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Influence of sodium butyrate administered *in ovo* on gene expression in the brain of broiler chickens*

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The gut-brain axis is a bidirectional interaction and information transfer system between the gastrointestinal tract and the central nervous system. It is also known that bioactive substances affect the colonization of the gut by bacterial microorganisms. The following research assessed the effect of sodium butyrate (SB) on the expression of genes related to innate immune response and small heat shock proteins in the brains of broiler chickens. For this purpose 1000 eggs were incubated and divided into 4 groups. Three were injected with either 0.1, 0.3, or 0.5% doses of SB and the last one only with saline. Samples were collected *post-mortem* on day 42 of rearing. RNA isolated from the brain was tested for gene expression using a panel of the following: *HSPB1, HSPB5, HSPB8, HSPB9, HSP70, IL-1β, IL-2, IL-4, IL-6, IL-8, IFNγ, IFNβ, TLR4, GR, BDNF, GSK3β, NR2A, CRH, NF-κB p65*. SB stimulation induced significant changes in gene expression levels, especially in the 0.3% SB group, where down-regulation of most genes is evident. It can be assumed that changes in the level of gene expression are determined by the concentration of SB in the injection dose.

KEY WORDS: broiler / gut-brain axis / small heat shock proteins / sodium butyrate

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With the ban on antibiotics used as growth promoters in European poultry diets, the search for adequate substitutes has begun. Growth promoters increase intestinal efficiency and broiler productivity. Promising substitutes are short-chain fatty acids **(SCFAs)** and their metabolites. Sodium butyrate, or butyric acid sodium salt (SB), which belongs to SCFAs, has gained interest as a substance that improves poultry production and affects the immune response and the gastrointestinal tract [Ghanim and Isihak 2022]. Earlier Jiang *et al*. [2015] reported that SB reduced cytokine induction and affected chickens' gastrointestinal tract and intestinal microbiota. However, publications on the effects of SB on the brain have appeared only recently, and their number is limited. Therefore, the exact mechanism of *in ovo* stimulation with SB on brain development and gene expression remains unclear.

The function of various intestinal bacteria is to produce SCFAs by fermenting the compounds that animals cannot digest. The indigestible residues include fiber, cellulose, sugar, and starch [Bortoluzzi *et al.* 2017]. SB, in particular, is produced by *Faecalibacterium prausnitzii*, *Clostridium* spp., and *Fusobacterium* bacteria [Zhu *et al*. 2021]. Once it is produced, intestinal cells can use SB as an energy source.

In addition, SB stimulates the differentiation and maturation of intestinal cells and could modify the intestinal barrier [Onrust *et al*. 2015]. It regulates the production of inflammatory cytokines by modulating intestinal immune cells (lymphocytes and macrophages). Consequently, SB has anti-inflammatory effects mediated by signaling pathways. In particular, it modulates pro-inflammatory cytokines by inhibiting *NF-kB* activation [Bortoluzzi *et al.* 2017].

Moreover, SCFAs, as bioactive compounds, are involved in signal transduction within the gut-brain axis. As a two-way system, the gut-brain axis involves the endocrine, immune, and nervous systems, enabling the host brain to impact the gastrointestinal tract [Ding *et al.* 2018]. On the other hand, the commensal bacteria-induced degradation of proteins and carbohydrates causes the production of additional neuroactive components, e.g., serotonin in the intestine [Józefiak *et al*. 2020].

More specifically, structural elements of the bacterial cell walls and bacterial metabolism products trigger the host immune response. They activate enteroendocrine cells, which affect the nervous system locally and systemically. The brain, in turn, influences microbiota composition, gut physiology, and the immune system. Neurotransmitters, including norepinephrine and epinephrine, stimulate the growth of beneficial microbial bacteria, such as *Bifidobacterium* spp. and/or *Lactobacillus* spp. [Beldowska *et al*. 2023] and the bacteria residing in the gut are a source of peripheral hormones and neurotransmitters, such as dopamine and 5-hydroxytryptamine (5-HT). These neurotransmitters transmit information about gut health through the fibers of the vagus nerve to the brainstem and other brain areas. Stress factors through the peripheral and central pathways activate the hypothalamic-pituitary-adrenal (HPA) axis [Beldowska *et al.* 2023]. As a result, the intestinal microbiota's composition and the intestinal epithelium's function are altered [Cao *et al*. 2021].

