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# Cannabidiol can affect morphology, morphometry, enzymatic and microbial activity of rabbit digestive system

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## Abstract

The present research aimed to evaluate the effects of the continuative dietary administration of a hemp oil extract containing cannabinoids (cannabidiol, **CBD**) on the macroscopic morphology, morphometry, and enzymatic activity of different intestinal tracts as well as on the production of short-chain fatty acids (**SCFAs**) in the cecum of growing rabbits. The research was performed on 16 rabbits randomly selected from 2 experimental groups (8 per group). In detail, 42 sixty-day-old New Zealand White × California rabbits (sex ratio 1:1, average weight 1621.3 ± 46.2 g) were homogeneously divided into 2 groups (21 animals/group), namely control and CBD. Both groups were fed the same commercial diet, but the CBD one was supplemented with 0.1 mL of hemp extract in coconut-based oil corresponding to 10 mg of CBD/animal/d. Up to 92 d of age (for 27 d), individual live weight and feed intake were measured weekly. At 92 d of age, 8 rabbits/group (sex ratio 1:1) were moved to a specialized slaughterhouse, and the gastrointestinal tract was separated from the carcass. Samples from 8 rabbits per dietary treatment were used for the histomorphological analysis of small and large intestines. In addition, duodenum, jejunum, ileum, and cecum were processed for enzymatic analysis. The caecal contents were used for the SCFAs determination. The administration of CBD did not affect feed intake and the final rabbits' whole body weight ($P > 0.05$), but some changes were detected in the gastrointestinal tract of the animals. CBD seemed to interfere with protein digestion, with a significantly lower activity of the enzymes related to peptides in the small intestine and a consequent increase of the fermentative activity of caecal microbiota. This effect, in combination with a general decrease of fermentative activity in the caecal content of rabbits submitted to CBD treatment, was responsible for a change in the SCFA proportion mainly regarding the reduction of butyrate production ($P < 0.01$) that resulted significant higher in CTR group compared to CBD. This last result is very important for intestinal health. Such fermentation activity modification was coupled with changes in the relative abundance of goblet cells in the colon. Overall, our findings suggest that a relatively long-term administration of CBD may affect digestion in rabbits, in particular at enzymatic and fermentative levels.

## Lay Summary

The study compares the characteristics of the small and large intestines in 2 groups of healthy meat rabbit feeding or not with a cannabidiol extract for 27 d. Feed intake and final weight are similar for both groups. However, cannabidiol significantly interfered with protein digestion in the small intestine and decreased the microbial activity in the cecum. These results suggest that cannabidiol is able to affect the physiological activity of both the small and large intestines in rabbits. For that reason, further research is necessary to clarify the impact of cannabidiol on the gastrointestinal tract of rabbits and its long-term use may require the monitoring of intestinal function.

**Key words:** cannabidiol, macroscopic morphology, morphometry, enzymatic activity, rabbit digestive system

**Abbreviations:** BCFA, branched-chain fatty acids; CBD, cannabidiol; CD, crypt depth; PUFA, polyunsaturated fatty acids; SCFAs, short-chain fatty acids; VH, villus height

## Introduction

In recent years, hemp (*Cannabis sativa* L.) has gained great attention in animal nutrition for its high biomass production (Rehman et al., 2021), nutritive value (Apetroaei et al., 2024), and nutraceutical properties due to the presence of several chemical compounds including flavonoids, terpenes, phytocannabinoids, amino acids, and polyunsaturated fatty acids (**PUFA**) (Russo, 2011). Among the phytocannabinoids, the cannabidiol (**CBD**), a non-psychoactive compound derived from cannabidiolic acid after heat exposure (Rupasinghe et al., 2020), showed interesting properties mainly due to a series of effects alleviating pain, anorexia, nausea, anxiety disorders, and insomnia in both human and animals (Shannon and Opila-Lehman, 2016; Della Rocca and Di Salvo, 2020).

The CBD acts by binding mainly CBR-2 cannabinoid receptors, included in the endocannabinoid system (Di Marzo et al., 2004), but also non-cannabinoid ones (vanilloid subtype 1, TRPV1; peroxisome proliferator-activated receptors, PPARs; serotonin receptor 5-HT1A, Boehm et al., 2023).

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By interacting with some cannabinoid receptors distributed throughout the gastrointestinal tract (Izzo and Sharkey, 2010), CBD contributes to maintain gut homeostasis by modulating immune tolerance, gastrointestinal motility, visceral pain, and inflammation (Gotfried et al., 2020).

Mainly, CBD studies have been performed on its antianxiety effect in humans and in animals; most of them regarded rats and mice, respectively at the doses 10 and 20 mg/kg, and companion animals (Hunt et al., 2023; Marliani et al., 2024). Similarly, the effect of CBD on gastrointestinal health has been mainly studied in human and/or in animal models, but focusing on symptoms due to anxiety, such as nausea, vomiting, and intestinal inflammation (Gotfried et al., 2020).

The use of hemp, cannabis, or CBD, on farmed animal was assessed in several species such as chicken broilers (Konieczka et al., 2020), laying hens (Kasula et al., 2021), quails (Yalcin et al., 2018), dairy cows (Kleinhenz et al., 2022), steers (Gibb et al., 2005), goats (Cozma et al., 2015), pregnant sows (Palade et al., 2019), rabbits (Jacobson et al., 2023) and was recently reviewed by Muedi et al. (2024). Most of the studies were generally addressed to evaluate the in vivo or postmortem animal performance and only a few of them focused on the effect of CBD on the intestinal tract of farmed animals, with contrasting results. For example, Konieczka et al. (2020) observed an increase in the activity of gut bacterial enzyme in broilers feeding a hemp extract with 12 % of CBD included in the diet at 15 g/kg, while Vispute et al. (2019) did not find any effect on performance, jejunal villus height (**VH**), and crypt depth (**CD**) of 42 d old broiler feeding 0.2% or 0.3% of hemp seeds. To our knowledge, no further research is available concerning the impact of the use of hemp or cannabinoids on the small and large intestines of farmed animals.

The combined effect of reducing anxiety and modulating gastrointestinal activity of CBD could be useful in meat producing rabbit (*Orictolagus cuniculus*) mainly farmed in cages (Trocino et al., 2022). In fact, the cage system aims to maximize sanitary conditions and optimize animal management but can have a negative impact on animal welfare (Siddiqui et al., 2024) with a possible impact on health. Within them, several studies showed that stress is associated with changes in the digestive system (Feng et al., 2022), focusing on intestinal-cerebral inter-communication, a pathway known as the brain–gut-microbiota axis (Dinan and Cryan, 2012; Mayer et al., 2014). Additionally, from a One Health perspective, improving gut health is very important in rabbits in which intestinal disorders with a clear pathogenic origin (e.g., *Escherichia coli*) or without obvious pathogen involved (e.g., syndromes like epizootic rabbit enteropathy) account for the majority of losses in meat rabbit production (van der Sluis et al., 2024).

Within this context and starting from the hypothesis that the CBD can have effects on the gastrointestinal tract directly due to the presence of its specific receptors or indirectly through the gut–brain axis, the present research aimed to evaluate the effects of the continuative dietary cannabinoids administration on the macroscopic morphology, morphometry, and enzymatic activity of different intestinal tracts as well as on the production of short-chain fatty acids (**SCFAs**) in the cecum in growing rabbits.

## Material and Methods

### Ethics approval

The experimental procedures were approved by the Ethical Animal Care and Use Committee of the University of Napoli Federico II (prot. N. 2019/0058989) and were realized according to the principles of the EC Directive 2010/63/UE, regarding the protection of animals used for experimental and other scientific purposes.

