

Noradrenergic, galaninergetic and nitrergic innervation of the jejunum of fallow deer (*Dama dama*) fed with basket willow (*Salix viminalis*)

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Summary

In recent years, there has been growing interest in exploring the potential health benefits of incorporating natural plant substances into the diets of farm animals. Basket willow (*Salix viminalis*), rich in flavonoids, tannins, and salicylates, has emerged as a candidate with analgesic, anti-inflammatory, and anthelmintic properties, making it appealing for integration into ruminant farming practices. This study aimed to analyze the effects of dietary supplementation with basket willow on the neuroanatomical properties of the small intestine in farmed fallow deer. Immunohistochemical methods and morphometric measurements were employed to assess changes in noradrenergic, galaninergetic and nitrergic innervation patterns. Twenty-four fallow deer were divided randomly, with 12 individuals placed in both a control group and an experimental group, with the latter receiving willow supplementation. The results revealed significant alterations in the morphology and innervation patterns of the enteric nervous system (ENS) in the experimental group, including a decrease in ganglion size and nitric oxide synthase (NOS) immunoreactive nerve fibers, and an increase in galanin immunoreactive neurons. These findings suggest a potential modulation of gastrointestinal function by willow supplementation. However, caution is warranted due to the high salicylate content in basket willow, which may pose risks to gastrointestinal integrity. Overall, while basket willow supplementation shows promise in enhancing animal farming methods, further research is needed to elucidate its mechanisms and optimize its use in ruminant farming practices.

Keywords: ruminants, salicylates, enteric nervous system, fallow deer, basket willow

In recent years, there has been growing interest in exploring the potential health benefits of incorporating natural plant substances into the diets of farm animals (34). Among these, basket willow (*Salix viminalis*) has attracted significant attention. Basket willow leaves and twigs contain a diverse range of chemical compounds, including flavonoids, tannins, and salicylates, which have been associated with both

beneficial and adverse effects on animals (9, 43, 44). Notably, basket willow is known for its analgesic, anti-inflammatory, antiviral properties, making it an intriguing candidate for integration into ruminant farming practices (30, 34, 44). Additionally, its anthelmintic activity has been demonstrated, offering a potential solution to internal parasite issues in pastured animals (9).

One of the natural and ecological ways to introduce such supplements into the diet of farm animals is the silvopastoral system. This system provides essential benefits like food provision during challenging conditions (e.g., droughts), shelter, and shade to improve animal welfare and performance (13, 19). Additionally, it promotes ecological benefits such as soil conservation, enhanced soil quality, and water retention (13). Basket willow, due to its fast growth, high germination capacity, short rotation cycle, and resilience to pests and diseases, stands out as an ideal candidate for such systems (15, 33, 43). However, despite the economic and environmental advantages of incorporating basket willow into animal husbandry, gaps persist in understanding its effects on animal health. Particularly, the impact on the enteric nervous system (ENS) of fallow deer remains largely unexplored. The ENS is a sophisticated neural network responsible for orchestrating various gastrointestinal functions, including peristalsis, secretion, absorption, and blood flow regulation (8, 12). Given its pivotal role in maintaining animal health and well-being, any disturbances in ENS function can lead to a spectrum of intestinal disorders (24, 26, 40).

The growing interest in fallow deer production, particularly in Central Europe, highlights the economic viability and quality of fallow deer meat (2, 6, 16, 39). Renowned for its low fat and cholesterol content, high protein levels, and significant energy value, fallow deer meat is highly sought-after (16, 21). Understanding the ENS of fallow deer could lead to effective strategies to optimize gut health and in consequence enhance meat production. As fallow deer are ruminants, insights from this research could also non-directly benefit the breeding of other domestic cattle and ruminants.

