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Effect of 4-thiazolidinone derivative and nimesulide on parietal intestinal microbiota of rats during induced inflammation process *in vivo*

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RT: Methodology; Investigation; Data curation; Formal analysis; Validation; Funding acquisition; Visualization; Writing — original draft.
LH: Visualization; Writing — original draft; Formal analysis.

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None to declare.

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Nonsteroidal anti-inflammatory drugs, which are widely used in the treatment of diseases accompanied by pain and fever, can cause diseases of the gastrointestinal tract and are associated with disturbances of the intestinal microbiota. The search for new compounds that could affect the community of microorganisms, exhibiting antimicrobial and anti-inflammatory effects, is an important task of modern medicine and veterinary medicine. One of the promising molecules that have such effects are 4-thiazolidinone derivatives. The aim of this study was to analyze the effect of the newly synthesized compound *Les6490* and drug nimesulide on the intestinal wall microbiota of rats *in vivo* under the conditions of Freund's adjuvant-induced inflammatory process. The study of the effect of the above-mentioned drugs on the intestinal microbiota *in vivo* was carried out on a biomodel of rats, which were intragastrically administered with the test substances for two weeks. The study material was the parietal mucos of the small intestine, the microbiome of which was studied using 16S rRNA sequencing. Metagenomic analysis made it possible to analyze the types of microorganisms in experimental groups with induced inflammation (groups A and AL) and without it (groups K, L, N). It was established that the composition of the microbiome of the intestinal tract of rats changes under the conditions of induced inflammation and under the action of the compound *Les6490* (groups A and L) in comparison with the control group (group K). The influence of *Les6490* on the intestinal tract microbiome composition in rats is similar to that of nimesulide, but the effect is more pronounced. The compound *Les6490* potentiates the growth of *Helicobacter* and has an effect against *Stenotrophomonas* in the group without induced inflammation (group L), but in the group of inflammation (group AL) no such effect is observed. The compound alone (not in inflammation models) leads to increased species diversity of the rat gut microbiome.

Key words: microbiome, inflammatory process, 4-thiazolidinone derivative, 16S rRNA sequencing, intestinal microbiota, rats

Introduction

The gut microbiota is a collection of microorganisms inhabiting the gastrointestinal tract (GI), which begins to form after birth and is characterized by age and population characteristics. It contains trillions of microorganisms belonging to hundreds of species, exceeding the number of cells in the human body [20]. It includes bacteria, fungi, viruses and other microorganisms. The intestinal microbiota participates in important physiological functions of the

gastrointestinal tract, including motility, biotransformation of nutrients, immunomodulation and development of immunotolerance, synthesis of many biologically active substances, in particular those that are not produced in the body and do not come from the outside. Metabolites produced by the intestinal microbiota, in particular short-chain fatty acids (SCFAs), participate in important biochemical and physiological processes — they provide the energy needs of the intestinal epithelium, regulate smooth muscle motility, influence the level of pituitary hormones, and prevent the malignant transformation of colonocytes [1]. The production of signaling molecules for neural circuits has also been revealed. These compounds play a key role in neurogenesis, mental and cognitive development, emotions and behavior, as well as in the progression of neuropsychiatric diseases [18, 19].

The intestinal microbiota produces some important biologically active activators of the immune system, on which the response to a possible pathogenic threat depends. Therefore, the microbiota of the intestinal canal is evaluated as an additional functionally active human organ [8]. Changes in the species composition and quantitative relationships between the components of the microbiota can act as an etiopathogenetic factor of diseases of the gastrointestinal tract and the other organs and systems, in particular, disrupt the regulatory mechanisms of immune protection [17, 37]. On the other hand, measures and drugs aimed at modulating the intestinal microbiota have a therapeutic effect [37]. Thus, the colonization of *Bacteroides fragilis* is associated with increased activity of regulatory T-cells, which can alleviate the course of autoimmune diseases [26, 31]. Several species of commensal gram-positive bacteria in the colon (the most common of them are *Faecalibacterium prausnitzii* and *Roseburia*) synthesize butyrate (one of the important SCFAs) [16], which intracellular accumulation is one of the protective mechanisms against carcinogenesis [27], or exhibits an anti-inflammatory effect in the mucous membrane [14]. Regulation of intestinal microbiota, in the direction of increasing the number of bacteria of the genera *Akkermansia* and *Bacteroides*, can facilitate the course of colitis [35]. Bacteria of *L. casei* CRL431 species contributed to the normalization of processes related to obesity, which was reflected in the indicators of specific biomarkers [17].