### Animals and diet

The research was performed on 16 rabbits randomly selected from 2 experimental groups (8 per group). In detail, 42 sixty-day-old New Zealand White × California rabbits (sex ratio 1:1, average weight 1621.3 ± 46.2 g) were homogeneously divided into 2 experimental groups (21 animals/group). The animals were raised in individual cages (25 cm length × 45 cm depth × 30 cm height) in a room under controlled environmental conditions (temperature 21 °C, relative humidity 62%). During the adaptation period (60–64 d of age), the groups fed ad libitum the same commercial diet. From 65 d of age, both groups continued to be fed with the basal diet, but the Cannabidiol (CBD) group (11 females, 10 males) received 0.1 mL of a hemp extract in coconut-based oil, corresponding to 10 mg of CBD/animal/d. The administration dose was chosen based on studies on laboratory rabbits (Rooney et al., 2022) and on the indication of the producer (Giantec, Isernia, Italy). The hemp extract was administered individually, once a day, by putting it on an alfalfa dehydrated meal-based wafer (15 g) and waiting until the wafer has been completely consumed. The control group (10 females, 11 males) received the same amount of alfalfa wafer with 0.1 mL of coconut oil but without the hemp extract. Up to 92 d of age (for 27 d), the live weight and feed intake were individually controlled weekly.

CBD extract was characterized using High-Performance Liquid Chromatography—Diode Array Detector (HPLC-DAD) according to the European Pharmacopoeia (Ph. Eur. 2.2.29). The results of the CBD extract analysis are reported in Table 1. The ingredients and chemical characteristics of both diet and wafer are reported in Table 2: the ingredients were obtained from the commercial tag; the chemical characteristics were determined according to AOAC (2002) official methods. The amount of digestible energy (**DE**) has been estimated according to the equation proposed by (Fernández-Carmona et al., 2002), DE (MJ kg – 1

**Table 1.** CBD extract high performance liquid chromatography analysis

| | |
|---|---|
| Total cannabidiol (CBD + CBA) | 10.48% |
| CBD | 9.42% |
| CBA | 1.06% |
| Total tetrahydrocannabinol (THC + THCA) | 0.19% |
| D9-Tetrahydrocannabinol | 0.19% |
| Tetrahydrocannabinolic acid | ND |
| D8-tetrahydrocannabidinol | ND |
| Total cannabigerol (CBG + CBGA) | 0.36% |
| Cannabigerol | 0.24% |
| Cannabigerolic acid | 0.11% |
| Cannabinol | 0.04% |
| Cannabichromene | 0.03% |
| Cannabidivarin | 0.07% |
| Cannabidivarinic acid | ND |

Abbreviation: ND, not detected.

**Table 2.** Chemical–nutritional characteristics and ingredients of the diet and wafer used along the trial

| | Basal diet[1] | Wafer[2] |
|---|---|---|
| Crude Protein, % AF | 15.6 | 11.8 |
| Crude Lipids, % AF | 3.2 | 1.5 |
| ADF, % AF | 16.5 | 17.9 |
| Crude ash, % AF | 6.6 | 8.4 |
| Ca, % AF | 0.34 | — |
| P, % AF | 0.46 | 0.13 |
| Na, % AF | 0.22 | 0.05 |
| Lysine, % AF | 0.66 | — |
| Methionine, % AF | 0.23 | — |
| DE, MJ/kg | 11.6 | 10.9 |

Abbreviations: ADF, acid detergent fiber, AF, as feed, DE, digestible energy.
[1]Ingredients (in a decreasing-amount order): wheat bran, sunflower meal, dehydrated beet pulp, wheat straw, soybean shells, barley, grape seeds meal, toasted soybean seeds, sugar cane molasses, dehydrated alfalfa meal, soybean oil, salt. Min-vit supplementation/kg: Vit A 9000 IU, Vit D3 900 IU, Vit E 30 mg, Mn 30 mg, Zn 42 mg, Cu 6 mg, Fe 45 mg, I 1.8 mg, Se 0.06mg.
[2]Ingredients (in a decreasing-amount order): dehydrated alfalfa hay, dehydrated alfalfa, heat-crushed barley, wheat bran, oats, corn flakes, sugar cane molasses, glucose and fructose syrup, maltodextrins, sucrose. Min-vit supplementation/kg: Vit A 367 IU, Vit D3 37.65, coline 2.21 mg, Vit B1 0.61 mg, Vit B2 0.59 mg, Vit E 9.80 mg, biotine 0.02 mg, folic acid 0.04 mg, niacin 2.45 mg, calcium D-pantotenate 0.04 mg, CuSO4 0.37 mg, Fe 0.49 mg, MnO 2.54 mg, $ZnSO_4$* $H_2O$ 2.13 mg, butylhydroxitolouene 3.00 mg.
DE (MJ kg – 1 DM) = 14.2 – 0.205 ADF + 0.218 EE + 0.057 CP.

DM) = 14.2 – 0.205 acid detergent fiber (**ADF**) + 0.218 ether extract (**EE**) + 0.057 crude protein (**CP**) ($R^2$ = 0.965, relative standard deviation = 0.494).

At 92 d of age, a total of 16 rabbits (8 rabbits per group; sex ratio 1:1) were moved to a specialized slaughterhouse, and the gastrointestinal tract was separated from the carcass. The stomach, duodenum, jejunum, ileum, cecum, colon, and rectum were separated, emptied, gently rinsed with saline solution, drained, and finally weighed and measured.

The pH of the cecum was measured, before removing its content, using a portable pH-meter (Model HI 9025; Hanna Instruments, Woonsocket, RI, USA), equipped with an electrode (FC 230C; Hanna Instruments); then, the caecal content was collected in plastic tubes and immediately frozen at –20 °C until analysis.

## Intestinal morphometry and goblet cells

Samples from 8 rabbits per dietary treatment (a total of 80 samples, corresponding to 16 rabbits × 5 samples of intestinal tracts) were used for the histomorphological analysis of small and large intestine. From each of the following tracts: duodenum, jejunum, ileum, cecum, and colon, a single sample (2 to 3 cm long) was collected around 5 cm from the beginning of the single tract. All the samples collected were fixed in Bouin's solution for 24 h at 4 °C, washed in 70% ethanol, and then processed according to Ratti et al. (2023). Briefly, samples, after being dehydrated using 80%, 95%, and 100% ethanol solutions, washed with xylene, and embedded in paraffin (Bio-Optica, Milano, Italy), were cut with a microtome (RM2125 RTS; Leica, Nussloch, Germany) to obtain transversal sections of 5 µm, collected at intervals of 200 µm. Sections were stained with Mayer hematoxylin and eosin Y (EE; Merck KGaA) to determine the intestinal histomorphology or with Alcian blue (Ab; Bio-Optica, Milano, Italy) to assess the relative abundance of goblet cells (calculated on a 10,000 µm$^2$ area) in the mucosal epithelium (Zarantoniello et al., 2023). In addition, a series of morphological parameters on the absorptive epithelium were measured i) VH, CD, and VH:CD ratio in the small intestine (duodenum, jejunum, and ileum); ii) tunica mucosa in the large intestine (cecum and colon).

All the measurements and the morphological considerations were conducted on 6 sections for each intestinal tract for each specimen. Sections were examined using a Zeiss Axio Imager.A2 microscope equipped with an Axiocam 105 combined color digital camera, whilst the image analysis was performed using the ZEN 2.3 software (Zeiss, Oberkochen, Germany).

