Neuroprotective Effects of Whey and Buttermilk-Based Formulas on a DSS-Induced Colitis Murine Model

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Abstract

Inflammatory bowel disease is a gut-brain axis disorder that comprises chronic inflammatory conditions affecting the gastrointestinal tract, where alterations in the mood of patients are common. Gut-brain axis is a bidirectional communication that link gut and brain. The close association between inflammatory bowel disease and neuroinflammation has far-reaching implications, as is increasingly recognized as a contributing factor to neuropsychiatric and neurodegenerative diseases. The increasing prevalence and high economic cost, together with the loss of life quality of people suffering from these diseases, point to the need to find alternatives to alleviate them. Exploring new therapeutic avenues prompts us to consider the potential benefits of milk fractions, taking advantage of the use of dairy by-products, such as whey and buttermilk. This study examines the impact of cow's whey- and buttermilk-based formulas supplemented with bovine lactoferrin and milk fat globule membrane on the expression of cytokines, as well as on the components of immune and serotonergic system of the brain in a murine model of DSS-induced colitis. Our results show the potential of these dairy by-products, especially whey, as functional foods in ameliorating neuroinflammation and safeguarding the central nervous system function amid the neurological complications induced or concomitant with intestinal inflammatory processes.

Key words: Dairy by-products; lactoferrin; ulcerative colitis; gut-brain axis; serotonin.

Introduction

Inflammatory bowel disease (IBD) comprises chronic inflammatory conditions affecting the gastrointestinal tract, including Crohn's disease and ulcerative colitis (Ananthakrishnan, 2015). Beyond its localized effects, mounting evidence suggests that intestinal inflammation exerts profound influences on the central nervous system (CNS) function, implicating a bidirectional communication known as the gut-brain axis (Mitchell et al., 2022). The gut-brain axis operates along descending and ascending pathways, each contributing to the intricate relationship between intestinal inflammation and CNS dysfunction. The descending pathway highlights the impact of psychological stress as a prominent risk factor for both irritable bowel syndrome and IBD (Ge et al., 2022). Conversely, the ascending pathway highlights the role of intestinal signals in modulating brain physiology, with alterations in gut microbiota composition, immune responses, and epithelial integrity influencing CNS function and behavior (Masanetz *et al.*, 2022). This close association between IBD and neuroinflammation has far-reaching implications, as it is increasingly recognized as a contributing factor to a spectrum of neuropsychiatric and neurodegenerative diseases (Kip and Parr-Brownlie, 2023). Indeed, patients with IBD face an elevated risk of developing psychiatric comorbidities, including depression and anxiety (Hu et al., 2021), while also showing associations with neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Kim et al., 2022). These neurological complications not only diminish the quality of life for IBD patients but also add complexity to treatment and management strategies. Understanding the multifaceted nature of IBD-associated neurological complications is crucial for developing targeted therapeutic interventions.

In addition to the complex interplay between intestinal inflammation and CNS dysfunction, emerging evidence underscores the pivotal roles played by pattern recognition receptors (PRRs) and the serotoninergic system in bridging the gap between gut inflammation and neurological manifestations (Layunta et al., 2021). Within the family of PRRs, Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) both play crucial roles in the innate immune response by recognizing pathogen-associated molecular patterns and

initiating immune responses, including the production of pro-inflammatory cytokines. (Akesolo et al., 2022). Studies conducted in preclinical models of IBD have shed light on the involvement of some PRRs in the amplification of neuroinflammatory responses within the brain. Furthermore, alterations in the availability and signaling of serotonin (5-HT), a neurotransmitter closely linked to mood, cognition and behavior regulation, have been implicated in the behavioral changes observed in colitis models (Jones et al., 2020). This includes disruptions in tryptophan metabolism, a precursor of 5-HT, as well as aberrant serotoninergic signaling pathways in response to systemic inflammation (Couch et al., 2013). Together, these findings underscore the intricate connections between intestinal inflammation, cytokine-mediated neuroinflammation, PRRs activation, serotoninergic dysfunction, and neurological manifestations, highlighting their collective significance in understanding and managing the neuropsychiatric and neurodegenerative complications associated with IBD.

