ANNALS OF ANIMAL SCIENCE
ISSN: 2300-8733, https://sciendo.com/journal/AOAS

ACCEPTED AUTHOR VERSION OF THE MANUSCRIPT:

Gene expression level and immunohistochemical localization of cannabinoid and cannabinoid-related receptors in the small intestine of Holstein bulls (*Bos taurus taurus*)

DOI: 10.2478/aoas-2024-0016

Cezary Osiak-Wicha[1], Siemowit Muszyński[2], Ewa Tomaszewska[3], Katarzyna Kras[1], Katarzyna Ropka-Molik[4], Mykola Zhyla[5], Marcin Bartłomiej Arciszewski[1♦]

[1]Department of Animal Anatomy and Histology, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20-950 Lublin, Poland
[2]Department of Biophysics, Faculty of Environmental Biology, University of Life Sciences, Akademicka 13, 20-950 Lublin, Poland
[3]Department of Animal Physiology, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20-950 Lublin, Poland
[4]Department of Animal Molecular Biology, National Research Institute of Animal Production, 32-083 Balice n. Kraków, Poland
[5]Department of Normal and Pathological Morphology and Forensic Veterinary Medicine, Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies, Pekarska 50, 79010 Lviv, Ukraine

♦Corresponding author: mb.arciszewski@wp.pl

Received date: 16 August 2023
Accepted date: 15 December 2023

To cite this article: (2024). Osiak-Wicha C., Muszyński S., Tomaszewska E., Kras K., Ropka-Molik K., Zhyla M., Arciszewski M.B. (2024). Gene expression level and immunohistochemical localization of cannabinoid and cannabinoid-related receptors in the small intestine of Holstein bulls (*Bos taurus taurus*), Annals of Animal Science, DOI: 10.2478/aoas-2024-0016

Gene expression level and immunohistochemical localization of cannabinoid and cannabinoid-related receptors in the small intestine of Holstein bulls (*Bos taurus taurus*)

Cezary Osiak-Wicha[1], Siemowit Muszyński[2], Ewa Tomaszewska[3], Katarzyna Kras[1], Katarzyna Ropka-Molik[4], Mykola Zhyla[5], Marcin Bartłomiej Arciszewski[1♦]

[1]Department of Animal Anatomy and Histology, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20-950 Lublin, Poland

[2]Department of Biophysics, Faculty of Environmental Biology, University of Life Sciences, Akademicka 13, 20-950 Lublin, Poland

[3]Department of Animal Physiology, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20-950 Lublin, Poland

[4]Department of Animal Molecular Biology, National Research Institute of Animal Production, 32-083 Balice n. Kraków, Poland

[5]Department of Normal and Pathological Morphology and Forensic Veterinary Medicine, Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies, Pekarska 50, 79010 Lviv, Ukraine

♦Corresponding author: mb.arciszewski@wp.pl

DOI: 10.2478/aoas-2024-0016

Abstract

The gastrointestinal tract plays a crucial role in nutrient absorption, secretion, and motility, ensuring proper digestion and overall homeostasis. Regulation of this complex system involves the coordination of various communication pathways, including neural and humoral mechanisms. One such mechanism is the endocannabinoid system (ECS), a signalling network comprising endogenous cannabinoids, receptors, and enzymes involved in the regulation of physiological processes in mammals and non-mammalian species. While extensive research has been conducted on the ECS in monogastric animals, limited information is available on its presence and distribution in cattle. This study aimed to investigate the distribution and localization patterns of cannabinoid receptors type 1 (CB1R) and type 2 (CB2R) and transient receptor potential vanilloid type 1 (TRPV1) in the bovine small intestine. The study included immunohistochemical analysis of intestinal tissue samples from Polish Holstein-Friesian breed bulls. Gene expression levels of *CNR1*, *CNR2*, and *TRPV1* genes, encoding CB1R, CB2R, and TRPV1, respectively, were quantified using qPCR analysis. The results showed that all three

receptors were expressed in the bovine small intestine, with *TRPV1* exhibiting a significant upregulation in the jejunum compared to the duodenum and ileum. Immunoreactivity for CB1R and CB2R was predominantly observed in neurons of the enteric plexuses, while TRPV1 immunolabeling was detected in both enteric neurons and duodenal Brunner's glands. These findings may establish an anatomical foundation for further investigations, lending support to the potential therapeutic efficacy of cannabinoid receptor agonists in alleviating gastrointestinal motility disorders associated with bovine enteropathies and optimizing milk production in dairy cattle.

Key words: endocannabinoid system, cattle, CB1R, CB2R, TRPV1

The gastrointestinal tract (GIT) plays a vital role in nutrient absorption, secretion, and motility, ensuring proper digestion and overall homeostasis. The regulation of this complex system is achieved through the coordination of various communication pathways, including neural and humoral mechanisms (Costa et al., 2000). One such mechanism is the endocannabinoid system (ECS), which is a signaling network comprised of endogenous cannabinoids, receptors, and enzymes involved in the regulation of various physiological processes in both mammals and non-mammalian species (De Petrocellis et al., 1999; Toschi et al., 2021). This intricate system has gathered significant attention due to its crucial role in not only regulating the GIT, but also maintaining homeostasis in numerous other systems, like immune (Acharya et al., 2017), endocrine (Hillard, 2015), and cardiovascular (O'Sullivan, 2015). In addition to its prominent role in modulating various physiological processes, the ECS exerts direct regulatory influence over a wide array of vital functions at the central level encompassing anxiety, feeding behavior and appetite regulation, emotional behavior, depression, cognition, memory formation and pain perception (Kunos et al., 2008; Skaper and Di Marzo, 2012).

Within the GIT, the ECS plays a pivotal role in modulating gastric acid secretion, gut motility, nutrient absorption, and the regulation of inflammatory responses (Storr et al., 2009; Izzo et al., 2012). As mentioned above, cannabinoid receptors, including CB1R, CB2R, and TRPV1, are key components of the ECS that mediate its physiological effects. Studies in various mammalian species (Silver, 2019), including humans (Wright et al., 2005), rodents (Izzo et al., 1999) and pigs (Toschi et al., 2021) have established the presence and functional significance of CB1R, CB2R, and TRPV1 in the GIT, however, they also showed that this presence and location of ECS receptors varies across species. In wider perspective, the discovery of the ECS has opened up new avenues for research and potential therapeutic applications. For example, the interaction between cannabinoids and the ECS has led to the development of cannabis-based medicines for conditions related to pain (Pertwee, 2000; Mackie, 2006; Fernández-Carvajal et al., 2022). Despite the growing knowledge in this field, limited research has been conducted on the location and distribution of these receptors in the

GIT of livestock animals, such as cattle. Because of their characteristics as ruminants, cattle have a unique structure of their GIT, even among other herbivores, which results in different physiology when compared to monogastric animals. Despite differences in the GIT structure between ruminants and other mammals, the small intestine, a section that remains largely similar across species, is of particular interest in cattle for medical reasons, because it is widely recognized that diarrheal diseases represent a significant burden among gastrointestinal disorders affecting cattle, with the small intestine serving as a prominent site for infection and inflammation (Cho and Yoon, 2014). In addition to their medical significance, recent years have witnessed a growing body of evidence highlighting the potential economic importance of the ECS receptors in dairy cattle (and therefore, in dairy industry). Very recent research (Myers et al., 2021; Myers et al., 2023) suggest that the activation of CB1R in adipose tissue diminishes lipolysis while concurrently promoting lipogenesis in non-lactating cows, thereby may exerting a beneficial effect on augmenting lactation in dairy cows. These findings underscore the potential for leveraging CB1R stimulation as a means to enhance milk production and ultimately contribute to improved economic outcomes in the dairy industry.

The objective of this study was to examine the mRNA gene expression levels of *CNR1*, *CNR2*, and *TRPV1* genes, which encode CB1R, CB2R, and TRPV1, respectively, and to investigate the immunohistochemical (IHC) distribution and localization patterns of these ECS receptors in the bovine small intestine, especially within the enteric nervous system (ENS). Based on existing literature, we hypothesized that these receptors would exhibit immunoexpression within the neuronal populations of the myenteric plexus (MP), submucosal plexus (SP), or both, which are integral components of the ENS. By exploring the spatial distribution of these receptors within the ENS, our study intended to contribute to a deeper understanding of their potential functional roles in gastrointestinal regulation and their implications for bovine health. Furthermore, it may pave the way for future investigations exploring the potential therapeutic applications of cannabinoid ligands and their interactions with other signaling pathways in promoting gut health and optimizing livestock production and optimizing milk production (milk yield) in dairy cattle.