## Digestive and brush border membrane enzymes

For the enzymatic analysis, the intestinal tracts (duodenum, jejunum, ilea, cecum) from 8 rabbits per group (a total of 64 samples, corresponding to 16 rabbits × 4 samples of intestinal tracts) were thawed and diluted with phosphate-buffered saline (1:5 w/v, pH 7.4). All samples were disrupted with tissue-lyser (Tissue Lyser II, Qiagen, Germany) at 30 Hz for 1 min and immediately centrifuged at 12,000 rcf for 20 min at 4 °C. The enzyme activity was measured in the supernatant. The hydrolysis of maltose and sucrose, by the maltase and the sucrase-isomaltase, was determined according to Tibaldi et al. (2006). Alkaline phosphatase was determined using a kit by Paramedical (Pontecagnano Faiano, SA, Italy) following the manufacturers' protocol. Leucine aminopeptidase was determined according to Vizcaíno et al. (2014). Trypsin and chymotrypsin activities were assayed using 0.5 mM BAPNA (N-α-benzoyl-DL-arginine-4-nitroanilide) as substrate according to Erlanger et al. (1961) and 0.2 mM SAPNA (N-succinyl-(Ala)2-Pro-Phe-p-nitroanilide) according to Delmar et al. (1979), respectively, in 50 mM Tris–HCl buffer, pH 8.5, containing 10 mM $CaCl_2$. For maltase and sucrase-isomaltase, 1 Unit (U) of enzyme activity was defined as the amount of enzyme that catalyzes the reaction of 1 µmol of substrate hydrolyzed per min at 340 nm. For alkaline phosphatase activity, 1 U of enzyme activity was defined as the amount of enzyme that releases 1 µg of p-nitrophenol per min at 405 nm, following the manufacturer calculation. For trypsin, chymotrypsin, and leucine aminopeptidase, the enzymatic release of p-nitroanilide (pNA) was measured at 405 nm, and 1 U of enzyme activity was defined as the amount of enzyme that releases 1 µmol pNA per min, using as extinction coefficient 8,800 M/cm. The enzymatic activity was expressed as U/g tissue. All assays were performed in triplicate, and the color reactions were read using Tecan Sunrise (Mannedorf/Zurich, Switzerland) Elisa microplate reader.

## Short-chain fatty acids

The caecal contents of the 16 rabbits (8 per group) were divided into 2 aliquots (each about 5 mL) and used for the SCFAs determination. The samples were diluted with oxalic acid (1:1, v/v) and SCFAs were measured by gas Cromatograph model TRACE 1310 (Thermo Scientific SpA, Rodano, Milan, Italy) equipped with NUKOL fused silica capillary column 30 m × 0.25 mm × 0.25 µm film thickness SUPELCO, SpA, Bellefonte, PA, USA.

The operating conditions were FID detector temperature: 200 °C, oven temperature: isotherm 135 °C, carrier: helium flow: 1.5 mL/min.

Each sample was quantified by comparing samples peaks area of each SCFA with the corresponding of an external standard composed by acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate.

### Statistical analysis

The sample size (16 rabbits, 8 per group, sex ratio 1:1) was proposed by the Ethical Animal Care and Use Committee of the University of Napoli Federico II, as the minimum number of slaughtered animals to obtain statistically significant results.

Standing these indications, the power of the statistical analysis was evaluated and confirmed through the Proc POWER (SAS, 2002), planning significance level between 0.05 and 0.1.

The normality of the data and error distribution were evaluated using the Shapiro–Wilk test (SAS, 2002). The random selection of the animals and samples assured the respect of the 4 assumptions of analysis of variance (**ANOVA**). The homoscedasticity (variance homogeneity) was evaluated by Levene's test SAS (SAS, 2002).

Data were processed with a 2-ways ANOVA by using the GLM procedure of SAS (2002), according to the following model:

$$Y_{ijk} = m + G_i + S_j + G \times S_{ij} + e_{ijk}$$

Where $Y$ is the single observation, $m$ the general mean, $G$ the effect of the group ($i$ = control or CBD), $S$ the effect of the gender ($j$ = female or male), $G \times S$ the interaction between the 2 main factors, $e$ the error.

Comparison among means was performed by Tukey's test (SAS, 2002) at $P < 0.05$.

## Results

### Feed intake and biometric measurements

The overall feed intake was not different ($P = 0.4589$) between control (161.9 ± 12.1 g/d) and CBD group (161.4 ± 11.6 g/d), whilst males ingested more feed than females (171.5 ± 12.9 and 151.8 ± 11.4 g/d, respectively, $P < 0.01$), with no effect of the interaction ($P = 0.5768$).

Table 3 shows the live weight of the rabbits at the end of the trial as well as the weight of the stomach and the weight and length of the small intestinal tracts expressed in absolute value (grams or cm) or in percentage (only for the weights) of the respective live weight. Very few differences can be observed between the control and CBD group. Control group had a lower ($P < 0.05$) duodenal weight expressed in grams or in percentage of the live weight. The length of jejunum was longer ($P < 0.01$) in the control than the CBD group. The weight of the entire small intestine was higher in the CBD group ($P < 0.05$) but only when measured in grams.

Regarding the effect of sex, the females had lower ($P < 0.05$) live weight at slaughter, duodenal weight (in grams and %), ileum length than the males. The weight of the small intestine was lower ($P < 0.01$) in the females but only when expressed in grams.

The interaction effect between diet and gender was significant ($P < 0.01$) only for ileum length and the entire small intestine weight (grams).

Table 4 reported weight and length of the different tracts and of the entire large intestine, expressed as absolute value or in percentage of live weight (for only weights). The control group had a heavier ($P < 0.05$) cecum both in grams and % of live weight than the CBD one. The colon weight was also greater ($P < 0.05$) in the control group but only in % of live weight.

Females showed a lower ($P < 0.05$) weight in grams of cecum and whole large intestine than the males.

**Table 3.** Live weight at 92 d, stomach and small intestine macroscopic morphology of rabbits ($n$ = 16, 8 per group) according to dietary treatment and gender

| | Control | CBD | Female | Male | RMSE | *P*-values | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Group | Gender | Interaction |
| Live weight, g | 2617.1 | 2688.3 | 2585.2$^{b}$ | 2700.2$^{a}$ | 165.4 | 0.0623 | 0.0325 | 0.7945 |
| Stomach, g | 31.00 | 28.21 | 29.29 | 29.93 | 2.66 | 0.1949 | 0.9021 | 0.1407 |
| Stomach, %LW | 1.18 | 1.05 | 1.13 | 1.11 | 0.095 | 0.1816 | 0.7823 | 0.1845 |
| Duodenum weight, g | 14.71$^{b}$ | 18.57$^{a}$ | 15.00$^{b}$ | 18.29$^{a}$ | 1.12 | 0.0123 | 0.0265 | 0.1731 |
| Duodenum weight, %LW | 0.56$^{B}$ | 0.69$^{A}$ | 0.58$^{b}$ | 0.67$^{a}$ | 0.047 | 0.0071 | 0.0123 | 0.2733 |
| Duodenum length, cm | 43.07 | 42.86 | 42.43 | 43.50 | 3.89 | 0.9769 | 0.6245 | 0.7652 |
| Jejunum weight, g | 28.14 | 29.29 | 26.71 | 30.71 | 2.99 | 0.5360 | 0.6771 | 0.0933 |
| Jejunum weight, %LW | 1.08 | 1.09 | 1.03 | 1.14 | 0.092 | 0.4614 | 0.1627 | 0.1320 |
| Jejunum length, cm | 81.29$^{A}$ | 75.30$^{B}$ | 78.73 | 77.86 | 5.71 | 0.0053 | 0.6813 | 0.8784 |
| Ileum weight, g | 9.57 | 8.71 | 9.00 | 9.28 | 0.916 | 0.1413 | 0.7560 | 0.4562 |
| Ileum weight, %LW | 0.37 | 0.32 | 0.35 | 0.34 | 0.029 | 0.1723 | 0.8665 | 0.0849 |
| Ileum length, cm | 29.14 | 27.86 | 25.57$^{b}$ | 31.43$^{a}$ | 1.95 | 0.2889 | 0.0180 | 0.0035 |
| SI weight, g | 52.43$^{b}$ | 56.57$^{a}$ | 50.71$^{b}$ | 58.59$^{a}$ | 3.93 | 0.0309 | 0.0029 | 0.0021 |
| SI weight, % LW | 2.00 | 2.10 | 1.96 | 2.17 | 0.19 | 0.6025 | 0.5231 | 0.7991 |
| SI length, cm | 153.5 | 146.01 | 146.7 | 152.8 | 10.19 | 0.1548 | 0.2404 | 0.4291 |

Abbreviations: CBD, cannabidiol group; LW, live weight; RMSE, root mean square error; SI, small intestine.
$^{A,B}$Values within a row with different superscripts differ significantly at $P < 0.01$.
$^{a,b}$Values within a row with different superscripts differ significantly at $P < 0.05$.