Exploring new therapeutic avenues prompts us to consider the potential benefits of natural components present in food. Thus, milk proteins and lipids have been attributed with relevant properties for human health (Hsieh et al., 2015; Anto et al., 2020). Although these milk components can be obtained from whole milk, in the actual world in which food waste must be avoided and circular economy is a condition for sustainability, the use of dairy by-products must be considered. Whey, obtained after the coagulation of casein during cheese manufacture, constitutes a significant portion of these by-products, with its production reaching approximately 160 million tons annually worldwide (Osorio-González et al., 2022). Buttermilk, the liquid phase released during cream churning in the butter-making process, is another valuable by-product (Conway, Gauthier and Pouliot, 2014). Whey and buttermilk have been traditionally considered as waste products and their improper disposal have generated a substantial environmental impact due to the high biological oxygen demand they present (Zandona et al., 2021). However, both whey and buttermilk possess excellent nutritional properties and they are also constituted by a combination of bioactive proteins, peptides, and lipids with numerous health benefits.

Additionally, whey and buttermilk are rich in B-group vitamins, minerals, sodium, potassium and lactose (Barukčić et al., 2019b).

The complex composition of whey and buttermilk and the differences between products depending on the manufacture process, make necessary to investigate on their biological activities and explore their potential application in functional products. Furthermore, it is necessary that future prospects will focus on marketing and consumer education to emphasize the nutritional value of dairy by-products and their beneficial health effects, as well as to address environmental concerns (Barukčić et al., 2019a).

Thus, this study aims to examine the impact of whey- and buttermilk-based formulas on the molecular expression of inflammatory cytokines, PRRs, and various serotonergic system components of the brain in a murine model of DSS-induced colitis. Our goal is to uncover the potential of these dairy by-products as functional foods in ameliorating neuroinflammation and safeguarding CNS function amid the neurological disorders induced by gut inflammation.

Materials and methods

Whey- and buttermilk-based formulas preparation

Two formulas were prepared using whey and buttermilk as bases enriched with native bovine lactoferrin (bLF) and MFGM (milk fat globule membrane). The bLF, characterized by an iron saturation level below 10%, was generously provided by Tatua Dairy Company (Morrinsville, New Zealand). The purity of bLF was checked by SDS-PAGE, which showed a single band corresponding to a protein of about 80 kDa and purity higher than 90%. Whey and buttermilk were obtained from raw bovine milk, which was supplied by the dairy company Villacorona (El Burgo de Ebro, Spain) and processed at the Food Science and Technology Pilot Plant of the University of Zaragoza (Veterinary Faculty), following established procedures (Abad et al., 2023). Both whey and buttermilk were lyophilized and stored at -20°C until they were used.

In the whey-based formula, 3.4 g of lyophilized bovine whey were rehydrated in 50 mL of Milli-Q water, considering that its dry matter was 6.8%. The buttermilk-based formula was prepared

using 50 mL of buttermilk obtained from cream subjected to mechanical agitation. The dry matter of liquid buttermilk used for the formula was determined by lyophilization and was 8.7%. In both formulations, bLF was added at a final concentration of 175 mg/mL and dissolved slowly under agitation at 4°C. Subsequently, 5 g of the MFGM precipitate, obtained by centrifuging 50 mL of buttermilk at 40,000 × g for 30 min, was incorporated into each solution and homogenized to ensure uniform distribution within the formulas, resulting in a concentration of 100 mg/mL. The product and protein concentrations present in the dairy fractions used in this study are specified in Table 1.

Animals

Male C57BL/6 mice (6–12 weeks) were purchased from Janvier Labs (Le Genest St. Isle, France) and were allowed to acclimate for 1 week prior to the experiments. The animals were housed in plastic cages, 4 mice/cage, under normal laboratory conditions (20 ± 2°C, 40–60% relative humidity and 12-h light/dark-cycle) with free access to standard chow and water. All procedures were conducted under Project Licence PI40/17 and approved by the Ethics Committee for Animal Experiments of the University of Zaragoza. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes. Housing and maintenance of the animals were carried out at the Centro de Investigación Biomédica de Aragón (CIBA), in compliance with previously described regulations and environmental conditions.