Material and methods

Animals

The biological material used in this study was obtained from the animals following their slaughter at a local slaughterhouse. A total of n = 5 bovine males from Polish Holstein-Friesian breed, aged between 20 and 24 months and weighting 768 ± 46 kg (mean ± SEM), were selected for tissue collection at the slaughterhouse. Cattle was provided with a diet adhering to the specifications outlined in the Polish Feeding Standard, formulated to fulfill the nutritional needs for total digestible nutrients and crude protein (Włodarczyk and Budvytis, 2011). Bulls were fasted for a period of 12 hours before slaughter, and according to available knowledge, short term fasting should not affect the tested receptors (Lee et al., 2020). The sample size was determined using general guidelines for morphological sciences (Henry et al., 2016). The selected animals had no known history of GIT disorders, and post-mortem examination showed

no macroscopic abnormalities in the structure and wall of the GIT organs. These stringent inclusion criteria ensured that the collected intestinal tissues samples were representative of healthy specimens, free from pre-existing gastrointestinal conditions or evident structural alterations. The carcasses, after dissection, were processed for commercial purposes and consumption. As all tissue collection procedures were performed postmortem, ethical review and approval from the Ethics Committee, according to the Polish law, were not required for this study.

Sample collection

Research material was obtained from animals immediately after culling. The small intestine sections were identified and marked for subsequent dissection. In an effort to ensure consistency in tissue sampling protocols, the duodenum was selected for sampling posterior to the bile ducts, the target point for jejunum was determined by measuring 2 meters posterior from the duodenal insertion point of duodenocolic fold, and the section anterior to the ileo-cecal junction of the ileum was chosen for ileum sampling. Aseptic techniques were employed during the collection of tissue samples to minimize contamination. The marked regions of the small intestine were excised using sterile instruments, and care was taken to avoid damage to the tissue. Small intestine segments measuring 2.5 cm in length were collected from the duodenum, jejunum, and ileum of each subject. The collected tissue segments were then placed in sterile containers and fixed with a 4% buffered formaldehyde solution with picric acid at pH = 7.0 (for 24 hours). For gene expression analyses, adjacent sections of small intestine segments were opened at the mesentery, rinsed with saline solution, and immediately snap-frozen in liquid nitrogen. All tissue samples were collected within 15 min following the death of the animal.

Tissue processing

The tissue samples used for IHC staining underwent a thorough and standardized preparation process. After two washes in 0.01 M phosphate-buffered saline (PBS) for 10 minutes each, the samples were transferred to a transparent container containing a 16% sucrose and 0.01% bacteriostatic sodium azide (Avantor Performance Materials Poland S.A., Gliwice, Poland) solution at a temperature of 4°C. The sucrose solution was regularly replaced with fresh solution on a daily basis until the tissue samples settled at the bottom of the container. Subsequently, following the final wash in PBS, the tissue samples were embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen using dry ice. Using a cryostat (HM 525 NX, Thermo Scientific, Waltham, MA, USA), the frozen sections were cut to a thickness of 10 μm. Every fourth section was then carefully placed onto adhesive glass slides (Superfrost Plus, Thermo Scientific, Waltham, MA, USA) and stored at -20°C until further IHC stainings.

Gene expression quantification

Intestinal samples were subjected to RNA extraction using the PureLink RNA Mini Kit (Invitrogen, Waltham, MA, USA) following the manufacturer's instructions. The isolated RNA

was treated with DNase I (PureLink DNase Set; Invitrogen) to eliminate genomic DNA contamination. Next, the total RNA concentration and potential protein/chemical contamination were measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), while the integrity of the RNA was assessed through 2% agarose gel electrophoresis. For cDNA synthesis, 250 ng of the total RNA was transcribed using TranScriba Kit (A&A Biotechnology, Gdańsk, Poland). The resulting cDNA was subjected to qPCR to examine the expression of levels of *CNR1*, *CNR2*, and *TRPV1* genes. To prevent DNA amplification, the primers were designed to target different exons (Table 1). qPCR analysis was carried out using the RT-PCR Mix SYBR Green (A&A Biotechnology, Gdańsk, Poland) on the QuantStudio 7 Flex system (Applied Biosystems, Waltham, MA, USA). Two reference genes, *RPS9* and *ACTB*, were used as endogenous controls due to their stable expression across all tested bovine tissue types and experimental conditions (Janovick-Guretzky et al., 2007). All primer designs – for tested genes and endogenous controls were performed using Primer3web (https://primer3.ut.ee), and the synthesized primers were obtained from Genomed (Genomed, Warszawa, Poland). Each sample was analyzed in triplicate technical replicates. The relative expression of *CNR1*, *CNR2*, and *TRPV1* genes in the examined small intestine segments was calculated using the -ΔΔCT cycle threshold method (Livak and Schmittgen, 2001), and treatment means are expressed relative to the geometric mean of the specific gene mRNA expression level in the duodenum.

Immunohistochemistry

IHC analysis was conducted to examine the immunolocalization of CB1R, CB2R, and TRPV1 in the small intestine. The whole process was divided into two days. On the first day, in order to retrieve the antigens for enhanced detection, the sections were thoroughly rinsed with 0.1 M PBS (pH = 7.3) and subsequently treated with a solution containing sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH = 6.0) for 8 minutes at 80°C (multicooker RMC-PM381-E Redmond, China). Following the heat-induced antigen retrieval, the sections were washed with 0.1 M PBS (pH = 7.3) to remove any residual sodium citrate buffer. To inactivate endogenous peroxidase activity, the sections were exposed to a 3% H_2O_2 for 10 minutes at room temperature (RT). Subsequently, the sections were washed with PBS to remove the H_2O_2 solution. To reduce nonspecific background staining, sections were incubated for 5 minutes in UltraVision Protein Block (Thermo Scientific, Waltham, MA, USA). The sections were then subjected to overnight incubation at 4^{o}C in humid chambers with primary antibodies specific to CB1R, CB2R, and TRPV1, diluted in antibody diluent (Emerald, Cell Marque Corp., Rocklin, CA, USA). Each section was incubated with only one antibody. All antibodies and their dilutions used in this study are listed in Table 2. After overnight incubation, following another washing in PBS to remove unbound primary antibodies, to detect the bound antibodies the sections were incubated with BrightVision two-step detection system of poly-HRP-anti Ms/Rb IgG (ImmunoLogic WellMed B.V., Duiven, Netherlands) for 1 hour at RT. Subsequent washes with PBS were performed. Immunolabeling was visualized by exposing the sections to 0.05% diaminobenzidine (DAB, #D5905, Sigma-Aldrich, St. Louis, MO, USA). Following the DAB exposure, the sections were washed in running water for 10 minutes then distilled water, counterstained with Mayer's hematoxylin (Patho, Mar-Four, Konstantynów Łódzki, Poland)

and washed again in running water and distilled water. The mounted sections were air-dried, dehydrated in ethanol, cleared with xylene, and cover slipped using Shandon Consul-Mount (Thermo Scientific, Waltham, MA, US). Digital high-resolution photomicrographs (1920x1200) of the bovine's small intestine were captured using an Olympus BX61 microscope (Olympus, Nagano, Japan) equipped with a digital camera (LC35, Olympus, Nagano, Japan). All the photomicrographs were captured using Cell^M 2.3 software (Olympus cellSens Standard) under consistent lighting conditions and settings for brightness and contrast by one person. The assessment of the specific binding of the antibodies used was performed using a control procedure. Sections that were not exposed to primary antibodies were stained normally as part of the first phase, acting as a negative control (diluent without the antibody was applied instead of primary antibodies). Notably, no affirmative immunoreaction was observed in any of the control sections.

Immunofluorescence

Immunofluorescence (IF) detection was carried out in two days, similar to previously described technique. Samples were prepared on the first day in similar way to the section described above, with the difference that incubation in H_2O_2 was not performed. Double staining was carried out using one of the primary antibodies against a specific receptor (CB1R, CB2R, TRPV1), combined with an antibody against HuC/HuD as a neuronal marker to detect the enteric neurons. After an overnight incubation at RT, sections were washed in PBS to remove unbound primary antibodies. To detect the bound antibodies the sections were incubated with a mixture of secondary antibodies for one hour in RT. Two secondary antibodies, Alexa Anti-Rabbit and Alexa Anti-Mouse, were used because of two different hosts of primary antibodies (all antibodies and dilutions are presented in Table 2). The slides were then washed in PBS three times 10 minutes each, and mounted using phosphate-buffered glycerol (pH = 8.2). The cryosections underwent meticulous examination by one observer, using the Olympus BX61 microscope (Olympus, Nagano, Japan). The microscope was equipped with precisely selected filter cubes, facilitating the distinction of the unique fluorochromes employed (Alexa Fluor 595, MWIY2, excitation/emission wavelength 545–580 nm; Alexa Fluor 488, MNIBA2, excitation/emission wavelength 470–490 nm). Images were then captured using the digital camera C11440-36U (Hamamatsu Photonics, Shizuoka, Japan).