## Histological analysis

Rabbits from both the experimental groups did not evidence major signs of inflammation or alterations of the histological architecture in all the gut tract analyzed. Particularly, as regards the small intestine (Figure 1), in control and CBD groups, all the tracts analyzed were characterized, along their whole length, by numerous villi lined by simple columnar epithelium with brush border. The goblet cells were found interspersed in the columnar absorptive epithelium, with a constant average population in duodenum and jejunum and a wider presence in the ileum. The center of the lamina propria within the villi contained small blood capillaries and may evidence clear spaces, identifying the lacteals. The crypts of Lieberkühn were present in the lamina propria in all the tract analyzed.

Considering the large intestine (Figure 2), both CTRL and CBD groups were characterized by the presence of mucosal folds instead of prominent villi. The cecum presented a simple columnar epithelium with diffused goblet cells interspersed within enterocytes. The colon showed a tunica mucosa organized in higher folds compared to cecum, with highly abundant and tightly packed goblet cells.

The morphometry and the goblet cells relative abundance of duodenum, jejunum, ileum, cecum, and colon of the rabbits are pictured in Table 5. In the duodenum, goblet cells were higher ($P < 0.01$) in the CBD group. In the jejunum, the VH/CD ratio was higher in the CBD group ($P < 0.05$), while the number of goblet cells was higher in the control group ($P < 0.05$). No differences between control and CBD group were observed in the morphometry and goblet cells of ileum and cecum; instead, the number of goblet cells in the colon was higher ($P < 0.05$) in the control group.

No differences were observed between genders. The interaction was significant (P < 0.05) for only duodenal villi height and ileal CD.

**Table 4.** Large intestine macroscopic morphology of rabbits ($n$ = 16, 8 per group) according to dietary treatment and gender

| | Control | CBD | Female | Male | RMSE | *P*-values | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Group | Gender | Interaction |
| Cecum weight, g | 36.57[a] | 34.13[b] | 33.43[b] | 37.29[a] | 2.21 | 0.0421 | 0.0123 | 0.9267 |
| Cecum weigh %LW | 1.40[a] | 1.27[b] | 1.29 | 1.38 | 0.11 | 0.0262 | 0.0685 | 0.8728 |
| Cecum length, cm | 21.57 | 21.50 | 21.50 | 21.57 | 1.93 | 0.9535 | 0.9236 | 0.4548 |
| Colon weight, g | 20.86 | 19.57 | 19.86 | 20.57 | 1.96 | 0.4810 | 0.7496 | 0.3723 |
| Colon weight, %LW | 0.80[a] | 0.73[b] | 0.77 | 0.76 | 0.06 | 0.0491 | 0.9296 | 0.5357 |
| Colon length, cm | 86.07 | 84.21 | 84.07 | 86.21 | 3.54 | 0.4276 | 0.3404 | 0.7508 |
| Rectum weight, g | 2.43 | 2.57 | 2.29 | 2.71 | 0.15 | 0.3275 | 0.2305 | 0.4353 |
| Rectum weight, %LW | 0.09 | 0.09 | 0.09 | 0.10 | 0.007 | 0.9851 | 0.9643 | 0.8569 |
| Rectum length, cm | 5.64 | 6.57 | 6.29 | 5.93 | 0.45 | 0.0827 | 0.2156 | 0.5402 |
| LI weight, g | 57.43 | 58.71 | 55.57[b] | 60.57[a] | 4.76 | 0.7873 | 0.0489 | 0.8043 |
| LI weight, %LW | 2.19 | 2.18 | 2.15 | 2.24 | 0.19 | 0.8025 | 0.0628 | 0.7991 |
| LI length, cm | 113.28 | 112.29 | 118.86 | 113.71 | 5.06 | 0.7894 | 0.5365 | 0.5177 |

Abbreviations: CBD, cannabidiol group; LI, large intestine; LW, live weight; RMSE, root mean square error.
[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

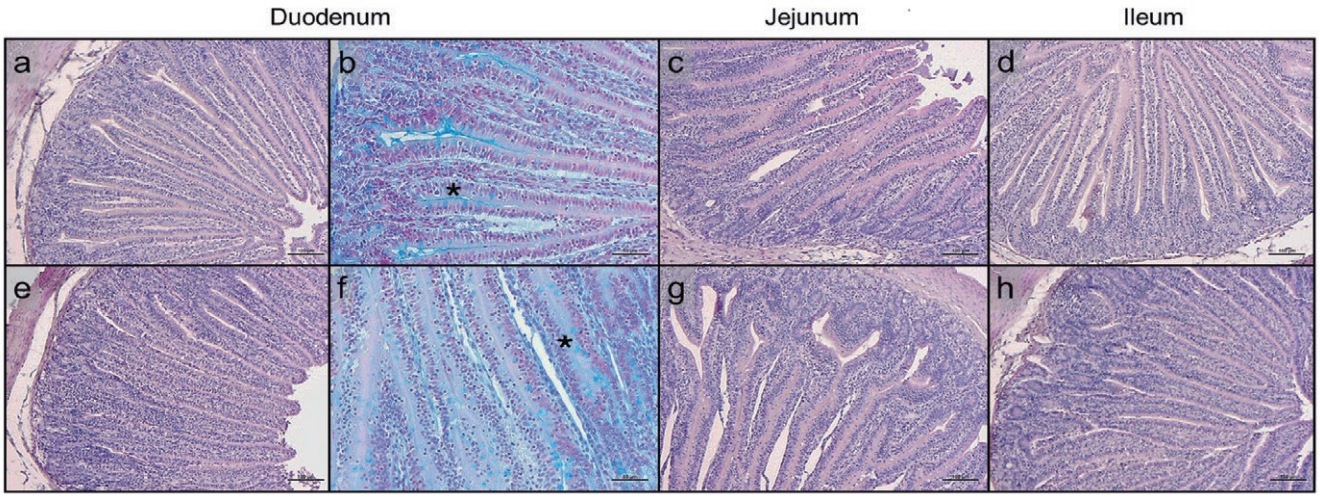


**Figure 1.** Example of histomorphology of small intestine (duodenum, jejunum, and ileum) of rabbits fed the experimental diets. (a to d) CTRL; (e to h) CBD. Scale bars: (a, c, d) and (e, g, h), 100 µm; (b) and (f), 50 µm. Asterisks indicate goblet cells in representative sections of duodenum from CTRL and CBD groups, respectively.

