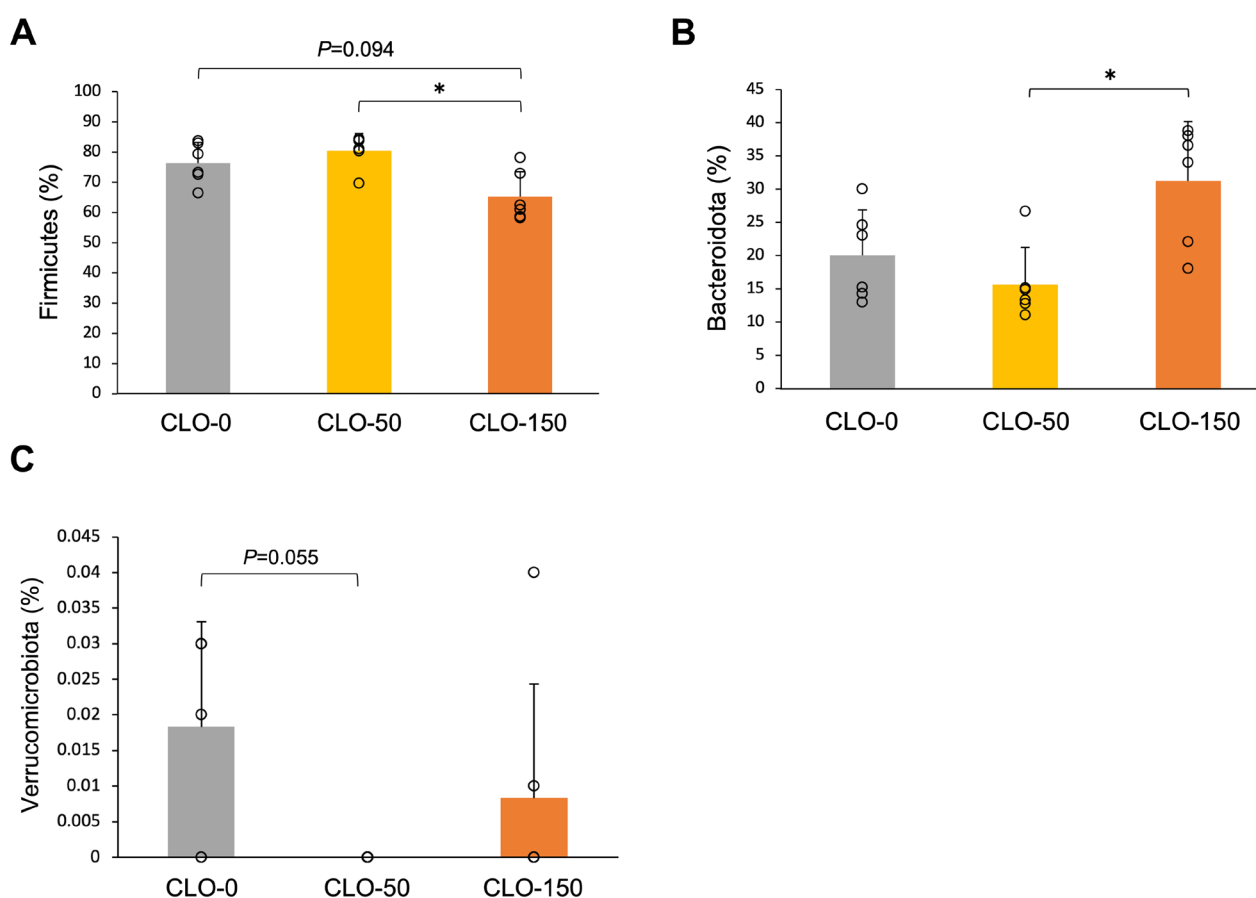


Fig. 1. A. Effect of clothianidin (CLO) exposure on the relative abundance of Firmicutes in the gut microbiota. CLO-150 tended to be lower than CLO-0 ($P = 0.094$) and significantly lower than CLO-50 ($P < 0.05$). B. Effect of CLO exposure on the relative abundance of Bacteroidota in the gut microbiota. CLO-150 was significantly higher than CLO-50 ($P < 0.05$). C. Effect of CLO exposure on the relative abundance of Verrucomicrobiota in the gut microbiota. CLO-50 tended to be lower than CLO-0 ($P = 0.055$). Values are mean + SD ($n = 6$ mice each), and circles show the values for individual mice ($*P < 0.05$).