The primary objective of this study is to analyze and evaluate potential changes in the nitrergic, galaninergic, and noradrenergic innervation patterns of the small intestine in farmed fallow deer following dietary supplementation with basket willow, employing immunohistochemical methods and classical morphometric measurements. We hypothesize that the inclusion of basket willow in the diet of farmed fallow deer will precipitate alterations in small intestine innervation and anticipate observing changes in the density and distribution of neural components within the ENS.

Tab. 1. Primary and secondary antibodies used in the study

Antibody	Host	Code	Dilution	Source
Primary antibody				
Anti-NOS	rabbit	160870	1:200	Cayman Chemical
Anti-Gal	rabbit	PA5-95500	1:1000	Invitrogen
Anti-TH	mouse	T2928	1:6000	Sigma-Aldrich
Secondary antibody				
Anti-mouse/Anti-rabbit	goat	DPVB-HRP	RTU*	ImmunoLogic

Explanation: *RTU = Ready To Use

Material and methods

The study was approved by the Local Ethics Committee (Resolution No. 24/2021). The experiment was conducted on male farm fallow deer at the Research Station of the Institute of Parasitology of the Polish Academy of Sciences in Kosewo Górne. Animals were randomly divided into two groups: control (n = 12) and experimental (n = 12). At the age of 9 months, the animals were grazed on a rotation basis with standard farm nutrition during winter seasons, and pasture green during summer seasons, both *ad libitum* (42). Animals from experimental group were additionally receiving chips of young willow shoots during winter seasons, and had an access to feeding basket willow (*Salix viminalis*) plots contiguous to the pasture area during summer seasons. Fallow deer were also granted unrestricted access to water and multi-component mineral licks Josera Phosphoreimer (Josera, Polska) throughout the year (41). Six individuals (21 months old) from both the control and experimental groups were randomly selected, fasted for 18 h and slaughtered at the local slaughterhouse.

Immediately after the slaughter, 2.5 cm segments of the jejunum were collected from each fallow deer. The gathered sections were rinsed with a physiological saline solution, fixed in 4% buffered formaldehyde (pH 7.0) for 24 h, then rinsed with tap water for 8 h. The samples were dehydrated in ascending ethanol series, cleared in xylene and embedded in paraffin at 60°C, overnight. Fixed cross-sections of the jejunum were then paraffin-embedded utilizing the modular embedding machine (MYR EC-350, Casa Álvarez Material Científico SA, Madrid, Spain), and sliced into 5 µm thick sections with a rotary microtome (HM 360, Microm, Walldorf, Germany). Every fourth section was placed on SuperFrost® Plus slides (Thermo Scientific, Menzel-Glaser, Braunschweig, Germany), and kept in an incubator (CG Wamed, Warsaw, Poland) at 37°C for 12 h.

Immunohistochemistry (IHC) analysis was conducted according to the protocol described by Osiak-Wicha et al. (27) with the additional steps of dewaxing the slides in xylene and rehydrating them in a descending concentration of ethanol. In short, the first day of IHC analysis included heat-induced epitope retrieval step in the sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH = 6.0) for 8 min at 80°C (multicooker RMC-PM381-E Redmond, China), incubation in 3% H₂O₂ for 10 min at room temperature (RT), incubation in UltraVision Protein Block (Thermo Scientific, Waltham, MA, USA) for 5 min at RT, and overnight incubation in 4°C with primary antibodies directed against nitric oxide synthase (NOS), galanin (Gal), and tyrosine hydroxylase (TH) (Tab. 1), diluted in antibody diluent (Emerald, Cell Marque Corp., Rocklin, CA, USA). Each section was incubated with only one primary antibody. On the second day sections were incubated with BrightVision two-step detection system of poly-HRP-anti Ms/Rb IgG (ImmunoLogic WellMed B.V., Duiven, Netherlands, Tab. 1) for 30 min at RT, and immunolabeling was visualized with 3,3'-diaminobenzidine (DAB substrate kit, ab64238, Abcam, Cambridge, UK). Phosphate-buffered saline (PBS, 0.1 M, pH = 7.3) was used to wash slides between each step. Following the DAB exposure, sections were rinsed with