To assess the co-localization of neuronal markers with CB1R, CB2R, and TRPV1 receptors, double-stained preparations were examined using fluorescence labeling techniques. Neurons were initially identified based on the presence of a specific fluorophore labeling the HuC/HuD antigen. Subsequently, the microscope filter was switched to detect the expression of a second antigen (CB1R, CB2R, or TRPV1) using a distinct fluorophore. This approach enabled the determination of the proportion of neurons exhibiting co-expression of specific antigen pairs. A minimum total of one hundred HuC/HuD immunoreactive (IR) neurons were quantified for each receptor marker in both the MP and SP. Data were collected from sections of all animals to ensure adequate representation. The percentages of HuC/HuD-IR neurons in examined intestinal sections were calculated.

Evaluation of the IHC reaction intensity

The evaluation of the IHC reaction intensity was performed based on the methods described previously (Cizkova et al., 2021). ImageJ software ver. 1.54f (https://imagej.nih.gov/ij/) was utilized for this purpose. The initial stage of the analysis involved color deconvolution using the IHC profiler plugin (Varghese et al., 2014). Subsequently, the immunostaining intensity of intestinal cells was quantified in the deconvoluted DAB images converted into a greyscale (8-bit). Manual selection of the regions of IHC reactions was conducted using the free-hand selection tool, and the staining intensity was assessed based on the "mean gray value" parameter. Ten measurements were taken from each section for each segment of the small intestine (duodenum, jejunum, ileum) for each cellular structure, where the reaction occurred (clearly visible staining). Mean values from each section were then calculated for a total of 30 mean value measurements per cellular structure (with the exception of Brunner's glands, which are found only in the duodenum). Pixel intensity values ranged from 0 to 255, with 0 representing the deepest shade (black) and 255 representing the lightest shade (white) of the color. Measurements were then converted into Optical Density (OD) using formula OD=-log(x/255) where x was measured mean grey value (Varghese et al., 2014). In order to classify the intensity levels, we employed automated thresholds in this investigation. Samples with OD values below 0.2 were categorized as negative, those falling within the range of 0.2 to 0.4 were deemed weak, samples with OD values ranging from 0.4 to 0.6 were considered moderate, and those surpassing 0.6 were classified as strong (Cizkova et al., 2021).

Statistical analysis

Results were represented by mean ± SEM. Multiple comparisons among segments were made using one-way ANOVA test followed by Bonferroni adjustment (for data showing equal SDs, as verified by Levene's test) or Welch ANOVA tests followed by Dunnett T3 adjustment (for data showing unequal SDs). The percentages of IR neurons and gene relative expression levels were compared between segments of the small intestine (duodenum, jejunum and ileum) for a given receptor (CB1R, CB2R, TRPV1) or mRNA gene expression (*CNR1*, *CNR2*, *TRPV1*). The significance of the overall differences was determined at a threshold of $P < 0.05$. All statistical analyses and graphs were performed using GraphPad Prism ver. 9.5.1 for Windows (GraphPad Software, San Diego, CA, USA, https://www.graphpad.com).

Results

Relative genes expression levels

To facilitate comparisons among the segments, all results were normalized to the expression level of a particular gene in the duodenum as the first segment of the small intestine. For *CNR1* and *CNR2* we observed no significant differences between all three segments of the small intestine. The analysis of *TRPV1* expression however yielded particularly intriguing results. Notably, *TRPV1* exhibited a significant upregulation in the jejunum when compared to the duodenum (Figure 1; $P = 0.003$), but not jejunum, also no differences in ileum in relation to other segments were observed.

Distribution of CB1R immunoreactivity

We observed mostly weak with some instances of moderate and strong CB1R-IR neurons in approximately half of neurons in the small intestine (on average 54.19 ± 5.67%) The majority of SP neurons were classified as weak CB1R-IR neurons, with a marginal presence of moderate to strong CB1R-IR neurons in the duodenum (Figure 2). Immunolabeling was confined to the cytoplasm of neurons with small diameter and smooth outline (Figure 3A-B). We noticed weak to moderate CB1R-IR cytoplasm of MP neurons (Figure 3D-F). Immunoreactivity was distributed relatively similarly between jejunum and ileum, whereas duodenal neurons showed mostly weak staining. We also observed weak to strong CB1R-IR cells in the smooth muscles of longitudinal layer of tunica muscularis with the DAB staining (Figure 3H-I), where the strongest staining occurred in the duodenum (moderate/strong). While the most CB1R-IR cells were observed in the jejunal and ileal neurons, there were no significant differences in the number of CB1R reactive neurons between the three sections of the small intestine (Figure 4A).

Distribution of CB2R immunoreactivity

We observed weak with a few instances of moderate and strong CB2R-IR neurons in approximately less than half of the intestine neurons (on average 42.97 ± 6.67%). Most of the CB2R immunoreactivity was concentrated in the cytoplasm of SP neurons with large diameter and smooth outline (Figure 5A-C). We didn't find CB2R-IR neurons in the MP neurons with the immunofluorescence staining, however using the DAB staining we detected a very weak immunoreactivity in two subjects. The observed CB2R immunoreactivity in MP neurons was granular, very scarce and diffused, and showed OD of mostly less than 0,2 so we deemed it as of no significance (Figure 5E). We also detected a few instances of weak CB2R-IR cells in the submucosal layer of the intestine wall (Figure 5B). Immunoreactivity was similar in all segments of the intestine, with a few moderate and strong cases in ileum (Figure 2). Highest number of CB2R-IR neurons was observed in the jejunum (Figure 4B; $P < 0.001$), while the number of reactive neurons in the rest of the small intestine was less than half.

Distribution of TRPV1 immunoreactivity

We observed a weak to strong TRPV1 immunoreactivity in the cytoplasm of the majority of bovine small intestine neurons (on average 63.19 ± 9.06%). The immunolabeling of TRPV1 exhibited a predominant distribution within the cell bodies of neurons, characterized by a smooth outline. Notably, neurons with larger diameter displayed heightened intensity of TRPV1 immunoreactivity while those with smaller diameters exhibited either faint or imperceptible labeling of the protein. We noticed similar distribution and immunolabeling in both MP neurons (Figure 6A-C) and SP neurons (Figure 6D-F), with predominantly weak and strong intensity, and a small number of moderately stained neurons (Figure 2). The strongest staining of SP neurons occurred mostly in the ileum, while MP neurons in the duodenum. We also observed weak to moderate TRPV1-IR cells in the longitudinal layer of the tunica muscularis (Figure 6G). We found a range of weak to strong TRPV1-IR cells in the Brunner's glands of duodenal mucosa (Figure 5H). We also observed weak to moderate staining in the endothelial cells of small blood vessels (Figure 6I), where the strongest staining occurred in the duodenum. Majority of TRPV1-IR neurons were found in the jejunum (74.39 ± 10.95%), while in duodenum and ileum we observed a TRPV1 immunoreactivity in slightly more than half on the neurons (Figure 4C; P = 0.013 and P = 0.015, respectively)

Discussion

The ECS plays a crucial role in regulating various gastrointestinal functions, including gastric acid secretion, gut motility, and inflammation (Izzo, 2004; DiPatrizio, 2016). In this study, we investigated the gene expression and immunohistochemical distribution of CB1R, CB2R and TRPV1 receptors in the small intestine of cattle, with the aim of contributing to the general knowledge about their potential roles in bovine gastrointestinal physiology. The selection of the small intestine as the primary area of investigation in this study stems from the previously mentioned reasons, as understanding the immunohistochemical localization of cannabinoid and cannabinoid-related receptors in this region may be of help for gaining insights into the potential involvement of these receptors in the modulation of immune responses, inflammatory processes, lactation, and overall gut health in dairy cows.