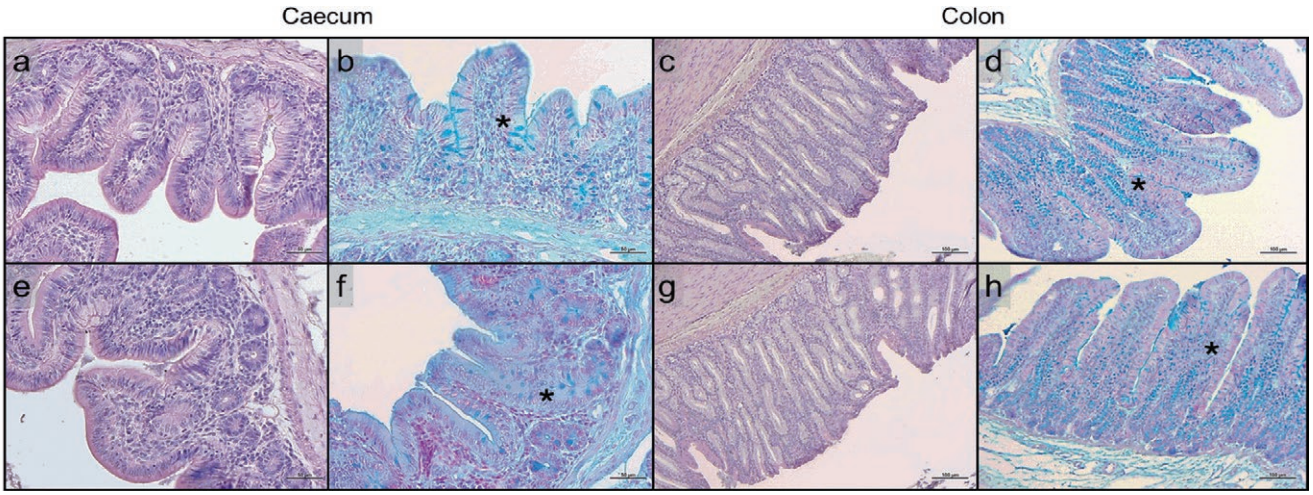


**Figure 2.** Example of histomorphology of large intestine (cecum and colon) of rabbits fed the experimental diets. (a to d) CTRL; (e to h) CBD. Scale bars: (a, b, d) and (e, f, h) 50 µm; (c) and (g) 100 µm. Asterisks indicate goblet cells in representative sections of cecum and colon from CTRL and CBD groups, respectively.

**Table 5.** Intestinal morphometry and goblet cells relative abundance (number of cells/10˙000 µm$^2$) in the different intestinal tracts of the rabbits ($n$ = 16, 8 per group) according to dietary treatment and gender

| | Control | CBD | Female | Male | RMSE | *P*-values | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Group | Gender | Interaction |
| Duodenum | | | | | | | | |
| Villi height, µm | 652.1 | 643.7 | 658.5 | 637.3 | 25.79 | 0.4212 | 0.1315 | 0.0108 |
| Crypt depth, µm | 104.0 | 99.64 | 104.4 | 99.23 | 9.12 | 0.3170 | 0.2574 | 0.9499 |
| VH/CD | 6.35 | 6.49 | 6.39 | 6.46 | 0.48 | 0.5563 | 0.7201 | 0.1927 |
| Goblet cells | 6.02$^{B}$ | 6.82$^{A}$ | 6.33 | 6.54 | 0.42 | 0.0049 | 0.1753 | 0.6011 |
| Jejunum | | | | | | | | |
| Villi height, µm | 574.1 | 572.8 | 567.9 | 579.0 | 17.38 | 0.9771 | 0.2617 | 0.8809 |
| Crypt depth, µm | 94.56 | 87.91 | 91.09 | 91.39 | 7.50 | 0.1268 | 0.8731 | 0.5519 |
| VH/CD | 6.12$^{b}$ | 6.57$^{a}$ | 6.33 | 6.36 | 0.37 | 0.0406 | 0.6218 | 0.4608 |
| Goblet cells | 7.07$^{a}$ | 6.69$^{b}$ | 7.04 | 6.72 | 0.32 | 0.0365 | 0.0596 | 0.1618 |
| Ileum | | | | | | | | |
| Villi height, µm | 529.8 | 542.9 | 541.8 | 531.0 | 21.47 | 0.3302 | 0.4508 | 0.3408 |
| Crypt depth, µm | 100.1 | 97.75 | 100.1 | 97.75 | 4.95 | 0.3344 | 0.6390 | 0.0262 |
| VH/CD | 5.33 | 5.59 | 5.44 | 5.48 | 0.31 | 0.1398 | 0.6390 | 0.1714 |
| Goblet cells | 11.35 | 10.88 | 11.20 | 11.02 | 0.62 | 0.1587 | 0.4698 | 0.4372 |
| Cecum | | | | | | | | |
| Tunica mucosa, µm | 240.9 | 258.5 | 250.9 | 248.5 | 23.08 | 0.1879 | 0.9915 | 0.7229 |
| Goblet cells | 5.82 | 6.30 | 5.99 | 6.17 | 1.02 | 0.3958 | 0.6641 | 0.5368 |
| Colon | | | | | | | | |
| Tunica mucosa, µm | 460.1 | 452.8 | 453.5 | 459.4 | 16.03 | 0.4661 | 0.5836 | 0.4876 |
| Goblet cells | 21.33$^{a}$ | 19.61$^{b}$ | 20.49 | 20.45 | 1.28 | 0.0294 | 0.6801 | 0.9160 |

Abbreviations: CBD, cannabidiol group; RMSE, root mean square error; VH/CD, villi height/crypt depth.
$^{A,B}$Values within a row with different superscripts differ significantly at $P < 0.01$.
$^{a,b}$Values within a row with different superscripts differ significantly at $P < 0.05$.

## Enzymatic analysis

The enzymatic activity evaluated in the small intestine and in the cecum is reported in Table 6. The duodenum control group showed higher activity of chymotrypsin and ALP ($P < 0.05$) than the CBD group while chymotrypsin, trypsin, L-ANP and ALP activities in the jejunum were higher ($P < 0.01$) in the control group. Also, in the ileum, saccharase, trypsin, L-ANP ($P < 0.05$), and chymotrypsin ($P < 0.01$) expressed higher activity in the control group, whilst in the cecum saccharase showed a higher activity ($P < 0.05$) in the CBD group.

The only significant effect of the sex was detected for saccharase, which activity was higher ($P < 0.05$) in females than in males in the cecum.

No effect of the interaction was observed.

**Table 6.** Digestive and brush border enzymes produced in the different intestinal tracts of the rabbits ($n$ = 16, 8 per group) according to dietary treatment and gender