Considering the bidirectional dependencies between the brain and the gut system, as well as the effects of SB on the intestinal barrier and nervous system, this study aims to demonstrate the impact of postbiotics injected *in ovo* during embryo development on gene expression in the brain, specifically changes in the expression of genes primarily related to the organism's immune response.

Material and methods

This experiment was conducted following the applicable regulations. The slaughter of the broilers was carried out in accordance with current regulations on handling animals during slaughter under Directive No. 2010/63/EU of September 22, 2010, concerning the protection of animals used for scientific purposes. The approval and consent of the Ethics Committee were not required. According to the Act of January 15, 2015, on the protection of animals used for scientific or educational purposes (item 266, Journal of Laws of the Republic of Poland), slaughtering to collect tissues and organs from animals is not a procedure.

Broiler chicken Ross 308 hatching eggs obtained from Hatchery (Drobex-Agro Sp. z o.o., Kuyavian-Pomeranian Voivodship Poland) were used for the study. The incubation was performed in a laboratory single-stage incubator (Jarson, Gostyń, Poland) and hatcher (Jarson, Gostyń, Poland). The incubation lasted 21 days and was divided into two stages. The first stage covered days 1-18 (incubator), and the second included days 19-21 (hatcher). The parameters in the incubator were: temperature – 37.7°C, ventilation - 50/60%, and humidity - 55/60%. In turn, the parameters in the hatcher were as follows: temperature – 37.5°C, humidity – 70%, ventilation – 80%. In detail, 1000 eggs were incubated according to the method described by Biesek *et al.* [2023].

Samples collection

On day 12 of incubation, the following doses of SB, molecular weight: 110.09 (Merck Life Science Poland), were administered *in ovo*: 0.1, 0.3, and 0.5%, each dissolved in 2.0ml saline. The dose was selected based on data from the literature. The control was the saline-injected group, 0.2 ml of 0.2 mmol/l. The eggs were divided into four groups of 250 eggs each, and the environmental conditions for all chickens were the same. After hatching, 60 chicks were selected from each group for rearing. On reaching day 42 of rearing, eight broilers were randomly selected from each group. The birds were killed, and the heads were taken directly onto dry ice in their entirety. The brains were isolated from the heads and immediately fixed in fixRNA stabilization buffer (fixRNA, EURx, Gdansk, Poland. They were stored at -20°C until further processing.

Gene expression

Total RNA was isolated from about 100 mg of the brain, which was homogenized in 0.2 ml of chloroform and 1 ml RNA Extracol (EURx, Gdansk, Poland) using a TissuesRuptor homogenizer (Qiagen GmbH, Hilden, Germany). RNA was purified from the solution and contaminant using a GeneMATRIX Universal RNA Purification Kit (EURx, Gdansk, Poland) following the manufacturer's instructions. Each RNA sample was quantitatively and qualitatively evaluated using the NanoDrop 2000 (Thermo Scientific Products, Wilmington, USA). Gene expression analysis was performed for a panel of genes selected based on literature data: *HSPB1*, *HSPB5*, *HSPB8*, *HSPB9*, *HSP70*, *IL-1β*, *IL-2*, *IL-4*, *IL-6*, *IL-8*, *IFNγ*, *IFNβ*, *TLR4*, *GR*, *BDNF*, *GSK3β*, *NR2A*, *CRH*, *NF*-κB *p65*. The gene expression analysis was performed by

Table 1. Primer sequences used in RT-qPCR reaction (F- Forward primer; R-Reverse primer)