The gut microbiome has been shown to be important for maintaining immune homeostasis, can influence local adaptive immune responses, and modulate systemic inflammation [9]. Many studies point to a microbial imbalance in the gut in autoimmune diseases. For example, studies have shown a decrease in the *Firmicutes*/*Bacteroidetes* ratio in patients with systemic lupus erythematosus (SLE) and type 1 diabetes [15]. The study [11] revealed an increase in the number of *Methanobrevibacter* and *Akkermansia* and a decrease in the number of *Butyricimonas* in patients with multiple sclerosis. The other studies have shown a decrease quantity in *Faecalibacterium* and an increase in *Eggerthella* and *Collinsella* in patients with rheumatoid arthritis (RA) [4]. Also, a number of authors indicate that the modulation of the intestinal microbiota has a positive effect on the course of RA [38, 17].

Nonsteroidal anti-inflammatory drugs (NSAIDs), which are widely used in the treatment of diseases associated with pain and fever, are capable of causing CKD diseases that are associated with disturbances of the intestinal microbiota, which is indicated in a number of scientific works analyzed in the literature review [33].

The search for the new compounds that could affect the microbiological community, exhibit antimicrobial and anti-inflammatory effects, is an important task of modern medicine and veterinary medicine. Derivatives of 4-thiazolidinone are one of the promising molecules that potentially possess the mentioned properties [13].

The purpose of the conducted research was to analyze the impact of 4-thiazolidinone derivative (*Les6490*) and nimesulide (NSAID) on the intestinal wall microbiota of rats *in vivo* during Freund's adjuvant-induced inflammatory process.

Materials and Methods

Chemistry

The synthesis of the investigated compound was conducted at the Department of Pharmaceutical, Organic, and Bioorganic Chemistry, Danylo Halytsky Lviv National Medical University.

The melting points were measured in open capillary tubes on a *BÜCHI B-545* melting point apparatus (*BÜCHI Labortechnik AG*, Flawil, Switzerland) and were uncorrected. The elemental analyses (C, H, N) were performed using the *Perkin-Elmer 2400 CHN* analyzer (*Perkin-Elmer*, Waltham, MA, USA) and were within ±0.4% of the theoretical values. The 500 MHz ^{1}H and 100 MHz ^{13}C NMR spectra were recorded on a *Varian Unity Plus 500* (500 MHz) spectrometer (*Varian Inc.*, Palo Alto, CA, USA). All spectra were recorded at room temperature, except where indicated otherwise, and were referenced internally to solvent reference frequencies. Chemical shifts (δ) are quoted in ppm and coupling constants (J) are reported in Hz. LC-MS spectra were obtained on a *Finnigan MAT INCOS-50* (*Thermo Finnigan LLC*, San Jose, CA, USA). The reaction mixture was monitored by thin layer chromatography (TLC) using commercial glass-backed TLC plates (*Merck Kieselgel 60 F254*, *Merck*, Darmstadt, Germany). Solvents and reagents that are commercially available were used without further purification. The thiazolidine-2,4-dione **(*i*)** and 1,3-diphenyl-1H-pyrazole-4-carbaldehyde **(*ii*)** and were prepared according to the methods described in [10, 37] respectively.

General procedure for the synthesis of 5-(1,3-diphenyl-1H-pyrazol-4-ylmethylene)-thiazolidine-2,4-dione *Les6490*. A mixture of 0.01 mol of thiazolidine-2,4-dione **(*i*),** 0.011 mol of 1,3-diphenyl-1H-pyrazole-4-carbaldehyde **(*ii*)** and 0.015 mol of ammonium acetate in 20 ml of toluene was heated under reflux for 5 h. Yellow crystalline precipitate was filtered off, washed with hexane,

and recrystallized from a mixture of DMF-ethanol (1:2). Yield: 85%, yellow crystal powder, mp 278–280°C (DMF-EtOH (1:2)). LC-MS (ESI+): m/z 348.0 (100.0%, $[M+H]^+$). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 7.40 (t, J = 7.4 Hz, 1H arom.), 7.47–7.59 (m, 6H, arom. + CH=), 7.63 (d, J = 7.3 Hz, 2H arom.), 8.00 (d, J = 8.0 Hz, 2H, arom.), 8.68 (s, 1H, CH, pyrazole), 12.52 (s, 1H, NH, thiazolidinone). ^{13}C NMR (101 MHz, DMSO-d_6): δ (ppm) 115.9, 119.8, 122.5, 123.1, 127.9, 128.4, 129.2, 129.4, 130.1, 131.8, 154.1, 167.5 (C=O), 167.9 (C=O) [37].