| | Control | CBD | Female | Male | RMSE | *P*-values | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Group | Gender | Interaction |
| Duodenum | | | | | | | | |
| Saccharase, µm/min/g | 2480.3 | 1977.2 | 2520.3 | 2252.6 | 635.1 | 0.0525 | 0.3455 | 0.6036 |
| Maltase, µm/min/g | 7361 | 4790 | 6763 | 5113 | 2440.9 | 0.0772 | 0.1760 | 0.9533 |
| Chymotrypsin, µm/min/g | 291.80[a] | 245.42[b] | 267.40 | 270.3 | 39.39 | 0.0498 | 0.7578 | 0.1356 |
| Trypsin, µm/min/g | 315.58 | 269.23 | 290.06 | 295.69 | 40.31 | 0.0553 | 0.8487 | 0.1356 |
| L-ANP, µm/min/g | 2813.6 | 1513.8 | 2636.3 | 1691.1 | 1033.5 | 0.0601 | 0.1518 | 0.6541 |
| ALP, µm/min/g | 71.20[a] | 31.77[b] | 63.64 | 39.34 | 20.22 | 0.0225 | 0.1205 | 0.6405 |
| Jejunum | | | | | | | | |
| Saccharase, µm/min/g | 2622.7 | 3145.9 | 2692.5 | 3161.9 | 558.6 | 0.4403 | 0.5686 | 0.0972 |
| Maltase, µm/min/g | 6981.5 | 5633.2 | 6257.1 | 6357.1 | 1127.2 | 0.0936 | 0.8918 | 0.3474 |
| Chymotrypsin, µm/min/g | 306.89[A] | 241.35[B] | 254.22 | 294.02 | 24.27 | 0.0042 | 0.2112 | 0.1255 |
| Trypsin, µm/min/g | 331.13[A] | 242.09[B] | 268.44 | 299.59 | 21.24 | 0.0002 | 0.2062 | 0.2009 |
| L-ANP, µm/min/g | 4480.8[A] | 2315.0[B] | 2864.1 | 4145.2 | 731.97 | 0.0008 | 0.0857 | 0.1180 |
| ALP, µm/min/g | 99.82[A] | 49.41[B] | 65.56 | 81.08 | 22.05 | 0.0076 | 0.5595 | 0.2721 |
| Ileum | | | | | | | | |
| Saccharase, µm/min/g | 1642.0[a] | 754.0[b] | 771.7 | 1502.5 | 600.7 | 0.0479 | 0.1432 | 0.4908 |
| Maltase, µm/min/g | 3514.3 | 2903.8 | 3050.1 | 3322.6 | 1227.6 | 0.0579 | 0.8060 | 0.4751 |
| Chymotrypsin, µm/min/g | 292.5[A] | 226.4[B] | 254.0 | 264.9 | 28.92 | 0.0031 | 0.5803 | 0.8871 |
| Trypsin, µm/min/g | 302.66[a] | 258.93[b] | 283.99 | 277.61 | 30.25 | 0.0432 | 0.7385 | 0.3627 |
| L-ANP, µm/min/g | 4460.0[a] | 2648.1[b] | 3526.7 | 3592.2 | 1011.3 | 0.0222 | 0.7460 | 0.1917 |
| ALP, µm/min/g | 53.37 | 33.14 | 43.99 | 43.12 | 11.34 | 0.0663 | 0.5002 | 0.8752 |
| Cecum | | | | | | | | |
| Saccharase, µm/min/g | 430.2[b] | 1058.0[a] | 987.7[a] | 403.1[b] | 638.7 | 0.0345 | 0.0483 | 0.4274 |
| Maltase, µm/min/g | 1061.1 | 377.3 | 1059.1 | 379.4 | 1231.5 | 0.3644 | 0.3671 | 0.3479 |
| Chymotrypsin, µm/min/g | 260.62 | 167.73 | 188.15 | 181.01 | 31.34 | 0.0536 | 0.4790 | 0.3603 |
| Trypsin, µm/min/g | 221.68 | 181.63 | 203.37 | 199.26 | 30.71 | 0.0602 | 0.5763 | 0.5726 |
| L-ANP, µm/min/g | 101.2 | 73.81 | 95.25 | 79.76 | 23.78 | 0.0812 | 0.2922 | 0.6567 |
| ALP, µm/min/g | 10.20 | 6.60 | 9.40 | 7.42 | 3.02 | 0.0723 | 0.2926 | 0.3169 |

Abbreviations: ALP, alkaline phosphatase; CBD, cannabidiol group; L-ANP, L-aminopeptidase; RMSE, root mean square error.
[A,B]Values within a row with different superscripts differ significantly at $P < 0.01$.
[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

## Caecal pH and SCFAs profile

The caecal pH and SCFAs profile of caecal contents are reported in Table 7. The control group had a lower caecal pH ($P < 0.01$) than the CBD while, acetate, propionate, butyrate, and isobutyrate were significantly higher in the control group when expressed in mmol/L. Thus, the total SCFAs resulted higher ($P < 0.01$) in the control than the CBD group. When the SCFAs were expressed as percentage of the total measured SCFAs, the acetate was not different between the groups, while the propionate, isobutyrate, isovalerianic, and valerianic acids were higher ($P < 0.01$) in the CBD group. Only butyrate showed higher ($P < 0.01$) percentages in the control group.

Gender only affected valerianic acid expressed in % of total SCFAs ($P < 0.05$), resulting higher in males than in females.

No effects of the interaction were observed for the data reported in Table 7.

## Effect of the interaction between diet and gender

The only significant effects of the interaction between the tested factors are reported in Table 8. CBD administration increased ($P < 0.01$) ileum length in females compared to the males, while no significant effects were detected between males and females of the control group. The weight of the entire SI increased in males of the CBD group compared to females ($P < 0.01$), while no differences were detected between the genders in the control group. The villi height in the duodenum of females fed CBD was higher than the males, while the opposite happened in the control group ($P < 0.05$). The CD in the ileum was minimized in males fed CBD ($P < 0.05$).

**Table 7.** Caecal pH and short-chain fatty acids (SCFAs) concentration (mmol/L and % of total SCFAs) in the caecal content of the rabbits (n = 16, 8 per group) according to dietary treatment and gender

| | Control | CBD | Female | Male | RMSE | *P*-values | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Group | Gender | Interaction |
| Ceacal pH | 6.57$^{B}$ | 6.94$^{A}$ | 6.73 | 6.79 | 0.19 | 0.0045 | 0.6877 | 0.9286 |
| Acetate, mmol/L | 29.64$^{A}$ | 19.45$^{B}$ | 25.91 | 23.17 | 3.77 | 0.0018 | 0.1274 | 0.2687 |
| Propionate, mmol/L | 2.97$^{a}$ | 2.40$^{b}$ | 2.86 | 2.51 | 0.31 | 0.0272 | 0.1083 | 0.0744 |
| Isobyturate, mmol/L | 0.13$^{B}$ | 0.20$^{A}$ | 0.18 | 0.15 | 0.032 | 0.0074 | 0.4359 | 0.8984 |
| Butyrate, mmol/L | 7.97$^{A}$ | 3.33$^{B}$ | 5.62 | 5.69 | 0.87 | 0.0009 | 0.5626 | 0.2985 |
| Isovalerianic, mmol/L | 0.18 | 0.24 | 0.22 | 0.21 | 0.05 | 0.0817 | 0.8403 | 0.4796 |
| Valerianic, mmol/L | 0.63 | 0.49 | 0.55 | 0.58 | 0.09 | 0.0630 | 0.8839 | 0.4892 |
| Total SCFAs | 41.52$^{A}$ | 26.13$^{B}$ | 35.35 | 32.30 | 3.38 | 0.0001 | 0.1520 | 0.1942 |
| Acetate, % tSCFAs | 71.41 | 74.31 | 73.99 | 71.73 | 3.05 | 0.1427 | 0.2823 | 0.3411 |
| Propionate, % tSCFAs | 7.14$^{B}$ | 9.19$^{A}$ | 8.28 | 8.05 | 0.71 | 0.0003 | 0.8730 | 0.0661 |
| Isobyturate, % tSCFAs | 0.31$^{B}$ | 0.81$^{A}$ | 0.58 | 0.54 | 0.20 | 0.0012 | 0.8205 | 0.9797 |
| Butyrate, % tSCFAs | 19.12$^{A}$ | 12.84$^{B}$ | 14.85 | 17.11 | 3.23 | 0.0059 | 0.4442 | 0.6445 |
| Isovalerianic, % tSCFAs | 0.45$^{B}$ | 0.97$^{A}$ | 0.69 | 0.73 | 0.27 | 0.0043 | 0.4661 | 0.6275 |
| Valerianic, % tSCFAs | 1.56$^{B}$ | 1.89$^{A}$ | 1.61$^{b}$ | 1.83$^{a}$ | 0.22 | 0.0097 | 0.0364 | 0.6895 |

Abbreviations: CBD, cannabidiol group; RMSE, root mean square error; tSCFAs, total short chain fatty acids.
$^{A,B}$Values within a row with different superscripts differ significantly at $P < 0.01$.
$^{a,b}$Values within a row with different superscripts differ significantly at $P < 0.05$.