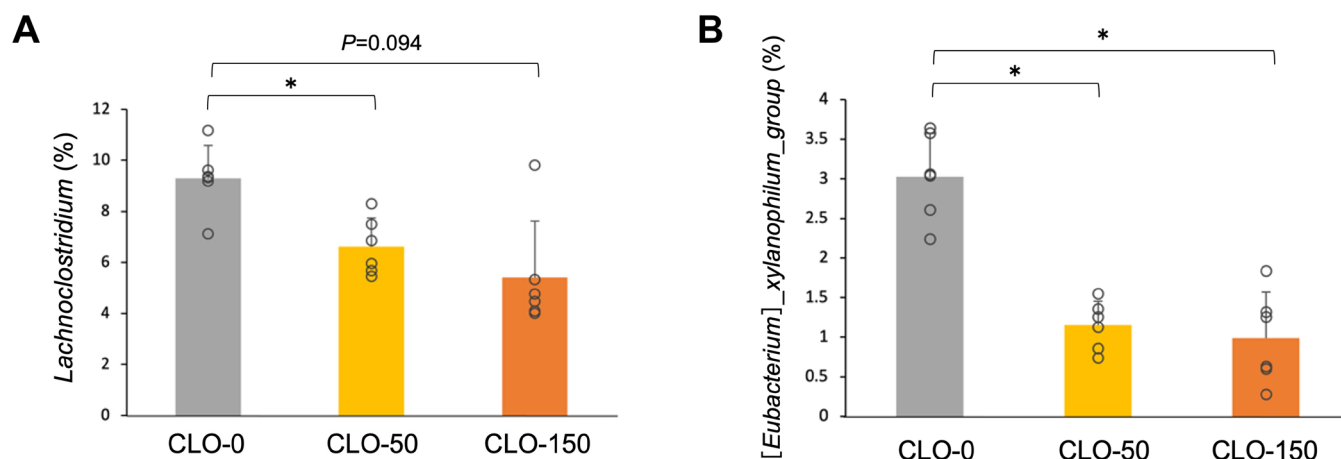


Fig. 2. A. Effect of clothianidin (CLO) exposure on the relative abundance of *Lachnospirillum* (phylum Firmicutes) in the gut microbiota. CLO-50 was significantly lower than CLO-0 ($P < 0.05$). CLO-150 showed a tendency to be lower than CLO-0 ($P = 0.094$). B. Effect of CLO exposure on the relative abundance of *[Eubacterium]_xylanophilum_group* (phylum Firmicutes) in the gut microbiota. CLO-50 and CLO-150 were significantly lower than CLO-0 ($P < 0.05$). Values are mean + SD ($n = 6$ mice each), and circles show the values for individual mice ($*P < 0.05$).

potential of CLO to affect the host through dysbiosis.

Plasma serotonin levels were significantly lower in CLO-150 than in CLO-0 or CLO-50 ($P < 0.05$) (Fig. 3). The gut microbiota is known to influence tryptophan metabolism in the host [34] and plays an important role in increasing blood serotonin levels by promoting serotonin synthesis by colonic enterochromaffin cells in the host [54]. In other words, the present results suggest that CLO-induced dysbiosis disrupts tryptophan metabolism in the intestine. Since tryptophan absorbed in the gut and transferred to the central nervous system (CNS) via the blood–brain barrier has the potential to contribute to serotonin synthesis in the CNS [38], disturbance of tryptophan metabolism in the gut may alter serotonin availability in the CNS. Serotonin is associated with CNS functions such as mood, behavior, sleep, and appetite; depression and anxiety disorders are associated with decreased serotonin availability in the CNS [29]. On the other hand, the relative abundance of Firmicutes in the gut microbiota was lower with CLO exposure (Fig. 1A). A decrease in the Firmicutes/Bacteroidota ratio has been reported in the gut microbiota of rats exposed to mother–infant separation stress and chronic unpredictable mild stress [6], and similar characteristics are observed in the gut microbiota of depressed patients [22]. Subchronic administration of CLO has been reported to increase anxiety-like behavior in mice [17], and CLO exposure during fetal and neonatal periods increases anxiety-like behavior in F1 and F2 generation mice [41]. The relationship between CLO and increased anxiety-like behaviors reported in these studies may be related to a decreased Firmicutes/Bacteroidota ratio in the gut microbiota or to the disturbance of tryptophan metabolism by dysbiosis.