Gene	Name	Primer sequences	Reference
HSPB1	Heat shock protein beta 1	F: CGTGCCCTTCACCTTCCTCA R: TGGGCAGCAGACGGAAGTATC	Basaki *et al*. 2020
HSPB5	Heat shock protein beta 5	F: CCAGGGCCATTCAAGAGCAC R: CGTGGCTTCCTTGAGCCAAC	Basaki *et al*. 2020
HSPB8	Heat shock protein beta 8	F: TTCAAGCCTGAGGAGCTGACG R:AAGGAGGCGAAGACAGTGATGG	Basaki *et al*. 2020
HSPB9	Heat shock protein beta 9	F: CCGCACGCAGAGACCATCTT R: CACATCCTGGCAGACGGAGAA	Basaki *et al*. 2020
HSP70	Heat shock protein beta 70	F: GGGAGAGGGTTGGGCTAGAG R: TTGCCTCCTGCCCAATCA	Fu *et al*. 2023
IL-1β	Interleukin 1β	F: GGAGGTTTTTGAGCCCGTC R: TCGAAGATGTCGAAGGACTG	Dunislawska *et al*. 2022
IL-2	Interleukin 2	F:GCTTATGGAGCATCTCTATCATCA R: GGTGCACTCCTGGGTCTC	Dunislawska *et al*. 2022
IL-4	Interleukin 4	F: GCTCTCAGTGCCGCTGATG R: GGAAACCTCTCCCTGGATGTC	Dunislawska *et al*. 2022
IL-6	Interleukin 6	F: AGGACGAGATGTGCAAGAAGTTC R: TTGGGCAGGTTGAGGTTGTT	Dunislawska *et al*. 2022
IL-8	Interleukin 8	F: AAGGATGGAAGAGAGGTGTGCTT R: GCTGAGCCTTGGCCATAAGT	Dunislawska *et al*. 2022
IFNγ	Interferon γ	F: ACACTGACAAGTCAAAGCCGC R: AGTCGTTCATCGGGAGCTTG	Dunislawska *et al*. 2022
IFNβ	Interferon β	F: ACCAGATCCAGCATTACATCCA R: CGCGTGCCTTGGTTTACG	Dunislawska *et al*. 2022
TLR4	Toll-like receptor 4	F: GAGAACCTCAATGCGATGC R: ATAGGAACCTCTGACAACG	Fu *et al*. 2023
GR	Glucocorticoid receptor	F: TGGGAACGCTCAACCTTTC R:GCTGCCACAAGTAAGAACACCATA	Yan *et al*. 2020
BDNF	Brain-derived neurotrophic factor	F: ACATCACTGGCGGACACTTT R: GTTACCCACTCGCTTGTGCT	Yan *et al*. 2020
GSK3β	Glycogen synthase kinase 3 beta	F: ACTGCAGTCCTATGGAGTTGA R: ATATGCCACATCCTCTGCACAT	Zhang *et al*. 2022
NR2A	N-methyl-d-aspartic acid receptor subunit 2A	F: CATCTTTGCCACTACGGG R: TCAGCCACAGGGTTTCTAAC	Yan *et al*. 2020
CRH	Corticosterone-releasing hormone	F: TCTCCCTGGACCTGACTTTC R: GCCTCACTTCCCGATGATT	Yan *et al*. 2020
NF-κB p65	Nuclear factor kappa-light-chain-enhancer of activated B cells	F: GTGTGAAGAAACGGGAACTG R: GGCACGGTTGTCATAGATGG	Bortoluzzi *et al*. 2017

qPCR with initial reverse transcription. cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific/Fermentas, Vilnius, Lithuania). The qPCR reaction was performed using LightCycler 480 II. The qPCR reactions mixture contained SG onTaq qPCR Master Mix (2x) (EURx, Gdansk, Poland), 1 μM of each primer (presented in Table 1) specific to the target gene (synthesized by Genomed S.A.), and 70 ng of cDNA. The thermal profile of the qPCR reaction was carried out as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and elongation at 72°C for 45 s. Relative gene expression was calculated by the $^{\Delta\Delta}C_T$ algorithm, and the amount of the target gene was calculated by the $2^{-\Delta\Delta CT}$ formula.

Statistical analysis

The value of the control group is a normalized value. The results in the research groups are the deviations obtained relative to the control. Statistical analysis was performed using one-way ANOVA. Significance thresholds of $P<0.05$, $P<0.01$ and P 0.001 were used. Dunett's test was used as a post-hoc test to compare several experimental groups with one control group. Thes statistical analysis was performed using SAS statistical software (SAS Enterprise Guide 8.3; SAS Institute Inc., Cary, NC, USA).