Animal treatments, induction of experimental colitis and sample collection

Animals were randomly divided into six groups (n=4 per group): 1) control group, 2) whey-based formula (W) group, 3) buttermilk-based formula (BT) group, 4) dextran sodium sulfate (DSS) group, 5) W + DSS group and 6) BT + DSS group.

All groups daily received 100 μL of water (control and DSS groups), or 100 μL of whey- and buttermilk-based formulas (W, BT, W+ DSS, and BT + DSS groups) by gastric gavage for 8 days.

Considering the concentrations of the starting formulas, the dose of each dairy fraction was as follows: whey at 6.8 mg/mouse, buttermilk at 8.7 mg/mouse, bLF at 17.5 mg/mouse, and MFGM at 10 mg/mouse.

An injury-repair model of ulcerative colitis was used (Valero *et al.*, 2020). At day 4, colitis was induced by switching to a 2.5% (w/v) solution of DSS (MW: 40,000 Da, Panreac, Lörrach, Germany) as drinking solution for 5 days. All animals were euthanized via cervical dislocation and brain and colon samples were removed and cleaned in an ice-solution of NaCl (0.9%). For the assessment of the damage caused to the colon, the length of the colon was measured using a digital calliper with a 0.01 mm resolution. Subsequently, the colon was opened longitudinally and rinsed with 0.9% saline solution. Colitis symptoms were assessed using a standardized scoring system: Number of adhesions (0: 0, 1: 1, 2: 2, 3: > 2), diarrhoea (0: normal, 1: loose stool, 4: water diarrhoea), tumefaction (0: absence, 1: moderate, 2: severe), stenosis (0: 0, 1: 1, 2: 2, 3: > 2), mucus (0: absence, 1: presence), haemorrhage (0: absence, 1: manifest bleeding), erythema (0: no macroscopic changes, 1: < 1 cm, 2: ≥ 1 cm), ulcers or erosions (0: 0, 1: < 1 cm, 2: ≥ 1 cm) and occult blood (0: no, 1: yes). A cumulative macroscopic damage score was then calculated for each animal.

Samples for RNA studies were collected in RNAlater from Qiagen (Hilden, Germany) and stored for one day at 4°C, being subsequently frozen at −80°C.

Histopathological study

Brain and colon samples were initially fixed in 10% buffered formalin, followed by dehydration, embedding in paraffin blocks, and sectioning into 4 μm-thick slices. These sections were then stained with hematoxylin–eosin. Subsequently, the examination of these samples was conducted by the Anatomopathological Service of the Veterinary Faculty of the University of Zaragoza, using a Nikon Eclipse Ci microscope, a Nikon DS-Ri 1 digital still camera, and a Nikon NIS calibrated digital image analysis system.

RNA isolation and real-time PCR

For brain RNA extraction, the samples were first homogenized using the Ultra Turrax T25 from IKA (Staufen, Germany), and total RNA was extracted using TRI® Reagent from Sigma-Aldrich (Saint Louis, MO, USA), following the manufacturer's protocol. The extracted RNA (1 µg) was used as a template for first-strand cDNA synthesis using the qScript cDNA SuperMix from Quantabio (Beverly, MA, USA). The cDNAs obtained by reverse transcription (RT) were used to determine the mRNA level by real-time PCR using Fast SYBR Green Master Mix from Thermo Fisher Scientific (Waltham, MA, USA).