Animals

The experimental work was performed on sexually mature non-linear white rats with an initial weight of 220±5.1 g, obtained from the vivarium of the Danylo Halytsky Lviv National Medical University. Before the experiments, the rats were acclimatized for a week. They were allowed *ad libitum* access to water and a standard rodent diet and housed in an air-conditioned experimental animal room (temperature: 22–24°C, humidity 50–65%, and a 12-h light/dark cycle).

Research was conducted in accordance with the provisions of the European Convention on the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes (Strasbourg, 2005), Directive 2010/63/EU Of The European Parliament And Of The Council and Law Of Ukraine No. 3447-IV on the Protection of Animals from Cruelty as amended by 440-IX dated 14.01.2020, according to protocol no. 10 dated 20.12.2021 of the meeting of the Commission on Ethics of Scientific Research, Experimental Developments and Scientific Works of the Danylo Halytskyi LNMU.

Modeling of the inflammatory process

In order to induce the inflammatory process, experimental animals were injected with Freund's adjuvant (AF) in a volume of 0.1 ml, subcutaneously in the plantar part of the hind limb [22, 24].

Study of gut microbiota

The study of the effect of the 4-thiazolidinone derivative and nimesulide on the gut microbiota *in vivo* was carried out on a rat biomodel. The animals of the experimental group (n=30) with normal feeding received nimesulide (NSAID) at a dose of 15.0 mg/kg and a derivative of 4-thiazolidinone — *Les6490* (5-(1,3-diphenyl-1H-pyrazol-4-ylmethylene)-thiazolidine-2,4-dione) at a dose of 10 mg/kg intragastrically once a day during 14 days.

Table. Groups of rats involved in the experiment

Group number	Group name	Group description in the experiment	Investigated by 16sRNA sequencing
1	A	Freund's adjuvant	1
2	AL	Freund's adjuvant + *Les6490*	1
3	K	Control animals	1
4	L	*Les6490*	1
5	N	Nimesulide	1

After that, the animals were removed from the experiment by decapitation against the background of inhalation anesthesia with diethyl ether. *Les6490* and nimesulide were dissolved in *Tween 80* before administration. The studied material was the parietal mucus of the transverse colon (parietal microbiota) collected under aseptic conditions.

Rats were randomly divided into 5 groups of 6 rats per group (table). A total of 30 rats were involved, material samples (one from each group) were sent for 16s RNA sequencing (*Novogene*, Beijing, China).

In order to induce the inflammatory process, animals of the first (A) and second (AL) groups were injected with Freund's adjuvant (AF) in a volume of 0.1 ml subcutaneously in the plantar part of the hind limb. After the appearance of signs of inflammation on the 5th day, the animals of the 2nd group were given the *Les6490* compound. Group 3 (K) was control (intact animals). Group 4, 5 — animals without induced inflammation, which were intragastrically injected with the *Les6490* compound (group L) and nimesulide (group N) [20] for 14 days.

Sequencing of 16S rRNA

DNA extraction, sequencing, and microbiome quantification were performed by *Novogene Bioinformatics Technology Co., Ltd*. To study the composition of the microbial community in each sample, operational taxonomic units (OTUs) were obtained by clustering with 97% identity on the effective tags of all samples and then identified. The amplicon was sequenced on an Illumina paired-end platform to generate 250 bp paired-end reads (Raw PE), then combined and preprocessed to obtain clean tags.

Total genomic DNA from the samples was isolated by the CTAB/SDS method. DNA concentration and purity were monitored on a 1% agarose gel. According to the concentration, DNA was diluted to 1 ng/μL with sterile water. 16S rRNA genes were amplified using specific primers (16S V4: 515F-806R, etc.). All PCR reactions were performed using *Phusion® High-Fidelity PCR Master Mix* (*New England Biolabs*).

Results and Discussion

The 4-thiazolidinone-bearing derivative *Les6490* **(*iii*)** has been synthesized by Knoevenagel condesation of 1,3-diphenyl-1H-pyrazole-4-carbaldehyde **(*i*)** and thiazolidine-2,4-dione **(*ii*)** with satisfactory yield (85%) and purity (fig. 1).

Metagenomic analysis by 16S rRNA gene sequencing made it possible to combine the obtained separate FASTQ files, and the filtered sequences provided readings of at least 400 nucleotide sequences. This made it possible to cluster similar variants and to single out separate taxonomic units of the species and genus of bacteria.