Crp1 levels in feces were significantly lower in CLO-50 and CLO-150 than in CLO-0 ($P < 0.05$) (Fig. 4A). Crp1 levels in cecal contents were significantly lower in CLO-50 than in CLO-0 ($P < 0.05$) (Fig. 4B). It has been shown that decreased α -defensin secretion is associated with dysbiosis. For example, decreased α -defensin secretion induces dysbiosis in depression model mice [43], and alcohol-induced dysbiosis is associated with decreased α -defensin expression [42]. Moreover, α -defensin administration has been shown to ameliorate dysbiosis in graft-versus-host disease model mice [13], while dysbiosis in Crohn's disease model mice has been linked to abnormal α -defensin secretion [40]. By preventing dysbiosis, α -defensins play a crucial role in safeguarding the host against various diseases. In fact, in humans, α -defensins such as HD-5 (Human α -defensin 5) play an important role in maintaining host health. HD-5 protein levels are reduced in the jejunum of obese individuals [19], and the expression of HD-5 and HD-6 (Human α -defensin 6) is decreased in the ileum of Crohn's disease patients [51]. It has been reported that administration of cigarette smoke condensate containing nicotine, an nAChR agonist like CLO, decreased the expression of *Crp1.4* and *RegIIIy* in the ileum and altered the intestinal microbiota [3]. In the present study, CLO exposure may have decreased α -defensin secretion and consequently altered the intestinal microbiota. In conclusion, the decrease in α -defensin secretion was suggested as a possible mechanism of CLO-induced dysbiosis.

On the other hand, Crp1 immunohistochemistry in the small intestine showed Crp1-positive reactions in the cytoplasm of Paneth

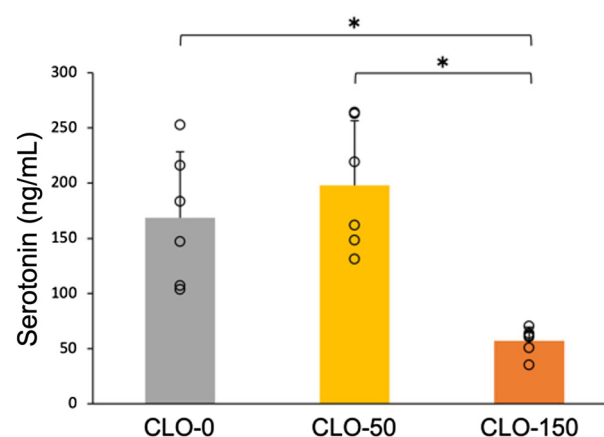


Fig. 3. Effect of clothianidin (CLO) exposure on plasma serotonin levels. CLO-150 was significantly lower than CLO-0 and CLO-50 ($P < 0.05$). Values are mean + SD ($n = 6$ mice each), and circles show the values for individual mice ($*P < 0.05$).