**Table 8.** Effect of the interaction between dietary treatment and gender

| Group | Gender | Ileum length, cm | SI weight, g | VH duodenum | CD ileum |
|---|---|---|---|---|---|
| Control | F | 29.3$^{B}$ | 52.7$^{B}$ | 640.3$^{b}$ | 97.7$^{a}$ |
| Control | M | 29.0$^{B}$ | 52.3$^{B}$ | 660.9$^{a}$ | 101.9$^{a}$ |
| CBD | F | 33.0$^{A}$ | 49.3$^{B}$ | 672.2$^{a}$ | 101.9$^{a}$ |
| CBD | M | 21.2$^{C}$ | 66.3$^{A}$ | 605.8$^{b}$ | 92.19$^{b}$ |
| RMSE | | 1.95 | 3.93 | 25.79 | 4.95 |
| *P*-value | | 0.0035 | 0.0021 | 0.0108 | 0.0262 |

Abbreviations: CBD, cannabidiol group; CD, crypt depth; RMSE, root mean square error; SI, small intestine; VH, villi height.
$^{A,B}$Values within a row with different superscripts differ significantly at $P < 0.01$.
$^{a,b}$Values within a row with different superscripts differ significantly at $P < 0.05$.

## Discussion

### Effect of cannabidiol

Recently, the European Commission stated that CBD can be considered as a novel food and, currently, 19 applications are under assessment at EFSA (EFSA, 2022). Importantly, the EFSA's expert panel observed that, among others, data on the effect of CBD on the gastrointestinal tract are scarce.

Standing to our knowledge this is the first research in which an in-depth study on the effects of CBD on intestinal morphology, morphometry, enzymatic and bacterial activities has been performed on healthy farmed animals.

In the present study, the use of cannabidiol at 10 mg/d in a late-growing stage of meat rabbits, from 65 to 92 d of age, did not modify feed intake and final weight but it was able to affect several aspects of morphology and morphometry as well as of microbial and enzymatic activity in the gastrointestinal tract. This is an interesting point, since our results at least suggest that some effects of cannabidiol administration on intestinal health of rabbits may occur in case of higher doses and or longer administration.

The main effect of the CBD administration on intestinal histomorphology was the relative abundance of goblet cells in the duodenum and their parallel decrease in the jejunum and colon, compared to the control group. Goblet cells are considered the major line of defense of the intestinal mucosa, through the production of mucus (Birchenough et al., 2015). However, their mode of action is different in the diverse intestinal tracts: i) in the small intestine they are adjacent to the enterocytes and supply the bicarbonate for proper mucin unfolding (Gustafson et al., 2012); ii) in the large intestine they might supply bicarbonate via its own bestrophin-2 bicarbonate transporter (Yu et al., 2010). Another interesting aspect is that goblet cells are under the direct regulation of the immune system (Oeser et al., 2015) since their number and activity increase during inflammatory intestinal conditions. In the present study, the number of goblet cells, at least for the cecum and colon (the only data we can find in the literature), was in line with the findings of other authors in rabbit (Grant and Specian, 2001; Ranjan and Das, 2021). In the small intestine, the role of the mucus produced by goblet cells is to protect the epithelium by trapping the chyme containing gastric and pancreatic digestive enzymes. Differently, in the colon, the mucus is very important to protect the epithelium from the microbiota covering the fecal matter (Gustafsson and Johansson, 2022) and, in rabbit, also the caecotrophs. The increased number of goblet cells in the duodenum may be suggestive of a higher needing to protect the small intestine from gastric secretions, but such hypothesis should be apparently in contrast with research in human and other animal species, in which CBD has been shown to reduce gastric acid secretions and motility (Gyires and Zádori, 2016).

Thus, because of the scarce literature available on the use of CBD in farmed animals and its effect on the digestive system,

the effect of CBD on gastric secretion should be deeply investigated in rabbits, since an increase could limit its potential use in this species.

The small intestine morphometry was also modified by CBD with an increase in VH/CD ratio in the jejunum, attributable to a reduction in the depth of the crypts (−7.03%) in comparison to the control, rather than to the difference in the VH (−0.23% in the CBD group). The VH/CD ratio is considered a powerful indicator of the absorptive ability of the digestive system (Addeo et al., 2022): in fact, a higher VH indicates an extended absorptive surface, whilst a shallow CD is usually associated with a slow turnover of the secretory cells from the intestinal crypt, which shows an efficient absorption of nutrients (Marcos et al., 2019). The jejunum, together with ileum, is the site of nutrient absorption from feed or caecotrophs in rabbits (Smith, 2020). For this reason, better conditions of absorptive areas, like those observed in CBD group, can have a positive impact on digestive health.

The enzymatic activity registered in the different tracts of the small intestine indicated a general reduced activity of the enzymes involved in peptide digestion such as L-ANP, chymotrypsin, and trypsin in the small intestine due to the administration of CBD. However, the reduced protease activity in our trial did not affect the final weight of the animals. Two are the possible explanations for this result: i) the late-growing stage (65 to 92 d of age) in which the CBD has been used, when the growth rate of rabbits slows down compared to previous periods (Birolo et al., 2022) and then also the relative protein requirement is reduced; ii) the length of the CBD treatment (27 d). The CBD treatment stimulates the saccharase activity that is more pronounced in the cecum than in the small intestine while the other enzyme activities exhibit similar values along the small intestine of both groups. The reduced activity of ALP in all the tracts of the small intestine suggests a modified integrity of the intestinal barrier in the rabbits fed CBD. This observation aligns with histological findings related to goblet cells relative abundance, which are considered a primary line of defense of the intestinal mucosa. ALP also plays a significant role in maintaining the intestinal barrier function (Santos et al., 2022). The decrease in intestinal ALP activity following CBD treatment may have been affected by the increased abundance of goblet cells. This relative abundance could potentially suppress ALP activity, as goblet cells are vital for mucosal defense and can alter the local microenvironment. This interaction is consistent with findings by Larrick and Mendelsohn (2020), who reported decreased ALP activity with compromised intestinal epithelial barrier integrity in mice, although they did not examine goblet cell abundance. Furthermore, Santos et al. (2022) reviewed several studies showing that gut-associated inflammation could be prevented by oral, intravenous, or via duodenal catheter of ALP supplementation. Nevertheless, further investigations are needed to elucidate the relationship between ALP activity in the context of CBD treatment.

The CBD strongly reduced the production of SCFAs in the cecum (modifying also the pH inside this organ) and this is suggestive for the reduction of the fermentative activity of the caecal microbiota, considering that SCFAs are the end products of fermentation of dietary fibers by the anaerobic intestinal microbiota (den Besten et al., 2013). Indeed, as clearly shown by our results, not only the amounts of the single SCFA were affected by the dietary CBD administration, but also their relative proportion on the total SCFAs amount. In this regard, the proportion of acetate, coming from the fermentation of structural carbohydrates, did not vary between the groups, despite its highest absolute value in the control group. Differently, the proportion of both butyric and propionic acids was reduced and increased, respectively, in the CBD group. Unfortunately, standing to our knowledge, no other research available in literature focused attention on SCFAs production due to the administration of CDB, then further insights are necessary to better understand this scenario. However, the butyrate, also coming from the fermentation of structural carbohydrates, plays a key role in the health of digestive system of rabbits, being the second SCFA produced by bacterial fermentation after the acetate. Butyric acid is considered the main enterocytes' energy source (Bovera et al., 2010) and it is also required for the proper development of the Gut-Associated Lymphoid Tissue (Mroz, 2005). Li et al. (2020) used sodium butyrate at 0.5% in rabbit diets as a replacement to antibiotics, founding that this dose was able to improve the intestinal health by regulating both the physical and immune barriers, as well as the intestinal microbiota. As a consequence, higher proportions of butyrate can help to maintain a better health status of the rabbit's digestive system. A decreased proportion of butyrate in CBD group could be attributed to a lower fermentative activity of butyrate-producing bacteria; similarly, the increase in propionate % on total SCFAs is suggestive of an increased activity of propionate-producing bacteria. The reduced fermentative activity can be due to a reduced number of bacteria, to a reduced enzymatic activity, or to a reduced amount of fermentable substrate availability. In this research we are not able to identify the specific effects of CBD on butyrate and propionate production in rabbit caecal content; in addition, standing our knowledge, no further studies are available in literature on this topic. Considering the lower relative abundance of goblet cells measured in the colon of rabbits fed CBD, it could be hypothesized a reduced number of bacteria in the caeco-colic tract, and a consequently lower number of these cells required to protect the intestinal epithelium (Gustafsson and Johansson, 2022).