The fig. 2 shows the alpha diversity of the intestinal microbiome by the number of detected species based on the Abundance-based coverage estimators index (ACE). The results show that with induced inflammation (group A)

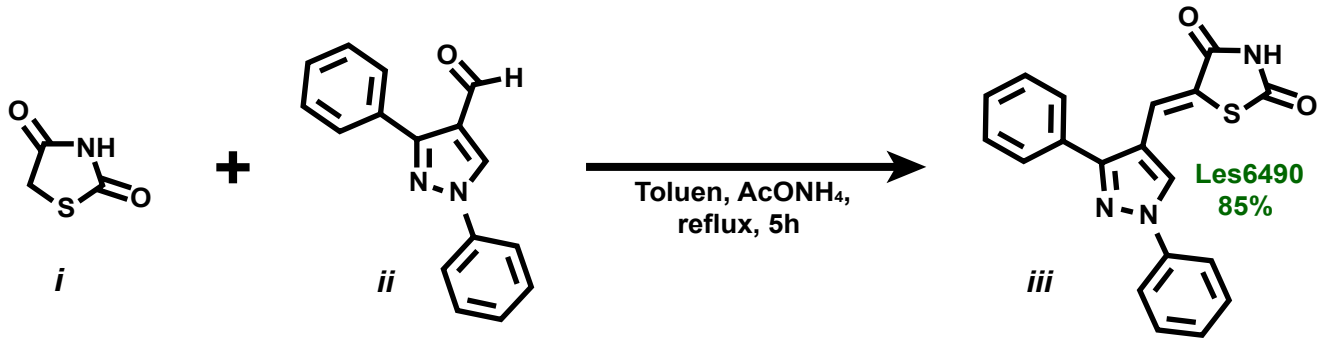

Fig. 1. Scheme of 5-(1,3-diphenyl-1H-pyrazol-4-ylmethylene)-thiazolidine-2,4-dione, *Les6490* synthesis

there is a decrease in the alpha diversity of the intestinal microbiota compared to the control, probably due to the action of pro-inflammatory cytokines [3]. In the AL group under the influence of *Les6490*, this effect is removed — alpha diversity in this group is higher than in the control, while in groups N (nimesulide) and L (*Les6490*) there is a slight increase in the index of alpha diversity.

The dendrogram of microbiota diversity at the level of types and classes (fig. 3) visualizes features close to those marked in fig. 2. In group A, *Proteobacteria* predominate with a significant decrease in the *Firmicutes* class compared to the control. At the same time, the indicators of groups A and AL are close to each other, but different from the control, which indicates the influence of the inflammatory process on the intestinal microbiome. The greatest alpha diversity was found in group L under the influence of *Les6490*, while nimesulide showed a much smaller effect (group N).

The results of the study of dominant taxa at the level of individual genera in the microbiomes of the intestinal canal of rats under experimental conditions are visualized in fig. 4. It is important that when using the indicated primers, about half of the genomes were identified.

Genomes of four genera of bacteria were identified in the control group. Representatives of the intestinal microbiota of the main genus, *Lactobacillus*, were found in the largest number. The genus *Stentotrophomonas* are weakly fermenting gram-negative bacteria that are found in water, soil, and plants, and can also cause opportunistic nosocomial infections [27]. The genomes of *Nitronomonas*, aerobic bacteria capable of oxidizing ammonia, and *Delftia* bacteria of the *Burkholderiacaeae* family found in water, soil and the intestinal tract of animals and humans, were also discovered. Since they are able to metabolize various substances — xenobiotics, so they are recommended for use in bioremediation systems. However, some variants have virulence factors and are isolated in opportunistic diseases in humans and animals [36].

In animals with induced inflammation (groups A and AL), bacteria of the genus *Lactobacillus* were not detected or were detected in minimal quantities (group AL in which animals received *Les6490* compound against the background of an inflammatory process).