distilled water, counterstained with Meyer's hematoxylin (Patho, Mar-Four, Konstantynów Łódzki, Poland), rinsed with tap water for 10 minutes, dehydrated with ascending concentrations of ethanol, cleared with xylene, cover-slipped with Shandon Consul-Mount (Thermo Scientific, Waltham, MA, US), and dried in the incubator (CG Wamed, Warsaw, Poland) at 37°C for 12 h. The specificity of the antibodies was tested both by a negative control, in which primary antibodies were replaced by the antibody diluent, and by the pre-absorption experiment in which primary antibodies were mixed with an excess of target synthetic protein before incubation. No positive immunoreaction was detected in any of the control sections.

Sections were viewed with light microscope (BX-51 DSU, Olympus, Tokyo, Japan) equipped with a digital color camera (DP-70, Olympus, Tokyo, Japan) and digital high-resolution photographs were captured using Cell^M 2.3 software (Olympus cellSens Standard) under consistent lighting conditions and settings for brightness and contrast by one person.

A minimum of 100 myenteric and submucosal neurons per animal were included in the analysis. The following parameters of myenteric and submucosal ganglia were measured: ganglion width, length, and surface area, as well as the surface area of immunoreactive (IR) nerve fibers within the intestine layers and ganglia. Additionally, the diameter and surface area of IR neurons were measured, along with the number of IR neurons present. The surface area of nerve fibers immunoreactive to NOS, TH, and Gal were calculated and expressed in micrometers relative to 100 micrometers of intestine layer and ganglion. The area occupied by NOS-IR, TH-IR, and Gal-IR neurons was quantified as a percentage of the total ganglion area. Furthermore, the number of NOS-IR, TH-IR, and Gal-IR neurons was expressed as a percentage of the total number of 100 neurons identified in the submucosal and muscular layers of each specimen.

Mean values and standard errors were utilized to present the findings, with comparisons made between the control and experimental groups. The normality of the data distribution was assessed using the Shapiro-Wilk test. Datasets concerning ganglion morphology were assessed using Student's t-test, while datasets concerning noradrenergic, cholinergic and nitrergic innervation were evaluated using one-way ANOVA, with Bonferroni's correction. In cases where the data did not follow a normal distribution, the Mann-Whitney U test, with continuity correction, was employed to compare means. For all tests, a p-value of < 0.05 was established as statistically significant. All calculations and graphs were made using GraphPad Prism ver. 9.5.1 for Windows (GraphPad Software, San Diego, CA, USA).

Results and discussion

In the small intestine of animals fed with basket willow a significant decrease in the width of the myenteric ganglions (MG) ($p < 0.05$; Fig. 1A) and area of the MG ($p < 0.001$; Fig. 1C) was observed in the experimental group compared to the control group. A significant decrease in NOS-immunoreactive (IR) nerve fibers area of tunica muscularis was observed compared to the control group ($p < 0.001$; Fig. 1D; Fig. 2A-B). Conversely, a significant increase in Gal-IR nerve fibers area of tunica muscularis was observed in the experimental group of animals compared to the control group ($p < 0.001$; Fig. 1D). A significant increase in the area occupied by Gal-IR neurons ($p < 0.001$; Fig. 1G) and the Gal-IR neurons ($p < 0.001$; Fig. 1H; Fig. 2E-F) in MG was noticed in the experimental group compared to the control group. With regard to the submucosal ganglions (SG), there was a noticeable decrease in the width ($p < 0.05$; Fig. 3A), length ($p < 0.05$; Fig. 3B) and area ($p < 0.05$; Fig. 3C) in the experimental group compared to the control group.