CB1Rs, primarily located in neuronal tissue of the central nervous system, mainly the brain, are also detected on peripheral nerve terminals and exhibit localization in extra-neural locations including the testis, eye, vascular endothelium, and spleen (Kendall and Yudowski, 2016). Beyond that, CB1R has been extensively characterized for its involvement in the regulation of neurotransmitter release, gastrointestinal motility, and gut-brain signaling (Pacher et al., 2006). Our findings regarding CB1R localization in the bovine intestine align with previous studies in various species like ferrets (Van Sickle et al., 2001), dogs (Galiazzo et al., 2018) and pigs (Toschi et al., 2021) indicating its involvement in the gut motility regulation. The localization of CB1R in the tunica muscularis suggests its potential role in modulating smooth muscle contractility within the gastrointestinal tract. Our observations of receptor presence in the tunica muscularis of the bovine small intestine are consistent with previous research findings, substantiating the notion that these receptors are consistently localized within this anatomical compartment across different species (Duncan et al., 2005; Duncan et al., 2008). However, it is worth noting that the density of CB1R immunoreactivity in bovine intestine was lower (54.19%) when compared to other animal species, such as horses (64%) (Chiocchetti et al., 2009), guinea-pigs (80%) (Furness, 2006) or even sheep, a small ruminant (62%) (Mazzuoli et al., 2007). This difference may reflect species-specific variations in CB1R distribution and density, which could influence the extent of CB1R-mediated regulatory effects on gut motility in cattle. The mRNA expression level of *CNR1*, a gene coding for CB1R, showed no differences between the segments of the small intestine, which is consistent with the observed CB1R-IR density using IHC. No segment-specific differences in *CNR1* expression and CB1R immunoexpression suggest the possibility of unified regulatory mechanisms for CB1R across the three segments of the bovine small intestine. Previous research has indicated the involvement of CB1R in modulating gastrointestinal motility and inflammation, and our results suggest similar sensitivity of all three segments to the regulatory effects of this receptor.

CB2R, on the other hand, is primarily located in immune cells, such as cells of macrophage lineage or leukocytes, and is known to modulate immune responses and inflammatory processes (Munro et al., 1993), with the help of endogenous cannabinoids, such as 2-arachidonoylglycerol (2-AG). The prevailing evidence indicates that 2-AG predominantly

exerts its influence on leukocyte recruitment functions, specifically involving chemokine release, fibronectin adhesion, and migration. This discernible effect of 2-AG in positively regulating immune cell recruitment stands as a significant pro-inflammatory mechanism attributed to endocannabinoids or cannabinoids, as consistently highlighted in the existing literature (Cabral and Griffin-Thomas, 2009; Basu and Dittel, 2011; Turcotte et al., 2016;). Our investigation of CB2R immunolocalization deviated from some approaches employed in other species Due to the inclusion of observations pertaining not only to MP neurons but also to SP neurons in our study, currently, a direct comparison of CB2R-IR SP neurons with other species is not possible. While CB2R immunoreactivity was not detected in the MP neurons, similar to findings in pigs (Toschi et al., 2021) or dogs (Galiazzo et al., 2018), which is contrary to the observations obtained in rats (Duncan et al., 2008) and humans (Wright et al., 2008), we observed CB2R immunoreactivity in the SP of bovine intestines. It is also worth noting that only the jejunal SP neurons showed the presence of CB2R at a level above 50%, while in the remaining segments their presence was scarce. This observation highlights the need to explore CB2R localization beyond the MP to gain a comprehensive understanding of its distribution and potential functional roles in the gastrointestinal tract of animals. The presence of CB2R in the SP suggests its involvement in immune modulation and inflammation regulation within this specific anatomical region. An interesting assumption also appeared in the work on the human large intestine, where researchers suggest a potential increase in the amount of CB2R during inflammatory processes (Wright et al., 2005; Wright et al., 2008). It should also be mentioned here that there is a difference in the localization of CB2R between cattle in our study and dogs, where in the latter they were also found in both layers of the tunica muscularis (Galiazzo et al., 2018), which may suggest a difference between herbivores and carnivores. In the case of *CNR2* expression, we noted no significant differences between small intestine sections, but with a similar data pattern to IHC, which is a modest yet consistent increase in the jejunum compared to both the duodenum and ileum. The relatively higher immunoreactivity of CB2R in the jejunum suggests its potential significance in this segment, possibly influencing immune responses, gastrointestinal secretions, and neuronal signaling. On the other hand, the similar immunoreactivity of CB2R in the duodenum and ileum hint at a common regulatory mechanism or functional relevance shared between these regions. Further investigation is required to elucidate the functional implications of CB2R localization in the intestines and its potential impact on immune responses in cattle.

In addition to CB1R and CB2R, we examined the localization and distribution of TRPV1 in bovine small intestine. The TRPV1 is a sensory receptor that plays a significant role in pain perception and the modulation of sensory neurotransmission. It is widely localized in the peripheral nervous system, including sensory neurons, as well as in various tissues and organs, including the gastrointestinal tract. Activation of TRPV1 by specific stimuli, such as heat, capsaicin, or acidic pH (Dhaka et al., 2009), leads to the generation and transmission of pain signals (Pertwee, 2001; Tominaga and Tominaga, 2005). In the gastrointestinal system, TRPV1 is involved in the regulation of gut motility, inflammation, and nociceptive signaling, contributing to the overall sensory and homeostatic functions of the digestive system. Our results demonstrated the presence of TRPV1 immunoreactivity in multiple anatomical structures, including the tunica muscularis, duodenal Brunner's glands, and the endothelium of

blood vessels. This distribution pattern is consistent with previous analyses of humans (Wright et al., 2005), pigs (Poonyachoti et al., 2002; Toschi et al., 2021), and rodents (Kono et al., 2013) intestine sections. The relatively higher density of TRPV1 immunoreactivity compared to CB1R suggests a potentially significant role for TRPV1 in bovine intestine. The presence of TRPV1 in Brunner's glands and the endothelium of blood vessels implies its involvement in glandular secretory functions and vascular regulation within the gastrointestinal microenvironment. Moreover, the localization of TRPV1 in the tunica muscularis supports its contribution to sensory perception and nociceptive signaling in bovine intestine (Tominaga et al., 1998; Zwick et al., 2002). The most striking finding in our study was the significant upregulation of TRPV1 mRNA gene expression as well as the percentage of TRPV1-IR neurons in the jejunum when compared to both the duodenum and ileum. TRPV1 is a well-known receptor involved in pain perception and the sensation of heat. The heightened expression of *TRPV1* in the jejunum may indicate its crucial role in sensing and responding to diverse environmental stimuli and dietary components in this segment. Additionally, the lower *TRPV1* expression in the duodenum, in comparison to the jejunum, suggests that the duodenum may have reduced responsiveness to certain sensory stimuli or nociceptive signals. Such segment-specific variations in TRPV1 expression and TRPV-IR neurons could be linked to differences in pain perception and visceral sensation along the small intestine. These findings contribute to proving the multifaceted roles of TRPV1 in gastrointestinal physiology, encompassing sensory processing, vascular regulation, and secretory functions.

Interestingly, apart from the significant difference in *TRPV1* expression between the duodenum and jejunum, there were no other significant differences in the expression of *CNR1*, *CNR2*, and *TRPV1* among the three segments of the small intestine. This finding suggests a relatively consistent mRNA expression of *CNR1* and *CNR2* across the duodenum, jejunum, and ileum, implying that these receptors may play broader roles throughout the small intestine. However, it is essential to consider that these receptors might still exhibit functional differences and responses to specific stimuli within each segment despite the lack of statistically significant variations. Additionally, the IHC results were found to be consistent with the mRNA gene expression patterns of *CNR1*, *CNR2*, and *TRPV1*. The immunoreactivity of CB1R and CB2R, corresponding to the gene expression levels of *CNR1* and *CNR2*, respectively, exhibited similar trends in the small intestine segments. These concordant results between mRNA gene expression and specific density of cannabinoid and cannabinoid-related receptors' localization in ENS neurons further validate the segment-specific variations in the ECS within the small intestine.

It's essential to also acknowledge the broader implications of dietary factors on cannabinoid receptors, even though our study did not specifically focus on that. Reference to articles exploring the effects of high-fat diet (HFD) on cannabinoid receptors in rodent models provides valuable context. Notably, these studies reveal that HFD induces significant alterations in CB1 and CB2 immunoexpression, disrupting fasting-induced increases in CB1R in the nodose ganglia and prompting changes in CB1 and CB2 gene expression in the skeletal muscle (Cluny et al., 2013; Crespillo et al. 2010). Furthermore, study on diet-induced obese mice, indicates that baseline intestinal transit was increased, and the efficacy of enhancing it through

cannabinoid CB1R antagonism was less than in lean mice (Izzo et al., 2009). This suggests a potential alteration in the responsiveness of CB1R to antagonism in the context of a HFD. Additionally, the levels of the endocannabinoid's anandamide and 2-arachidonoylglycerol in the small intestine were reduced and increased, respectively, after the HFD. Although our study did not involve a HFD, the observed modifications in CB1 and CB2 expression in the bovine small intestine underscore the potential impact that any diet, not exclusively HFD, may exert on cannabinoid receptors. Moreover, the cumulative evidence from our study, coupled with insights from research on HFT in rodent models, suggests that the dietary composition, including a potential HFD, may exert a discernible impact on cannabinoid receptor expression in the small intestine of high-producing dairy cows. This recognition emphasizes the importance of considering dietary influences on ECS dynamics, laying the groundwork for future investigations into the nuanced relationships between different diets, ECS, and bovine gut health.