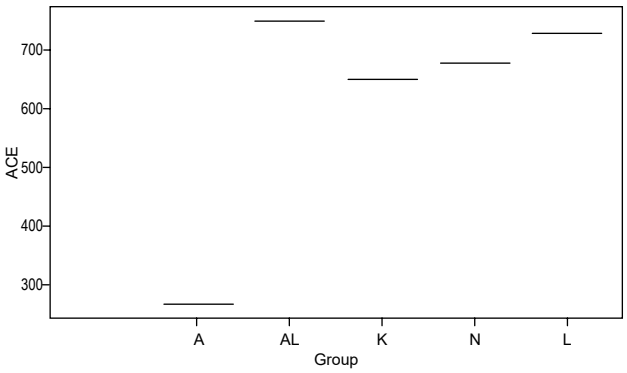

Fig. 2. Results of the ACE alpha-diversity index

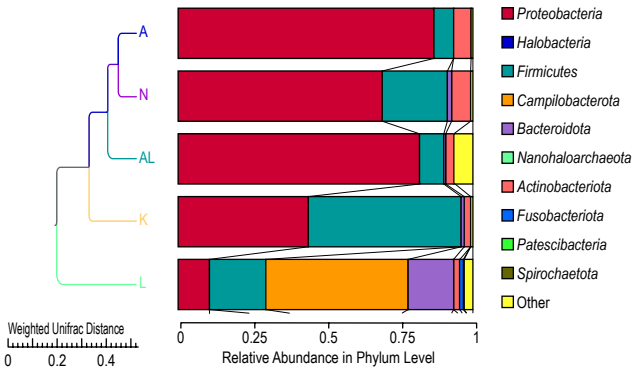

Fig. 3. Species diversity results at the phylum and class levels

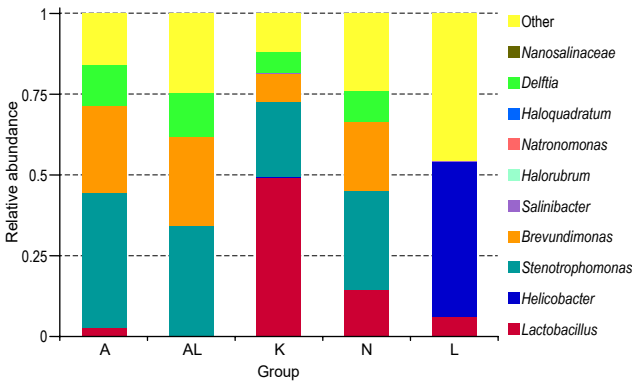

Fig. 4. Relative abundance of dominant tax

The most pronounced changes were found in animals of group L that received *Les6490*. Compared to the control, the relative number of bacteria of the genera *Lactobacillus* and *Stenotrophomonas* decreased. However, in this group an increase in the number of *Helicobacter* was noted, which is not observed in the other studied groups. *Helicobacter* species easily colonize the surface of the gastrointestinal tract due to microaerophilic metabolism, spiral shape and special motility [29]. Depending on their location in the gastrointestinal system, they are divided into gastric *Helicobacteria* such as *Helicobacter pylori*, and entero-hepatic *Helicobacteria*, which mainly colonize the intestine and the hepatobiliary system and play a protective role in the development of certain autoimmune processes [25]. In addition, *Helicobacter* can induce the production of antibacterial peptides that counteract potentially harmful bacteria [34], or compete with bacteria for the same ecological niche. Bacteria of the *Delftia* genus were not detected in the animals of this group. In animals of groups A, AL and N, the number of these bacteria was approximately the same, although slightly higher than in the control. Therefore, the fact of the absence of genetic sequences in bacteria of the *Delftia* genus isolated from animals that received the drug *Les6490* (group L) requires the additional study. The obtained results indicate an alteration of the composition of the intestinal microbiome in the animals of this group.

The composition of the microbiota of animals from group N that received nimesulide was also characterized by certain features. A relatively smaller number of lactobacilli was noted in the intestinal microbiome of these animals compared to the control and group L. In contrast to the L group, the composition of the N group was dominated by bacteria of the genera *Stenotrophomonas* and *Brevundimonas* characterized by the synthesis of carotenoids, which can act as antioxidants, but it is very difficult to synthesize them chemically [21]. However, their number did not differ significantly from groups A and AL. Therefore, the studied compound *Les6490* and nimesulide differed in their effect on the composition of the microbiota in rats under the experiment conditions.