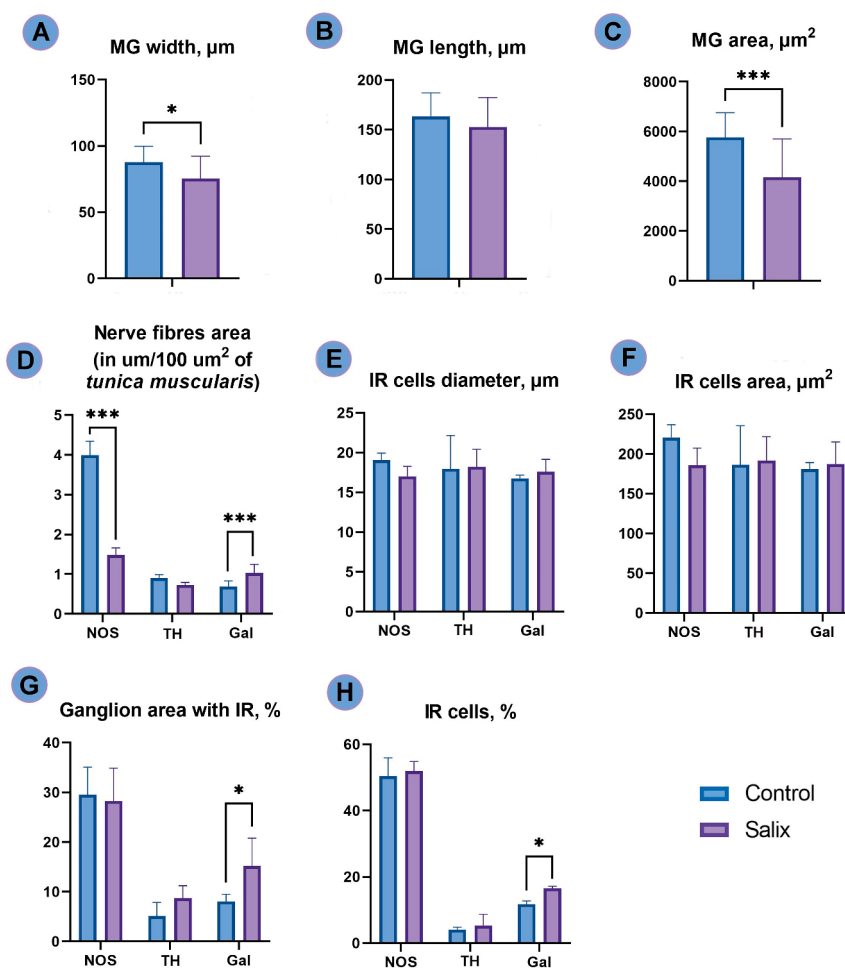


Fig. 1. The effect of basket willow feeding on the morphology of the Myenteric Ganglion (MG) and the immunoexpression of nitric oxide synthase (NOS), tyrosine hydroxylase (TH) and galanin (Gal) in fallow deer: (A) myenteric ganglion width, (B) myenteric ganglion length, (C) myenteric ganglion area, (D) nerve fibres area, (E) immunoreactive (IR) cells diameter, (F) immunoreactive cells area, (G) ganglion area, (H) immunoreactive cells percentage. Explanations: Asterisks (*) indicate significant differences between control and experimental groups (* $p < 0.05$; *** $p < 0.001$).

A significant decrease in NOS-IR nerve fibers area of submucosa was also observed in experimental group compared to the control group ($p < 0.001$; Fig. 3D). Additionally, a significant increase in TH-IR cells ($p < 0.05$; Fig. 3E; Fig. 2C-D) and area of

TH-IR cells ($p < 0.05$; Fig. 3F) was observed in the experimental group of animals compared to the control group. A significant increase in the experimental group was also observed in the case of ganglion area occupied by NOS-IR and Gal-IR cells ($p < 0.05$; Fig. 3G