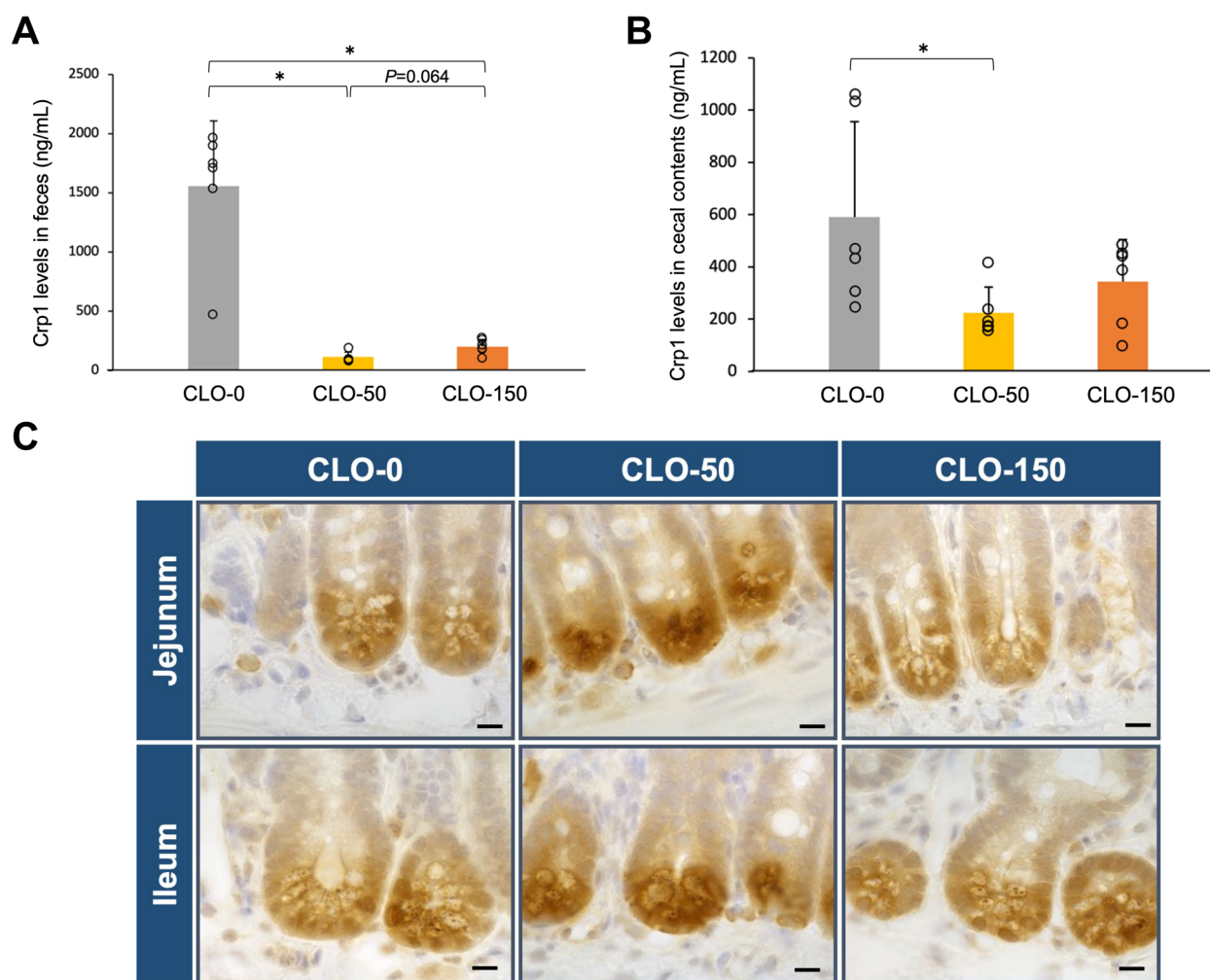


Fig. 4. **A.** Effect of clothianidin (CLO) exposure on Crp1 levels in feces. CLO-50 and CLO-150 were significantly lower than CLO-0 ($P<0.05$), and CLO-150 tended to be higher than CLO-50 ($P=0.064$). **B.** Effect of CLO exposure on Crp1 levels in cecal contents. CLO-50 was significantly lower than CLO-0 ($P<0.05$). Values are mean + SD ($n=6$ mice each), and circles show the values for individual mice ($*P<0.05$). **C.** Representative immunohistochemistry of Crp1 in jejunum and ileum. Compared to CLO-0 and CLO-150, CLO-50 showed a strong positive reaction throughout the cytoplasm. Scale bars=10 μ m.