Results and discussion

Administration of SB *in ovo* caused statistically significant differences in the level of gene expression in the brain of the broiler chickens, specifically in the expression of innate immune response genes (Fig. 1), heat shock proteins (Fig. 2), and other genes (Fig. 3). In the 0.1% SB group, there was a decrease in *IL-2* (-1.04), *IFNβ* (-1.36), *GSK3β* (-0.75), and *HSPB1* (-0.94) genes compared to the control. Significant changes occurred in the 0.3%SB group for the following genes: *HSPB1* (-2.32), *HSPB5* (-2.28), *HSPB8* (-1.72), *HSPB9* (-2.39), *HSP70* (-1.52), *IL-1β* (-1.71), *IL-2* (-1.49), *IL-4* (-1.56), *IL-6* (-1.55), *IL-8* (-1.52), *IFNγ* (-1.59), *IFNβ* (-2.55), *BDNF* (-1.39), *GSK3β* (-1.45), *NR2A* (-1.22). Significant decreases in the expression also occurred in the group with the highest SB concentration of 0.5%: *IL-1β* (-1.38), *IL-2* (-0.98), *IL-8* (-1.08), *IFNγ* (-1.37), *IFNβ* (-2.25), *HSPB1* (-2.59), *HSPB5* (-2.62), *HSPB8* (-1.45), *HSPB9* (-1.73), *GR* (-2.2), *BDNF* (-1.55), *GSK3β* (-1.6), *NR2A* (-1.62), *NF-*κB *p65* (-1.04). The 0.1% SB group shows increased NR2A (0.06) gene and TLR4 (0.86) expression. The results indicate that SB has different effects on gene expression in the brains of broiler chickens, depending on the dose. Decreased levels of heat shock proteins such as *HSPB1* and *HSPB5* at different SB concentrations indicate a variable host response to stress. In addition, a decrease in the expression level of pro-inflammatory cytokines such as *IL-1β*, *IL-2*, *IL-6,* and *IL-8* indicates a beneficial effect of SB. In particular, this effect is noticeable in the 0.3% group.

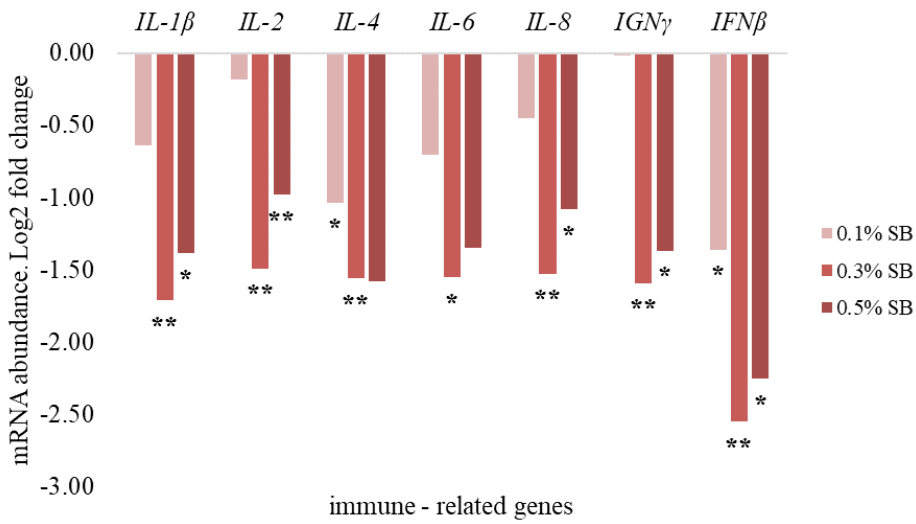

Fig. 1. Expression of immune-related genes in the brain, after *in ovo* stimulation of sodium butyrate in three doses.. Relative gene expression was calculated by the $^{\Delta\Delta}C_T$ algorithm, and the amount of the target gene was calculated by the $2^{-\Delta\Delta CT}$ formula. Significances obtained as a result, *P≤0.05, **P≤0.01, ***P≤0.001.