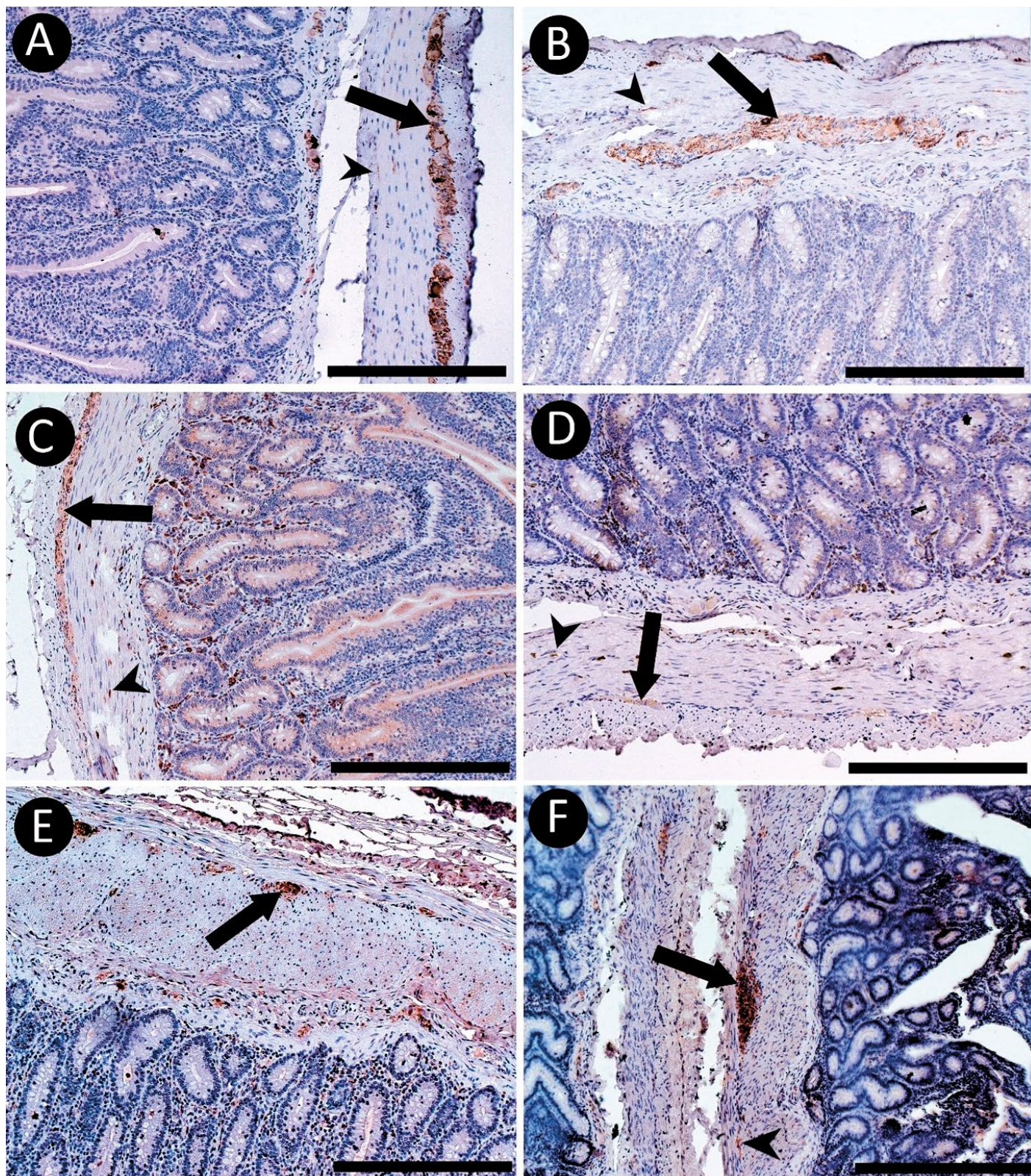


Fig. 2. Immunoections to biologically active substances studied found in ENS of the small intestine of the control (A, C, E) and experimental fallow deer (B, D, F). Pair (A-B) presents the immunoreactivity to nitric oxide synthase (NOS), pair (C-D) immunoreactivity to tyrosine hydroxylase (TH) whereas pair (E-F) immunoreactivity to galanin (Gal)