Overall, our study may contribute to enhancing our understanding of the localization and distribution of CB1R, CB2R, and TRPV1 receptors in the small intestine of dairy cows. The close similarities between the gene expression data and IHC results strengthens the validity of our findings and emphasizes the significance of the enteric nervous system in maintaining gut homeostasis and responding to various external stimuli. The observed differences compared to other animal species underscore the importance of considering species-specific variations in the ECS within the GIT. Future studies should explore the functional implications of these receptors in dairy cow intestines, elucidating their roles in gastrointestinal regulation and potential therapeutic applications for enhancing dairy cattle health and productivity.

Conclusions

In summary, our study unveils receptor distribution in Holstein bulls small intestines. CB1R, mostly in neurons, showed segment-specific localization, potentially influencing motility. CB2R, in immune cells, increased moderately in the jejunum, implying immune involvement. Elevated TRPV1 in the jejunum highlights sensory and dietary roles. Variations from other species stress species-specific considerations. Further research may clarify receptor functions, contributing to the mitigation of gastrointestinal disorders and enhancing dairy cattle well-being, and productivity.

Conflict of interest

All the authors hereby declare no conflict of interest.

Author contribution

All the listed authors have made a substantial, direct, and intellectual contribution to the work. All the authors approved the manuscript.

References

Acharya N., Penukonda S., Shcheglova T., Hagymasi A.T., Basu S., Srivastava P.K. (2017). Endocannabinoid system acts as a regulator of immune homeostasis in the gut. Proc. Natl. Acad. Sci., USA, 114: 5005–5010.

Basu S., Dittel B.N. (2011). Unraveling the complexities of cannabinoid receptor 2 (CB2) immune regulation in health and disease. Immunol. Res., 51: 26–38.

Cabral G.A., Griffin-Thomas L. (2009). Emerging role of the cannabinoid receptor CB2 in immune regulation: therapeutic prospects for neuroinflammation. Expert. Rev. Mol. Med., 11: e3.

Chiocchetti R., Bombardi C., Mongardi-Fantaguzzi C., Venturelli E., Russo D., Spadari A., Montoneri C., Romagnoli N., Grandis A. (2009). Intrinsic innervation of the horse ileum. Res. Vet. Sci., 87: 177–185.

Cho Y.I., Yoon K.J. (2014). An overview of calf diarrhea – infectious etiology, diagnosis, and intervention. J. Vet. Sci., 15: 1–17.

Cizkova K., Foltynkova T., Gachechiladze M., Tauber Z. (2021). Comparative analysis of immunohistochemical staining intensity determined by light microscopy, ImageJ and QuPath in placental Hofbauer cells. Acta Histochem. Cytochem., 54: 21–29.

Cluny N.L., Baraboi E.D., Mackie K., Burdyga G., Richard D., Dockray G.J., Sharkey K.A. (2013). High fat diet and body weight have different effects on cannabinoid CB(1) receptor expression in rat nodose ganglia. Auton Neurosci., 179: 122–130.

Costa M., Brookes S., Hennig G. (2000). Anatomy and physiology of the enteric nervous system. Gut, 47: iv15–iv19.

Crespillo A., Suárez J., Bermúdez-Silva F.J., Rivera P., Vida M., Alonso M., Palomino A., Lucena M.A., Serrano A., Pérez-Martín P., Macias M., Fernández-Llébrez P., Fonseca F.R. (2010). Expression of cannabinoid system in muscle: effects of high fat diet and CB1 receptor blockade. Biochem. J., 433: 175–185.

De Petrocellis L., Melck D., Bisogno T., Milone A., Di Marzo V. (1999). Finding of the endocannabinoid signaling system in Hydra, a very primitive organism: Possible role in the feeding response. Neuroscience, 92: 377–387.

Dhaka A., Uzzell V., Dubin A.E., Mathur J., Petrus M., Bandell M., Patapoutian A. (2009). TRPV1 is activated by both acidic and basic pH. J. Neurosci., 29: 153–158.

DiPatrizio N.V. (2016). Endocannabinoids in the gut. Cannabis Cannabinoid Res., 1: 67–77.

Duncan M., Davison J.S., Sharkey K.A. (2005). Review article: Endocannabinoids and their receptors in the enteric nervous system. Aliment. Pharmacol. Ther., 22: 667–683.

Duncan M., Mouihate A., Mackie K., Keenan C.M., Buckley N.E., Davison J.S., Patel K.D., Pittman Q.J., Sharkey K.A. (2008). Cannabinoid CB2 receptors in the enteric nervous system modulate gastrointestinal contractility in lipopolysaccharide-treated rats. Am. J. Physiol. Gastrointest. Liver Physiol., 295: G78–G87.

Fernández-Carvajal A., Fernández-Ballester G., Ferrer-Montiel A. (2022). TRPV1 in chronic pruritus and pain: Soft modulation as a therapeutic strategy. Front. Mol. Neurosci., 15: 930964.

Furness J.B. (2006). The enteric nervous system. Blackwell Publishing: Malden, MA, USA.

Galiazzo G., Giancola F., Stanzani A., Fracassi F., Bernardini C., Forni M., Pietra M., Chiocchetti R. (2018). Localization of cannabinoid receptors CB1, CB2, GPR55, and PPARα in the canine gastrointestinal tract. Histochem. Cell Biol., 150: 187–205.

Henry B.M., Tomaszewski K.A., Walocha J.A. (2016). Methods of evidence-based anatomy: a guide to conducting systematic reviews and meta-analysis of anatomical studies. Ann. Anat., 205: 16–21.

Hillard C.J. (2015). Endocannabinoids and the endocrine system in health and disease. In: Endocannabinoids, Pertwee R.G. (ed.). Springer Cham., Switzerland, pp. 317–339.

Izzo A.A. (2004). Cannabinoids and intestinal motility: Welcome to CB2 receptors. Br. J. Pharmacol., 142: 1201–1202.

Izzo A.A., Capasso R., Aviello G., Borrelli F., Romano B., Piscitelli F., Gallo L., Capasso F., Orlando P., Di Marzo V. (2012). Inhibitory effect of cannabichromene, a major non-psychotropic cannabinoid extracted from *Cannabis sativa*, on inflammation-induced hypermotility in mice. Br. J. Pharmacol., 166: 1444–1460.

Izzo A.A., Mascolo N., Pinto L., Capasso R., Capasso F. (1999). The role of cannabinoid receptors in intestinal motility, defaecation and diarrhoea in rats. Eur. J. Pharmacol., 384: 37–42.

Izzo A.A., Piscitelli F., Capasso R., Aviello G., Romano B., Borrelli F., Petrosino S., Di Marzo V. (2009). Peripheral endocannabinoid dysregulation in obesity: relation to intestinal motility and energy processing induced by food deprivation and re-feeding. Br. J. Pharmacol., 158: 451–461.

Janovick-Guretxky N.A., Dann H.N., Carlson D.B., Murphy M.R., Loor J.J., Drackley J.K. (2007). Housekeeping gene expression in bovine liver is affected by physiological state, feed intake, and dietary treatment. J. Dairy Sci., 90: 2246–2252.

Kendall D.A., Yudowski G.A. (2016). Cannabinoid receptors in the central nervous system: their signaling and roles in disease. Front. Cell. Neurosci., 10: 294.

Kono T., Kaneko A., Omiya Y., Ohbuchi K., Ohno N., Yamamoto M. (2013). Epithelial transient receptor potential ankyrin 1 (TRPA1)-dependent adrenomedullin upregulates blood flow in rat small intestine. Am. J. Physiol. Gastrointest. Liver Physiol., 304: G428–G436.

Kunos G., Osei-Hyiaman D., Liu J., Godlewski G., Bátkai S. (2008). Endocannabinoids and the control of energy homeostasis. J. Biol. Chem., 283: 33021–33025.

Lee J.Y., Lee G.J., Nakamura A., Lee P.R., Kim Y., Won C.H., Furue H., Oh S.B. (2020). Involvement of cannabinoid type 1 receptor in fasting-induced analgesia. Mol. Pain, 16: 1744806920969476.

Livak K.J., Schmittgen T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods, 25: 402–408.

Mackie K. (2006). Cannabinoid receptors as therapeutic targets. Ann. Rev. Pharmacol. Toxicol., 46: 101–122.