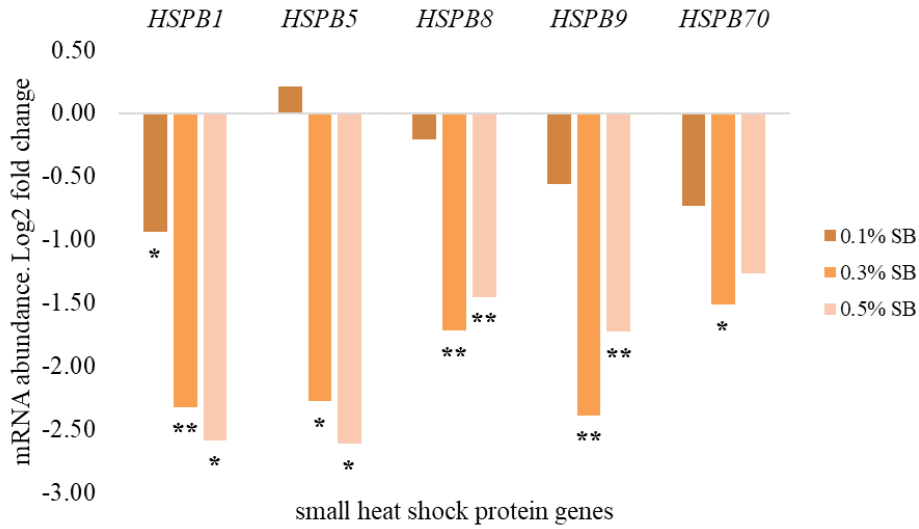

Fig. 2. Expression of small heat shock protein genes in the brain, after *in ovo* stimulation of sodium butyrate in three doses. Relative gene expression was calculated by the $^{\Delta\Delta}CT$ algorithm, and the amount of the target gene was calculated by the $2^{-\Delta\Delta CT}$ formula. Significances obtained as a result, *P≤0.05, **P≤0.01, ***P≤0.001.

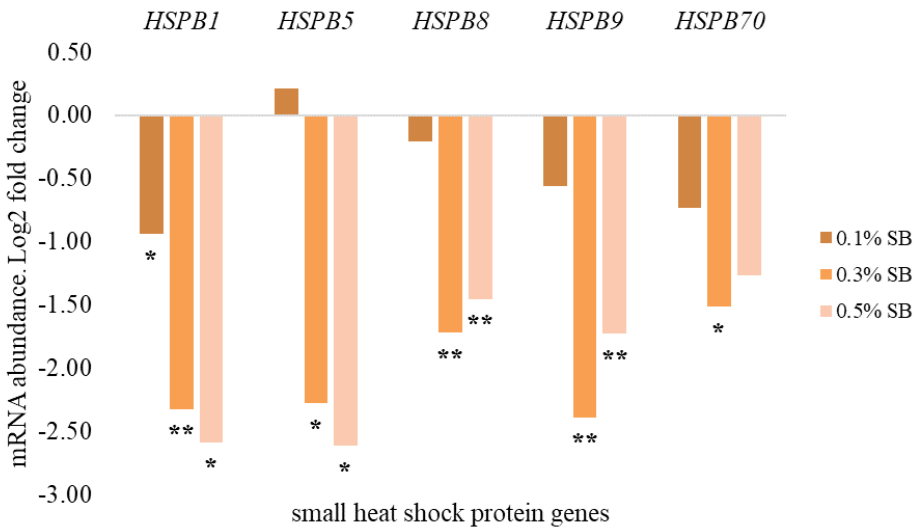

Fig. 3. Expression of genes in the brain, after *in ovo* stimulation of sodium butyrate in three doses. Relative gene expression was calculated by the $^{\Delta\Delta}C_T$ algorithm, and the amount of the target gene was calculated by the $2^{-\Delta\Delta CT}$ formula. Significances obtained as a result, *P≤0.05, **P≤0.01, ***P≤0.001.

Many studies indicate that small heat shock proteins (SHSPs) are stress proteins that are not expressed in the absence of stress conditions. The results obtained by Basaki et al. [2020] showed that *HSPB1*, *HSPB5*, *HSPB8*, and *HSPB9* are expressed in both standard and thermally manipulated chick brains. HSP levels increase in response to stress and return to baseline levels when stimulants are removed [Basaki *et al.* 2020]. The reduction in the expression of the *HSPB1*, *HSPB5*, *HSPB8*, *HSPB9,* and *HSP70* genes obtained in the above studies for all tested groups may indicate the beneficial effect of SB, which most likely reduced the stress in broiler chickens. The data obtained by Wang *et al.* [2018] showed a positive impact of the use of dietary prebiotics on improving brain immunity in broilers. This impact is manifested by a reduction in pro-inflammatory cytokines, including *IL-6* and *IL-8*, at both the transcriptional and protein levels and reduced up-regulated *HSP70* and *TLR4* gene expression during exposure to stressful conditions. The studies mentioned above showed reduced gene expression of the cytokines *IL-1β, IL-2*, *IL-4*, *IL-6,* and *IL-8*, with a particularly significant decrease in expression occurring in the 0.3% SB group for each of the mentioned interleukins.