Explanations: In all micrographs arrows point to ganglionic enteric neurons immunoreactive to the studied substance whereas, arrowheads indicate nerve fibers immunoexpressing one of the studied neurotransmitter/neuropeptide. Scale bar: 100 μ m

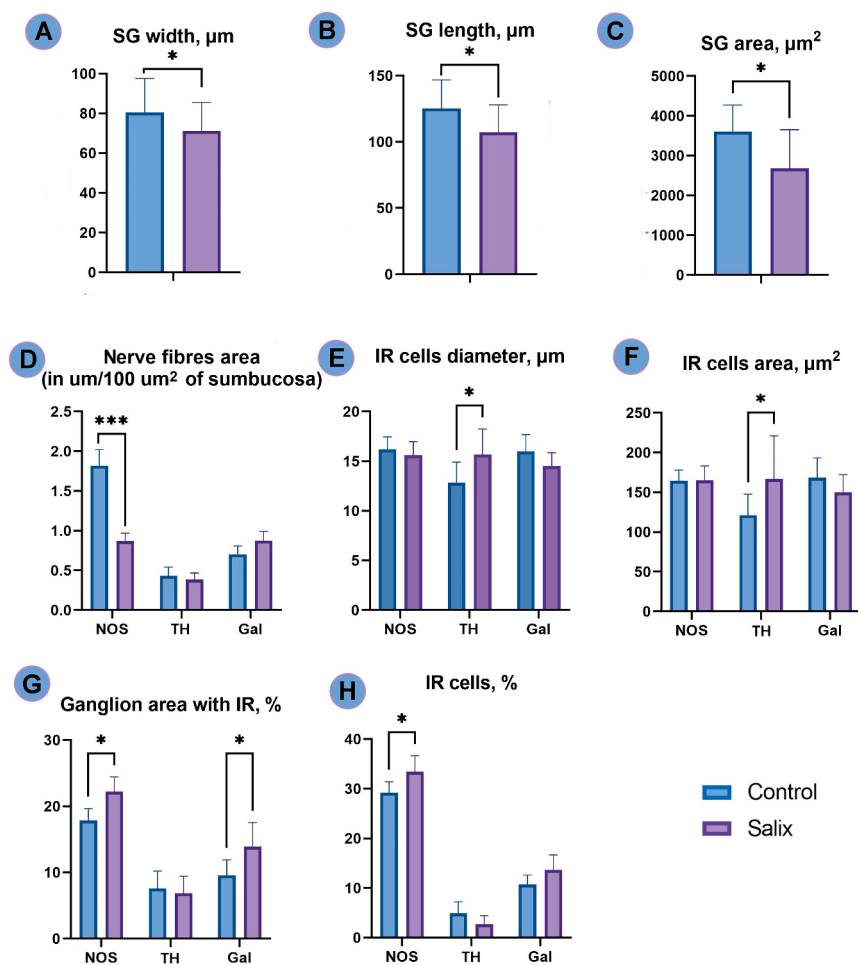


Fig. 3. The effect of basket willow feeding on the morphology of the Submucosal Ganglion (SG) and the immunoexpression of nitric oxide synthase (NOS), tyrosine hydroxylase (TH) and galanin (Gal) in fallow deer: (A) submucosal ganglion width, (B) submucosal ganglion length, (C) submucosal ganglion area, (D) nerve fibres area, (E) immunoreactive (IR) cells diameter, (F) immunoreactive cells area, (G) ganglion area, (H) immunoreactive cells percentage. Explanations: Asterisks (*) indicate significant differences between control and experimental groups (* $p < 0.05$; *** $p < 0.001$).

for both). There was also a significant increase in NOS-IR cells percentage ($p < 0.05$; Fig. 3H) in the experimental group compared to the control group. No other changes were observed.