Mazzuoli G., Mazzoni M., Albanese V., Clavenzani P., Lalatta-Costerbosa G., Lucchi M.L., Furness J.B., Chiocchetti R. (2007). Morphology and neurochemistry of descending and ascending myenteric plexus neurons of sheep ileum. Anat. Rec., 290: 1480–1491.

Munro S., Thomas K.L., Abu-Shaar M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. Nature, 365: 61–65.

Myers M.N., Abou-Rjeileh U., Chirivi M., Parales-Girón J., Lock A.L., Tam J., Zachut M., Contreras G.A. (2023). Cannabinoid-1 receptor activation modulates lipid mobilization and adipogenesis in the adipose tissue of dairy cows. J. Dairy Sci., 106: 3650–3661.

Myers M.N., Zachut M., Tam J., Contreras G.A. (2021). A proposed modulatory role of the endocannabinoid system on adipose tissue metabolism and appetite in periparturient dairy cows. J. Anim. Sci. Biotechnol., 12: 21.

O'Sullivan S.E. (2015). Endocannabinoids and the cardiovascular system in health and disease. Handb. Exp. Pharmacol., 231: 393–422.

Pacher P., Bátkai S., Kunos G. (2006). The endocannabinoid system as an emerging target of pharmacotherapy. Pharmacol. Rev., 58: 389–462.

Pertwee R.G. (2000). Cannabinoid receptor ligands: Clinical and neuropharmacological considerations, relevant to future drug discovery and development. Exp. Opin. Investig. Drugs., 9: 1553–1571.

Pertwee R.G. (2001). Cannabinoid receptors and pain. Prog. Neurobiol., 63: 569–611.

Poonyachoti S., Kulkarni-Narla A., Brown D.R. (2002). Chemical coding of neurons expressing δ- and κ-opioid receptor and type I vanilloid receptor immunoreactivities in the porcine ileum. Cell Tissue Res., 307: 23–33.

Silver R.J. (2019). The endocannabinoid system of animals. Animals, 9: 686.

Skaper S.D., Di Marzo V. (2012). Endocannabinoids in nervous system health and disease: the big picture in a nutshell. Philos. Trans. R. Soc. Lond. B Biol. Sci., 367: 3193–3200.

Starowicz K.M., Cristino L., Matias I., Capasso R., Racioppi A., Izzo A.A., Di Marzo V. (2008). Endocannabinoid dysregulation in the pancreas and adipose tissue of mice fed with a high-fat diet. Obesity (Silver Spring), 16: 553–565.

Storr M.A., Keenan C.M., Zhang H., Patel K.D., Makriyannis A., Sharkey K.A. (2009). Activation of the cannabinoid 2 receptor (CB2) protects against experimental colitis. Inflamm. Bowel Dis., 15: 1678–1685.

Tominaga M., Caterina M.J., Malmberg A.B., Rosen T.A., Gilbert H., Skinner K., Raumann B.E., Basbaum A.I., Julius D. (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron, 21: 531–543.

Tominaga M., Tominaga T. (2005). Structure and function of TRPV1. Pflug. Arch. Eur. J. Physiol., 451: 143–150.

Toschi A., Galiazzo G., Piva A., Tagliavia C., Mazzuoli-Weber G., Chiocchetti R., Grilli E. (2021). Cannabinoid and cannabinoid-related receptors in the myenteric plexus of the porcine ileum. Animals, 11: 63.

Turcotte C., Blanchet M.R., Laviolette M., Flamand N. (2016). The CB2 receptor and its role as a regulator of inflammation. Cell. Mol. Life Sci., 73: 4449–4470.

Van Sickle M. D., Oland L.D., Ho W., Hillard C.J., Mackie K., Davison J.S., Sharkey K.S. (2001). Cannabinoids inhibit emesis through CB1 receptors in the brainstem of the ferret. Gastroenterology, 121: 767–774.

Varghese F., Bukhari A.B., Malhotra R., De A. (2014). IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. PLoS One, 9: e96801.

Włodarczyk R., Budvytis M. (2011). Proper feeding of high-yielding cows – how to fullfil their productive potential (in Polish). Życie Weter., 86: 771–776.

Wright K., Rooney N., Feeney M., Tate J., Robertson D., Welham M., Ward S. (2005). Differential expression of cannabinoid receptors in the human colon: Cannabinoids promote epithelial wound healing, Gastroenterology, 129: 437–453.

Wright K.L., Duncan M., Sharkey K.A. (2008). Cannabinoid CB2 receptors in the gastrointestinal tract: a regulatory system in states of inflammation. Br. J. Pharmacol., 153: 263–270.

Zwick M., Davis B.M., Woodbury C.J., Burkett J.N., Koerber H.R., Simpson J.F., Albers K.M. (2002). Glial cell line-derived neurotrophic factor is a survival factor for isolectin B4-positive, but not vanilloid receptor 1-positive, neurons in the mouse. J. Neurosci., 22: 4057–4065.

Received: 16 VIII 2023

Accepted: 15 XII 2023

Table 1. Primers used in the study

Gene	Primer sequences (5’ to 3’)	Product length	GeneBank accession number
CNR1	F: GGCGAGGGAGCTTCTCCCG R: ATTTGGATGCCATGTCGCTTT	225	NM_001242341.2

CNR2	F: GCCAATGGCTCCAGCGATGG R: CACACTGGCCAGAAAGTCGGC	228	NM_001192303.1
TRPV1	F: CACGTACATCCTCCTGCTCA R: CGAAAGGCCTTCCTCATACA	156	XM_015458647.2
ACTB	F: TCCCTGGAGAAGAGCTACGA R: AGGTAGTTTCGTGAATGCCG	133	NM_173979.3
RSP9	F: CCTCGACCAAGAGCTGAAG R: CCTCCAGACCTCACGTTTGTTC	64	NM_001101152.2

Table 2. Primary and secondary antibodies used in the study

Antibody	Host	Code	Dilution	Source
Primary antibody				
Anti-CB1R	Rabbit	BS-1683R	1:100	ThermoFisher
Anti-CB2R	Rabbit	ab3561	1:200	Abcam
Anti-TRPV1	Rabbit	ACC-030	1:200	Alomone Labs
Anti-HuC/HuD	Mouse	A-21271	1:400	ThermoFisher
Secondary antibody				
Anti-Mouse/Rabbit	Goat	DPVB-HRP	RTU[1]	ImmunoLogic
Alexa 488 Anti-Mouse	Mouse	A-11029	1:800	ThermoFisher
Alexa 595 Anti-Rabbit	Rabbit	A-21207	1:800	ThermoFisher

RTU = Ready to use.

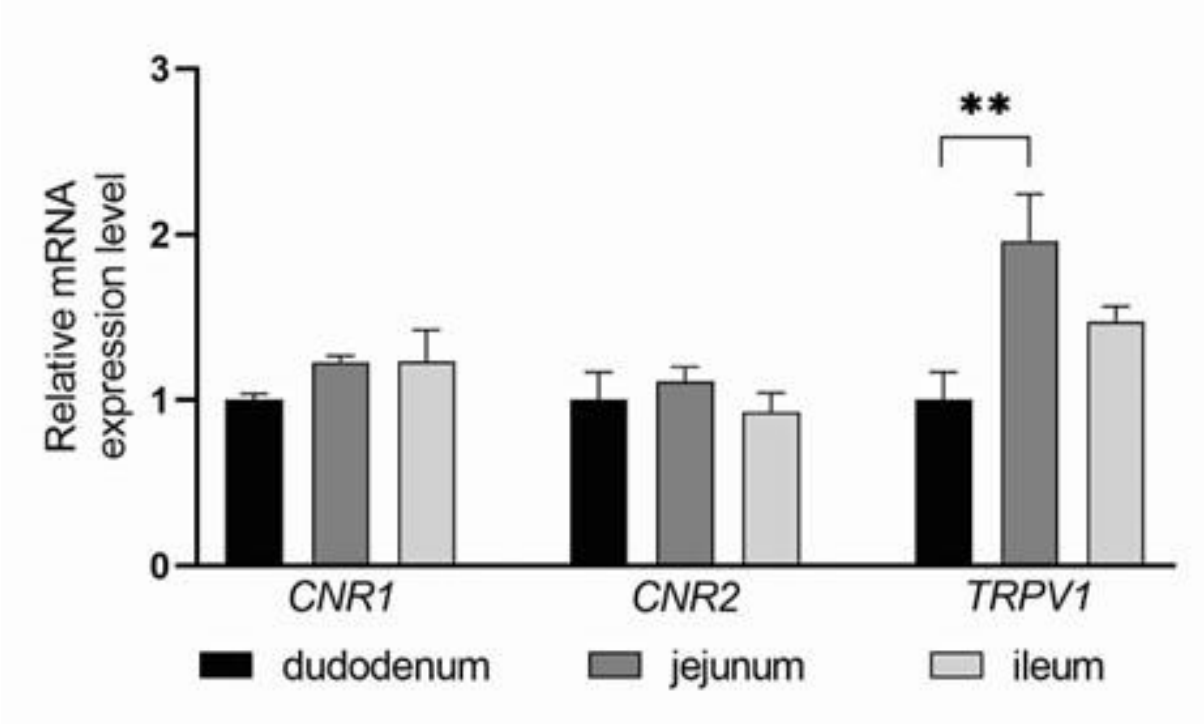

Figure 1. The relative expression of *CNR1*, *CNR2*, and *TRPV1* genes in the examined small intestine segments bovine small intestine (duodenum, jejunum and ileum). Gene expression was normalized by the geometric mean of the *RSP9* and *ACTB* housekeeping genes and presented as relative to the expression level duodenum. Asterisks (*) indicate significant differences between small intestine segments (**P<0.01).