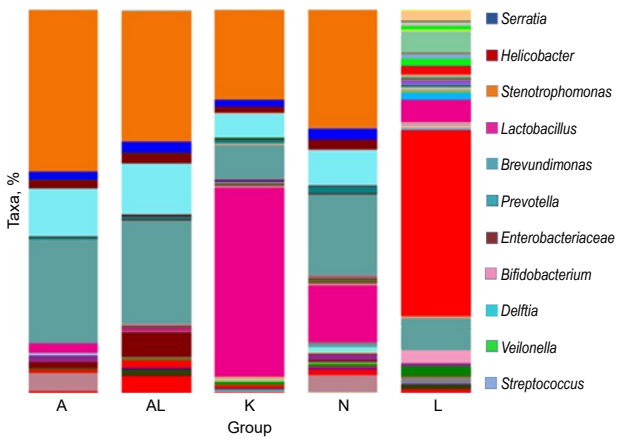

Fig. 5. Taxonomic composition of the intestinal microbiome in rats

The results of the study of the relative amounts of bacterial taxa at the species level are shown in fig. 5. When comparing these results with the previous ones, it should be taken into account that the composition of higher-order taxa may include a different number of lower taxa. However, such properties of microbiota as competitive ability, production of signaling molecules and pathogenicity factors, etc., are manifested precisely at the level of individual species or variants of one species of microorganisms. The results of visualization of the relationships between species and genera in the intestinal microbiome of experimental animals showed that the greatest diversity was found in group L rats, that received the compound *Les6490*. Bacteria of the genus *Streptococcus*, *Prevotella*, *Veillonella*, *Helicobacter* were found in this group, which are absent or found in slightly smaller quantities in the other groups of animals. On the other hand, in this group, the number of bifidobacteria is reduced and bacteria of the *Serratia* genus are absent. In group N, compared to group L, the number of bifidobacteria is higher, but lower than in the control group. In the groups of animals with induced inflammation, the number of bifidobacteria is also significantly lower than in the control and in group N. It should be taken into account that bifidobacteria are considered and used as probiotics, which have the ability to suppress the growth of pathogenic bacteria and regulate the immune response. Thus, the present studies show the development of changes in the components of the microbiome of the intestinal canal both during the induced inflammatory process and during inflammation against the background of the introduction of *Les6490*. But the most expressive changes were found in animals under the influence of the newly synthesized substance *Les6490* and, partially, nimesulide.

The composition of the microbiomes of the intestinal tract changes under the conditions of induced inflammation and under the action of the newly synthesized compound *Les6490* in comparison to the control group. The compound *Les6490* affects the microbiome composition of the intestinal tract of rats in a similar way to the NSAID nimesulide, but this effect is more pronounced. The compound *Les6490* potentiates the growth of *Helicobacter*, inhibits *Stenotrophomonas* and generally promotes the development of greater species diversity of the gut microbiome.

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Вплив похідної 4-тіазолідинону та німесуліду на парієтальну кишкову мікробіоту щурів за індукованого запального процесу *in vivo*

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Нестероїдні протизапальні препарати, які широко застосовуються у лікуванні хвороб, що супроводжуються болем та гарячкою, здатні спричиняти захворювання шлунково-кишкового каналу і асоціюються з порушеннями кишкової мікробіоти. Пошук нових сполук, які б могли впливати на спільноту мікроорганізмів, проявляти протимікробну та протизапальну дію, є важливим завданням сучасної медицини та ветеринарії. Одними із перспективних молекул, які проявляють такі ефекти, є похідні 4-тіазолідинону. Метою дослідження було проаналізувати вплив новосинтезованої сполуки *Les6490* та німесуліду на пристінкову мікробіоту кишки щурів *in vivo* за умов індукованого запального процесу ад'ювантом Фрейнда. Експериментальне дослідження проводили на щурах, яким інтрагастрально впродовж двох тижнів вводили досліджувані речовини. Матеріалом для дослідження слугував пристінковий слиз тонкої кишки, мікробіом якого вивчали за допомогою секвенування 16S рРНК. Метагеномний аналіз дав можливість проаналізувати види мікроорганізмів у дослідних групах з індукованим запаленням (групи A та AL) та без запалення (групи K, L, N). Встановлено, що склад мікробіому кишкового тракту щурів змінюється в умовах індукованого запалення та за дії сполуки *Les6490* (групи A та L) якщо порівнювати з контрольною групою (група K). Вплив сполуки *Les6490* на склад мікробіому кишкового каналу щурів подібний до німесуліду, але її дія є вираженішою. Сполука *Les6490* сприяє збільшенню кількості бактерій роду *Helicobacter* та пригнічує ріст *Stenotrophomonas* у групі без індукованого запалення (група L), натомість у групі із запаленням (група AL) такого ефекту не спостерігали. Сама по собі сполука (не в моделях із запаленням) призводить до збільшення видової різноманітності мікробіому кишки щурів.

Ключові слова: мікробіом, запальний процес, похідна 4-тіазолідинону, секвенування 16S рРНК, мікробіота кишківника, щури

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