Toll-like receptor 4 (*TLR4*) is among the receptors that recognize threat-related signals from damaged cells and pathogens. It responds rapidly by activating innate immune responses. *TLR4*, as a microbial detection receptor, is responsible for recognizing pathogen-derived molecules, including liposaccharides (LPS). The LPS-TLR4 pathway has been used as a molecular biomarker for brain injury [Fu *et al.*

2023]. SB supports the modulation of the intestinal mucosa, increases the production of SCFAs, and regulates gene expression.

Moreover, SB activates the expression of Toll-like receptors 4. This substance has antioxidant, immune-strengthening, and anti-inflammatory functions. One of the actions of SB is to increase protein digestibility. The *TLR4* protein is responsible for coding the *TLR4* gene. The protein activation leads to an intracellular signaling pathway and inflammatory cytokine production that stimulates the innate immune system as reported by Bawish *et al*. [2023]. These authors demonstrated variable levels of TLR4 gene expression depending on the dose of SB administered. Our studies did not show any statistically significant changes in TLR4 gene expression. An increase in expression occurred in the 0.1% group, while in the other two groups the gene expression decreased slightly compared to the control.

The hypothalamus is a master regulator in the central brain, involved in stress and fear responses via the HPA axis. Yan *et al*. [2020] described how environmental and bedding enrichment influenced the gut microbiota of chickens, affecting their behavior and gene expression. They observed a decreased expression of stress-related genes, including corticosterone-releasing hormone (CRH) and glucocorticoid receptor (GR), in the brain. A decrease in both of these genes is also evident in the case of our studies described. *GR* gene expression significantly decreased in the SB 0.5% group by as much as -2.5 levels relative to controls. Such results are consistent with those described in an earlier Yan *et al*. [2020] work, in which *GR* and *CRH* gene expression increased in response to stress. Thus, it can be concluded that the negative expression of these genes obtained indicates the absence of stress factors affecting the hens and the positive immunomodulatory character of SB. Yan *et al.* [2020] also wrote that increased expression of brain-derived neurotrophic factor (*BDNF*) and N-methyl-d-aspartic acid receptor subunit 2A (*NR2A*) genes was accompanied by reduced stress levels and increased transcription factor pathways of gut microbiota. SB is key in promoting gut integrity and maintaining the balance of microbial diversity and abundance [Miao *et al*. 2022], while gut microbiota can modulate neurodevelopmental gene expression in the brain. Genes involving brain-derived neurotrophic factor (BDNF) have been shown to play an important role in brain development, including neural cell differentiation. *BDNF* is a master regulator of neural circuit development in the central nervous system. Altered levels of *BDNF* expression have been shown in brain-related disorders [Razeghi *et al.* 2023]. The results obtained after SB stimulation show a significant decrease in *BDNF* expression in the 0.3 and 0.5% SB groups, but a decrease in *NR2A* expression also for these two groups. This phenomenon can be explained by gene specificity.

Conclusion

As our own studies indicate, the direct effect of early stimulation of the intestinal microbiota in chickens indirectly influences changes in gene expression in the brain.

SB positively affects the expression level of genes, which is responsible for reducing the effects caused by a stress factor and is related to the immune response. The obtained results show differences related to the dose of the administered substance. A dose of 0.1% SB shows little or no difference compared to the control, suggesting it is too low. In turn, 0.5% dose causes a significant reduction in the expression of all genes, which may cause disruptions in the functioning of the body. The dose of 0.3% showed the most optimal effect. The analysis of brain gene expression after *in ovo* stimulation with SB in three different doses can be considered an important research direction. However, further research is necessary to obtain more results and select the most beneficial dose.

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