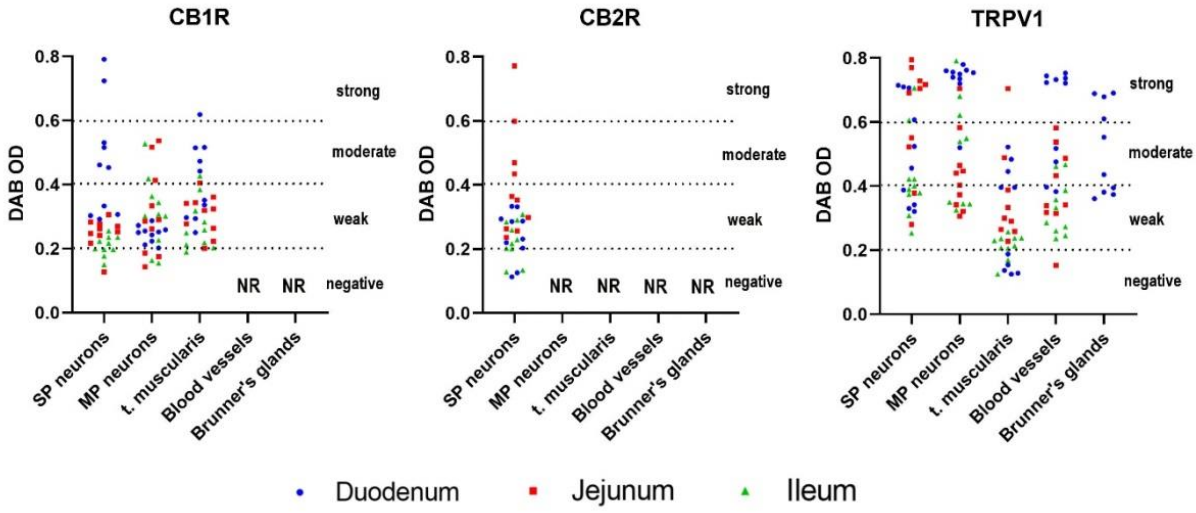

Figure 2. Summary of the intensity of CB1R, CB2R and TRPV1 immunoreactivity measured as the Optical Density (OD) in different cellular structures and represented as mean values: submucosal plexus (SP) neurons, myenteric plexus (MP) neurons, tunica muscularis, blood vessels and duodenal Brunner's glands of the bovine intestine. Structures where no clearly visible staining occurred were marked as No Reaction (NR)

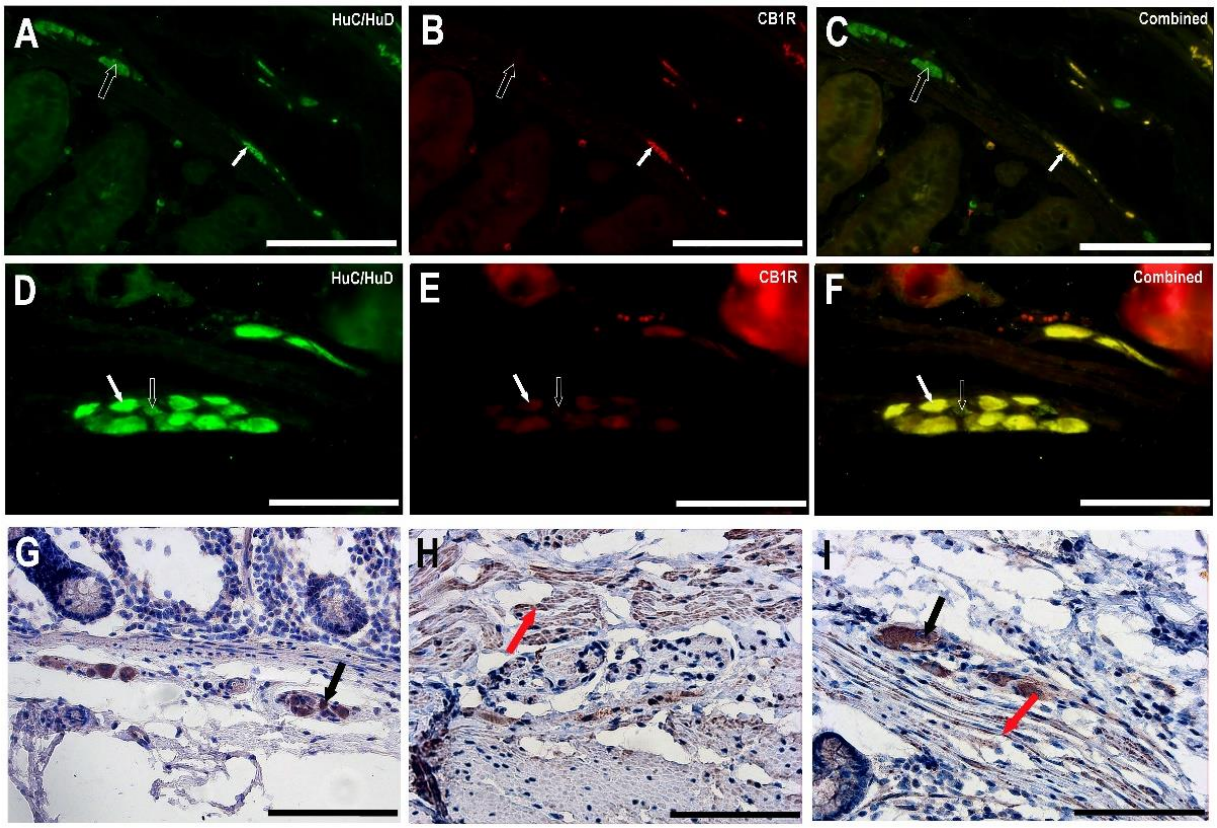

Figure 3. CB1R immunoreactivity in the bovine small intestine. HuC/HuD immunoreactive neurons in the submucosal plexus of duodenum (A), CB1R immunoreactive neurons in the submucosal plexus of duodenum (B), combined image (C), HuC/HuD immunoreactive neurons in the myenteric plexus of duodenum (D), CB1R immunoreactive neurons in the myenteric plexus of duodenum (E), combined image (F), DAB stained image of submucosal plexus in duodenum (G), DAB stained image of myenteric plexus in duodenum (H), DAB stained image of myenteric plexus in duodenum (I). White arrows indicate CB1R immunoreactive neurons, open white arrows indicate neurons that were CB1R negative, black arrows indicate CB1R immunoreactive neurons with DAB staining, red arrows indicate immunoreactivity in the tunica muscularis. Scale bars: 20 μm