Looking at the branched-chain fatty acids (**BCFA**), the sum of the isobutyric, isovaleric, and valeric acids amounts, produced in the cecum in both groups, was very similar (0.94 mmol/L vs. 0.93 mmol/L, respectively for control and CBD group) but the sum of their proportion on total SCFAs was strongly different (2.32% vs. 3.367%, respectively, for CONT and CBD group). BCFAs derive from the fermentation of some amino acids such as valine, leucine, and isoleucine. For that reason, a higher proportion of the BCFA in the SCFAs profile of rabbit caeca is suggestive of a greater fermentation activity on the amino acid substrate (Addeo et al., 2022). However, the similar absolute values of the BCFA in control and CBD groups indicated a similar fermentation of the amino acids in both groups, and the high proportion was the result of a reduction of the other fermentative activities, particularly that of acetate, propionate, and butyrate-producing bacteria.

Within the possible mechanisms for the changes in caecal fermentation parameters,

we hypothesize a change in intestinal motility, through a mechanism called "chymus jam" pathway (van der Sluis et al, 2024). Stress and stimulation of the sympathetic nervous system are reported to negatively affect normal functioning of the *fusus coli*, thereby slowing down peristalsis and inhibiting gut motility and digestion (Rees Davies and Rees Davies, 2003). Reduced

gut motility has been suggested to result in a longer retention of digesta in the cecum (Oglesbee and Lord 2020), filled by the suppling of nutrients from the ileum and by the action of the *fusus coli* that continuously sends back digesta (van der Sluis et al, 2024). This results in a prolonged retention of digesta in the cecum that induces a higher caecal pH, and lower acetate and higher butyrate levels (Gidenne et al., 2015). In particular, a lower but longer over time entrance of the digesta from ileum in the cecum could be responsible for a lower but longer over time SCFAs production. SCFAs levels in cecum and in blood are considered as trigger factors in the initiation of the caecotrophy cycle (Carabaño and Piquer, 1998). Thus, persistence of SCFAs production can induce an accumulation of digesta from colon in the cecum that could explain a lower production of SCFAs and a higher proportion of BCFA in the CBD group considering that digesta moving from the colon to cecum have low crude fiber but high protein content (Carabaño et al., 2010).

Another hypothesis is the difference in substrate availability. With a low digestion of protein in the small intestine it is reasonable to assume that more protein reached the cecum to be fermented, leading to an increased caecal $NH_3$ and pH levels (Morisse et al. 1985). In this regard, it is interesting to note that Tazzoli et al. (2013) suggested that high caecal protein contents could benefit the entire microbial population, but also some of the pathogenic strains to a higher extent.

Several researches on animal models (mainly mice) indicated an effect of cannabinoids in reducing gastrointestinal motility (Brierley et al., 2023). On the other hand, Capasso et al. (2009), reported the absence of effects on gut motility in mice after cannabidiol exposure. Indeed, based on our knowledge, our results represent the first report on the effect of CBD on the gastrointestinal tract of healthy rabbits.

### Effect of gender

Male rabbits ingested a high amount of feed and had a higher live weight at slaughter than females, according to the results by Blasco and Gómez (1993) who observed that male rabbits were, in general, heavier than females.

Very few data are available in the literature on the effect of gender on intestinal morphology, morphometry, and enzymatic activity. In our research, males had higher weight of duodenum and higher length of the ileum. In both cases, the differences between the sexes were detected when the data were expressed both as absolute values and as percentage of the live weight. Regarding the fermentative and enzymatic activities in the cecum, the only valerianic acid % of total SCFAs was higher in males and saccharase higher in females.

The effect of the interaction between the 2 tested factors was significant for few morphological and morphometric traits of the only small intestine. In general, CBD may be able to affect the length of the ileum in both sexes.

The CBD also reduced villi height and CD, respectively in the duodenum and ileum, but, in both cases, no significant effect of the interaction was observed in the VH/CD, thus suggesting a good surface absorption condition.

### Limitations and future directions of this study

Our research reports for the first time data on the effects of CBD on the intestine of rabbits used for meat production. However, we must point out a couple of limitations: firstly, the number of animals tested for each group (8). Even if it was assessed as effective by statistical tests, certainly a greater number of samples could allow us to highlight differences that did not emerge in this preliminary research. Another point concerns the timing of CBD administration. As preliminary research, we chose to administer CDB to rabbits with a mature digestive system for 27 d at the dose indicated by the producer, but obviously different choices could be made. Thus, different dosages as well as different therapeutic protocols, such as longer administration with suspension times, should be tested. Despite its limitations, we believe that this work paves the way for further research that aims to validate these preliminary results, using more animals and of different ages, in order to have a more complete picture and better understand the mechanisms underlying the results obtained.

## Conclusion

The administration of cannabidiol at 10 mg/d/head in growing rabbits for a relatively long period (27 d, from 65 to 92 d old) did not affect feed intake and the final rabbits' whole body weight. Despite that, some changes were detected in the gastrointestinal tract of the animals. CBD seemed to interfere with protein digestion, with a significant lower of activity of the enzymes related to peptides in the small intestine, with a consequent increase of in fermentative activity of caecal microbiota on these molecules. This effect, in combination with a general decrease of fermentative activity in the caecal content of rabbits submitted to CBD treatment, was responsible for a change in the SCFAs proportion mainly regarding the reduction of butyrate production, which is very important for the intestinal health. Such modification in the fermentation activity was coupled with changes in the relative abundance of goblet cells in the colon. Due to the scarcity of studies on this topic, further research is necessary to clarify the impact of CBD on the gastrointestinal tract of rabbits. In particular, different dosages as well as different therapeutic protocols (longer administration including suspension times) should be assessed. Indeed, to our knowledge, this is the first in-depth study on this topic. Overall, our findings suggest that a relatively long-term administration of CBD in rabbits may affect digestion in rabbits, in particular at enzymes and fermentative levels.

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## Conflict of interest statement

The authors declare no conflict of interest.

## Author contributions

Nadia Musco (Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Writing—review & editing), Giulia Pascon (Data curation, Investigation), Nicola Francesco Addeo (Investigation), Matteo Zarantoniello (Data curation, Investigation, Writing—review & editing), Mariarosaria Lanzieri (Investigation), Ike Olivotto (Supervision, Writing—review & editing), Francesca Tulli (Supervision, Writing—review & editing), Valeria Iervolino (Investigation), Ruggero Amato (Conceptualization), Pietro Lombardi (Validation, Writing—review & editing), and Fulvia Bovera (Conceptualization, Formal analysis, Methodology, Project administration, Supervision, Writing—original draft)

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