The findings of this study may shed light on the potential impact of dietary supplementation with basket willow on the neuroanatomy of the small intestine in farmed fallow deer. Our results revealed significant alterations in the morphology and innervation patterns of the ENS following exposure to basket willow, suggesting a potential modulation of gastrointestinal function in these animals.

One notable observation was the significant decrease in the MP ganglions width and area in animals fed with basket willow compared to the control group. This reduction in ganglion size may indicate a remodeling of the ENS architecture, potentially influencing its regulatory capabilities within the small intestine (5, 37). Furthermore, the decrease in NOS-IR nerve fibers in the tunica muscularis of the experimental group suggests a downregulation of nitrergic signaling,

which could affect smooth muscle function and intestinal motility (28). NOS demonstrates anti-adhesive properties and has been scientifically validated for its ability to impede neutrophil recruitment within inflammatory regions (3). This anti-adhesive effect extends to the inhibition of pro-inflammatory molecule production and the downregulation of adhesive compound expression. Decreased levels of NOS-IR nerve fibers can compromise these functions, potentially leading to heightened cell adhesion, increased neutrophil infiltration, elevated pro-inflammatory signaling, and augmented expression of adhesive molecules within inflamed tissues (3). In addition, changes in the morphology and innervation of the SP ganglions were also evident in the experimental group. A decrease in ganglion size and NOS-IR nerve fibers area suggests a similar downregulation of nitrergic signaling in the SP ganglions, potentially impacting secretory and vasomotor functions. Preclinical studies have demonstrated that NOS helps maintain gastric mucosal integrity, inhibits leukocyte adherence to blood vessel walls, and promotes the repair of nonsteroidal anti-inflammatory drugs induced damage (18). Given that willow contains salicylates, understanding the modulation of nitrergic signaling and its protective role in gastrointestinal function could be particularly relevant in contexts involving willow ingestion.

Conversely, an increase in the diameter and area of immunoreactive TH-IR cells indicates a potential upregulation of adrenergic signaling, which may increase smooth muscle tone and modulate blood flow regulation. However, it should be mentioned that TH is found mostly in fibers of external origin (extrinsic), unlike NOS and Gal (intrinsic) (29). TH plays a crucial role in regulating gut motility by catalyzing the conversion of tyrosine to L-dihydroxyphenylalanine (L-DOPA), a precursor in the synthesis of dopamine. Dopamine, synthesized from L-DOPA, acts as a neurotransmitter in the ENS and modulates gut motility by influencing smooth muscle contraction. The activity of TH, therefore, directly impacts the availability of dopamine, which in turn regulates gastrointestinal muscle tone and movement (7). A significant decrease in the MP ganglions width and area observed in animals fed with basket willow compared to the control group could be alarming, as it suggests a potential neurosuppressive effect of the willow on the ENS (35). Further investigations are warranted to elucidate the specific mechanisms by which basket willow might be

exerting this effect. One possibility is that the willow contains bioactive compounds that target and inhibit the proliferation or survival of enteric neurons (10). Additionally, willow might influence the expression of neurotrophic factors essential for enteric neuronal maintenance. The potential consequences of a reduced MP ganglions width and area could be manifold. Impaired gut motility might manifest as constipation or gastroparesis, while secretory dysfunction could lead to diarrhea or dehydration (25). Disruptions in blood flow regulation could compromise nutrient absorption and exacerbate intestinal injury. Therefore, a thorough evaluation of the gastrointestinal effects of basket willow is necessary to determine its safety and suitability for various applications.