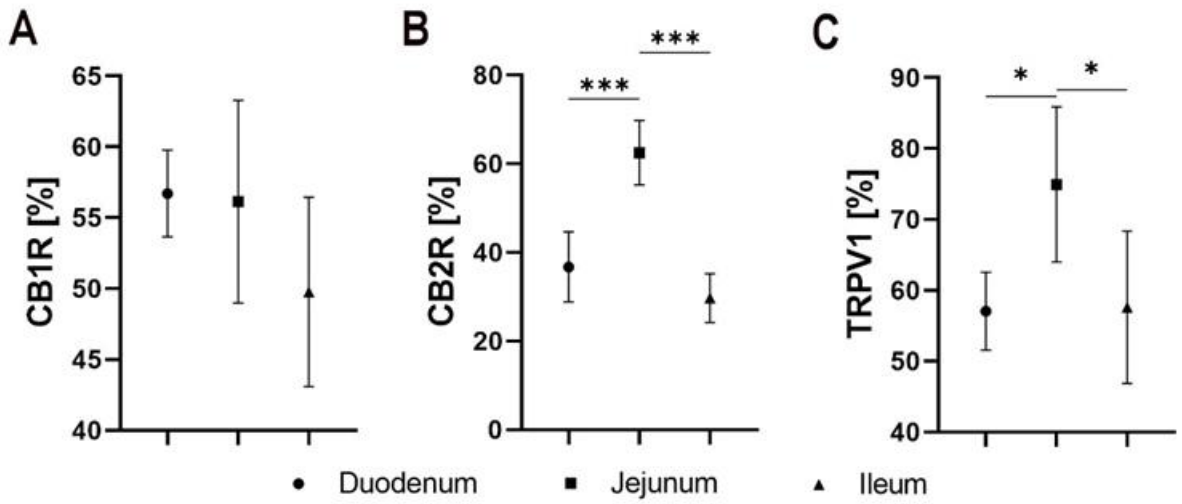

Figure 4. Summary of the number of CBR, CB2R and TRPV1 immunoreactive neurons in different sections: (A) duodenum, (B) jejunum, (C) ileum of bovine small intestine. Asterisks (*) indicate significant differences between small intestine segments (*P<0.05; ***P<0.001)

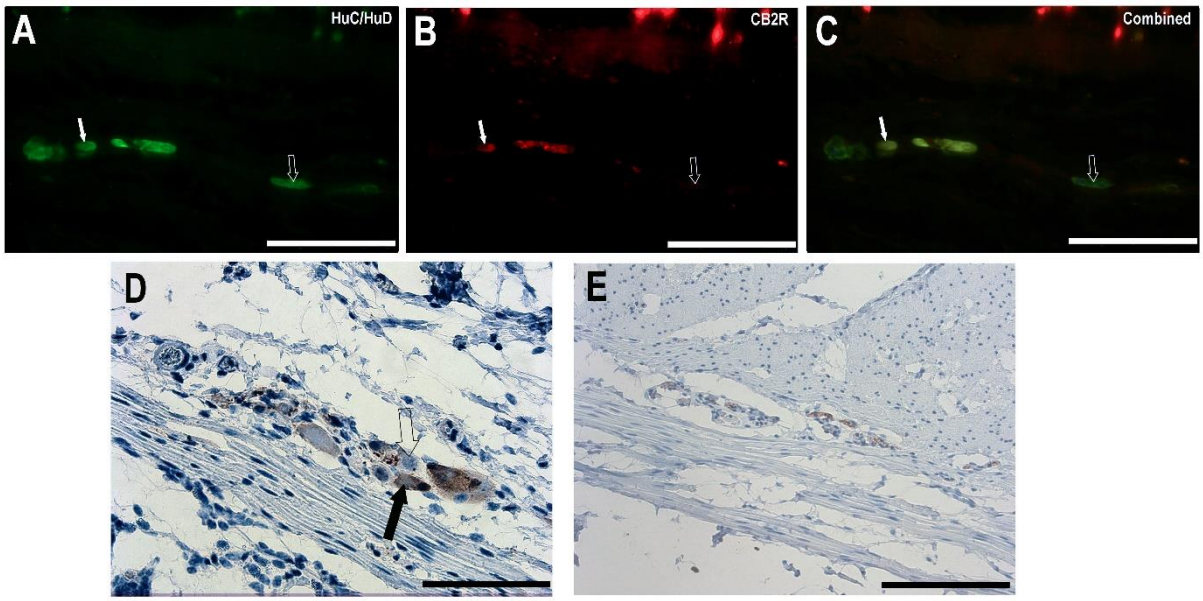

Figure 5. CB2R immunoreactivity in the bovine small intestine. HuC/HuD immunoreactive neurons in the submucosal plexus of jejunum (A), CB2R immunoreactive neurons in the submucosal plexus of jejunum (B), combined image (C), DAB stained image of submucosal plexus in duodenum (D), DAB stained image of myenteric plexus in duodenum (E). White arrows indicate CB2R immunoreactive neurons, open white arrows indicate neurons that were CB2R negative, black arrows indicate CB2R immunoreactive neurons with DAB staining, open black arrows indicate neurons that were CB2R negative in DAB staining. Scale bars: 20 μm

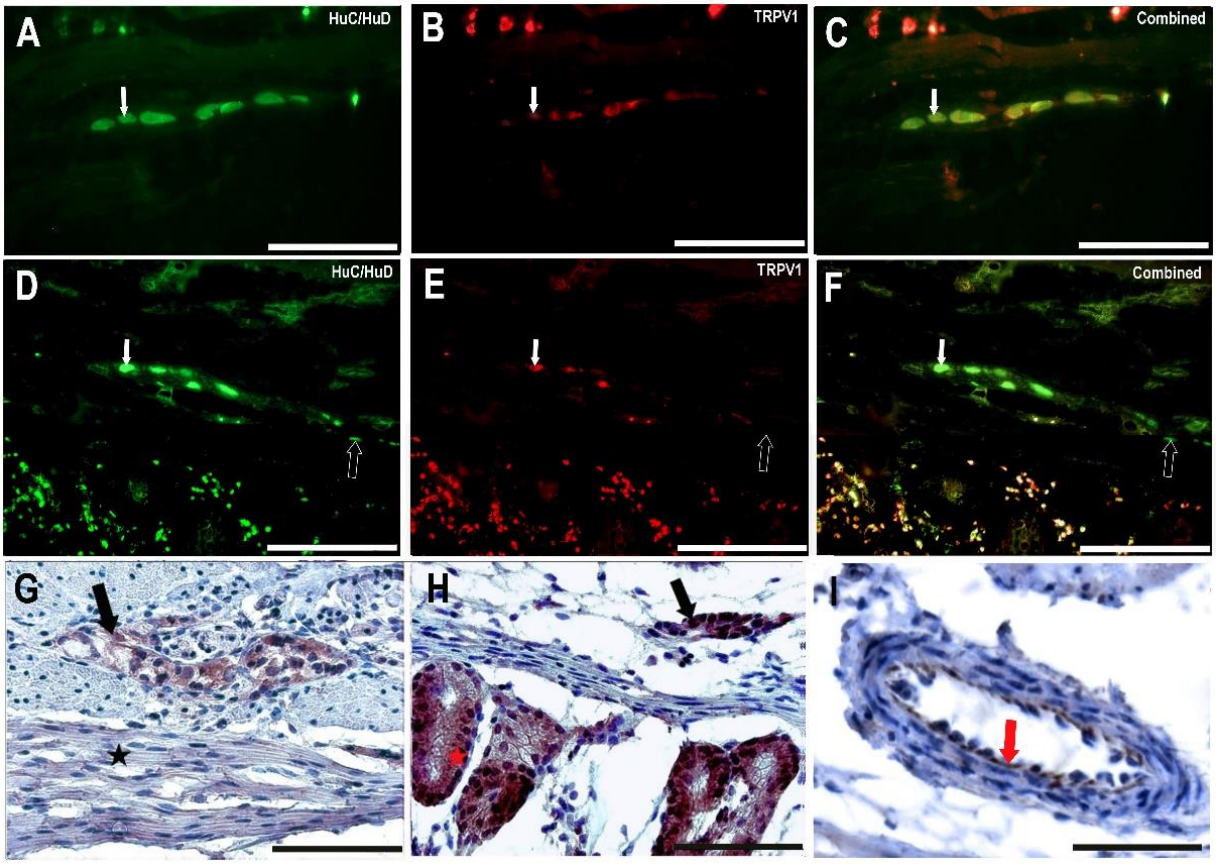

Figure 6. TRPV1 immunoreactivity in the bovine small intestine. HuC/HuD immunoreactive neurons in the myenteric plexus of jejunum (A), TRPV1 immunoreactivity in the myenteric plexus of jejunum (B), combined image (C), HuC/HuD immunoreactive neurons in the submucosal plexus of jejunum (D), TRPV1 immunoreactivity in the submucosal plexus of jejunum (E), combined image (F), DAB stained image of myenteric plexus in duodenum (G), DAB stained image of submucosal plexus in duodenum (H), DAB stained image of blood vessels in duodenum (I). White arrows indicate TRPV1 immunoreactive neurons, open white arrows indicate neurons that were TRPV1 negative, black arrows indicate TRPV1 immunoreactive neurons with DAB staining, black star indicates immunoreactivity in the tunica muscularis, red star indicates immunoreactivity in the Brunner's glands, red arrow indicates immunoreactivity in the endothelium of blood vessels. Scale bars: 20 μm (A–H), 50 μm (I)