cells in the duodenum, jejunum, and ileum. Staining was most pronounced in CLO-50, with the strongest intensity observed in the jejunum and ileum (Fig. 4C, Supplementary Fig. 5). The lower levels of Crp1 in feces and cecal contents of CLO-50 compared to CLO-0 (Fig. 4A and 4B) suggest that CLO inhibited the release of α -defensins from Paneth cells, resulting in intracellular storage of α -defensins and a decrease in α -defensin secretion. The mechanism by which CLO inhibits the release of α -defensins from Paneth cells is unknown; further studies are needed. In addition, Crp1 immunohistochemistry of CLO-150 in the jejunum and ileum showed a trend toward fewer positive granules compared to CLO-0, but the findings were not similar to those of CLO-50, and no concentration-dependent changes were observed (Fig. 4C). Because CLO-150 was administered with CLO at a concentration approximately three times higher than that of NOAEL, it is possible that CLO-150 might have induced the reduction in α -defensin secretion through a mechanism distinct from that of CLO at the NOAEL concentration in mice. For example, it is possible that the same trend as CLO-50 was not observed in Crp1 immunohistochemistry of Paneth cells because high concentrations of CLO caused an abnormality in the synthetic pathway of α -defensins, resulting in decreased synthesis and secretion of α -defensins themselves.

The present study is the first to demonstrate that CLO reduces α -defensin secretion from Paneth cells, suggesting that reduced α -defensin secretion is one of the mechanisms underlying CLO-induced dysbiosis (Fig. 5). α -Defensins serve a dual role in maintaining the composition of the intestinal microbiota and safeguarding the host against intestinal pathogens, thereby playing a pivotal role in the host's intestinal innate immunity [5]. In fact, Matrilysin-deficient (MAT^{-/-}) mice lacking mature Crps rapidly developed *Salmonella* infections when exposed to *Salmonella typhimurium*, and the oral 50% lethal dose was one-tenth that of MAT^{+/+} mice [52]. Thus, CLO may make the host more vulnerable to infection by invading microorganisms. Further studies are needed to clarify the exact toxicity of CLO.

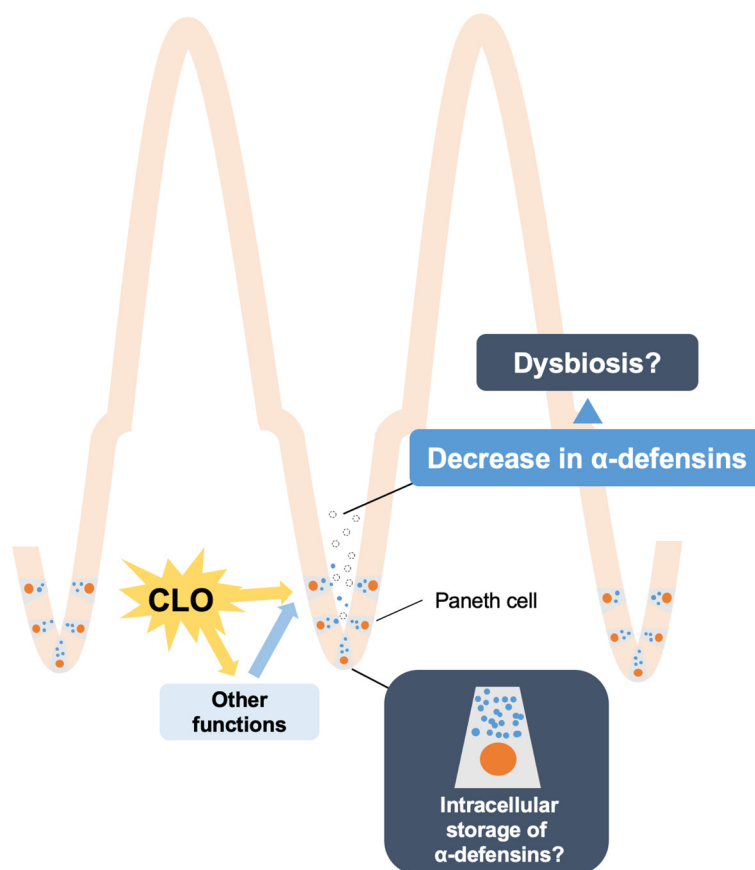


Fig. 5. Hypothesis of the mechanism by which clothianidin (CLO) exposure induces dysbiosis. CLO acts directly or indirectly on Paneth cells to decrease α -defensin secretion, which may be the cause of CLO-induced dysbiosis. Furthermore, CLO may decrease the ability of Paneth cells to release α -defensins, leading to intracellular storage of α -defensins.

CONFLICT OF INTEREST. The authors declare that there are no conflicts of interest.

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