Interestingly, an increase in Gal-IR nerve fibers and neurons within the MP ganglions was observed in animals supplemented with basket willow. Galanin is known to modulate gastrointestinal motility by acting as a neurotransmitter, activating three different G-protein-coupled receptors which are expressed in the smooth muscle cells of the gastrointestinal tract (GIT), and also enhancing or inhibiting the release of neuroactive substances. Additionally, it may directly influence smooth muscle cells by activating receptors located on their surface (4, 14, 22). Thus, its increase indicates that it may serve as a mechanism for gut maintenance or regulation function in response to changes induced by the willow supplementation. In the study by Moore et al. (23), which focused on beef cattle grazing dry, sparse summer pastures supplemented with willow under drought conditions, a reduction in liveweight loss was observed in cattle receiving willow supplementation. This suggests that willow supplementation may have a beneficial effect on gastrointestinal function and overall animal health under challenging environmental conditions. When considering these findings together, it is possible to speculate that the increase in galanin expression observed in our study could be part of the mechanism through which willow supplementation exerts its effects on gastrointestinal function. Given that galanin is an orexigenic peptide, involved in promoting feeding behavior and appetite regulation (22), its upregulation may indicate a compensatory response to enhance nutrient absorption and utilization in response to the stressors.

Our findings align with previous studies implicating basket willow in modulating gastrointestinal function through its effects on neurotransmitter systems. For instance, Ramos et al. (30) demonstrated the anti-inflammatory properties of basket willow, which may influence neuronal activity within the ENS. Similarly, Lin et al. (20) reported the analgesic effects of basket willow, suggesting a potential role in modulating pain perception in the gastrointestinal tract. However, it is essential to interpret these findings with caution, as the

precise mechanisms underlying the observed changes in ENS morphology and function remain elusive. Additionally, it's noteworthy that basket willow has been documented to possess anti-parasitic properties (9). Given the presence of parasites in the intestines of deer, as well as the lower observable number of parasites in the experimental group (although the level of parasitic infestation was not measured), it's plausible to consider that parasitic infestation could induce inflammation in the gastrointestinal tract. This inflammation could contribute to the observed increase in Gal, as it is known to be associated with inflammatory reactions (17). Furthermore, salicylates, commonly found in *Salix* species like salicylic acid, have significant effects on the ENS (3, 32). Basket willow has notably high concentrations of salicylates, with levels reaching 2200 mg/kg in branches and 3000 mg/kg in the bark. In contrast, other grazing plants like meadowsweet flowers contain significantly lower levels at 0.95 mg/kg (11). Excessive salicylate exposure can impact various organ systems, including the central nervous system, by disrupting oxidative phosphorylation and inhibiting Krebs cycle enzymes (31, 36). Emerging evidence suggests adverse effects associated with low-dose salicylate administration on the GIT. Frequent and prolonged exposure to salicylates has been linked to ulcers, gastrointestinal bleeding, and intestinal perforation (18, 38). Notably, salicylate toxicity can result from acute ingestion or chronic exposure, leading to adverse effects in both cases on the central nervous system, pulmonary function, and gastrointestinal integrity (1).

In conclusion, our investigation underscores the potential advantages of incorporating willow supplementation in ruminant farming practices. The observed alterations in the neuroanatomy of the small intestine suggest a promising avenue for enhancing gastrointestinal function and overall animal health. Willow supplementation has previously exhibited promise in mitigating livestock weight loss, reducing parasite burdens, and potentially modulating neurotransmitter activity, as evidenced by the upregulation of galanin expression. However, it is imperative to acknowledge the potential adverse effects associated with willow supplementation, particularly its high salicylate content. Excessive exposure to salicylates may pose risks to gastrointestinal integrity and overall animal well-being. Nonetheless, it is essential not to disregard the potential benefits of willow supplementation solely based on these adverse effects. Instead, a comprehensive evaluation of the risks and benefits should be conducted, considering factors such as dosage, duration of supplementation, and specific animal health conditions. Further research is warranted to elucidate the underlying mechanisms governing both the advantageous and detrimental effects of willow supplementation in the feed of ruminants as well as farming practices.

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