Quantification of expression of the different mRNA analyzed in brain mice samples was performed using the Step One Plus Real-Time PCR System from Applied Biosystems (Foster City, CA, USA), with GAPDH and HPRT1 as housekeeping genes. The specific primers used are detailed in Table 2. The fluorescence raw data was analyzed by the Applied Biosystem Step One Software v2.3 from Applied Biosystems (Foster City, CA, USA). Thus, the mRNA relative expression was calculated as $\Delta\Delta Ct = \Delta Ct_{control} - \Delta Ct_{treatment}$ being $\Delta Ct = Ct_{gene} - Ct_{calibrator}$. GAPDH and HPRT1 were used as calibrators. Finally, the levels of relative gene expression were converted and expressed as fold difference ($=2^{-\Delta\Delta Ct}$).

Statistical analyses

The results were expressed as the mean ± the standard error of the mean (SEM), and differences between groups were statistically analyzed using the computer-assisted Prism GraphPad Program (Prism version 8.01, GraphPad Software, San Diego, CA). One-way analysis of variance (ANOVA) followed by Bonferroni's (when parametric distribution is observed), Kruskal–Wallis tests (non-parametric) or unpaired t-tests were used to detect differences. Previously, normal distribution was confirmed with the Kolmogorov-Smirnov test. Significance level was set to $p < 0.05$.

Results

Macroscopic damage and histopathological alterations induced by DSS in mouse colon.

DSS administration resulted in a significant reduction in colon length compared to the control group, highlighting severe colonic damage (Figure 1A). This was further evidenced by a substantial increase in macroscopic damage observed in DSS-treated mice (Figure 1B). To provide a more detailed view of the damage, a histopathological analysis was conducted on colon sections from the control and DSS-administered groups (Figures 1C-D). The histopathological examination revealed significant lesions in the distal colon, characterized by infiltration of inflammatory cells and pronounced edema (Figure 1D), confirming severe lesions characteristic of the DSS-induced colitis.

Inflammatory state in the brain of mice treated with DSS

Histopathological study of brain sections from animals belonging to different experimental groups was conducted. Relevant brain structures (cerebral cortex, cerebellum, and hypothalamus) were examined for histological integrity, presence of cellular infiltrates or any signs of inflammatory response. No significant differences were observed histopathologically between the control group and the DSS-treated mice (Figure 2A), both in the whole brain (a-b), and in different regions such as the frontal cortex (c-d)). In addition, histopathological observations revealed no evident signs of inflammation or alterations in the brain structure or morphology in any of the other groups (data not shown).

Despite the above results, mRNA expression of interleukins IL-1β and IL-6, as well as TNF-α in the brain, was determined as inflammatory markers. As shown in Figure 2B, DSS treatment significantly increased the mRNA expression of IL-1β, -6, and TNF-α compared to the control, with IL-6 showing the most pronounced increase (6-fold change), suggesting that inflammatory intestinal states induced by DSS are also reflected in the brain of these animals.

Effect of whey- and buttermilk-based formulas on cytokine and PRR expression in the brain of mice with DSS-induced colitis

To assess the effect of whey- and buttermilk-based formulas on DSS-induced neuroinflammation, the mRNA expression of IL-1β, IL-6, TNF-α and several PRR in the brain of the different groups

231 of animals treated with whey or buttermilk formulas, with or without treatment with DSS, was
232 determined. As shown in Figure 2B, whey and buttermilk formulas did not modify the
233 inflammatory cytokine expression. However, the pre-administration of whey formula in DSS-
234 treated animals significantly reduced the expression of all these cytokines compared to that of the
235 DSS group, reaching nearly the expression levels of the control. On the other hand, the pre-
236 administration of buttermilk formula also reduced the expression of all cytokines, but it was
237 significant only for IL-6.

238 The results revealed a substantial and significant increase in the expression of all analyzed Toll-
239 like receptors (TLRs), including TLR 1, 2, 3, 4, 5, 6, 7, 8, 9, and 11, as well as NOD-like receptors
240 NOD1 and NOD2 in brain tissue, in response to DSS-induced colitis, as shown in Figure 3. This
241 upregulation exhibited a relatively uniform pattern across the diverse array of receptors.

242 Interestingly, pre-administration of both whey- and buttermilk-based formulas attenuated the
243 expression of these TLRs and NOD receptors compared to the group treated solely with DSS.
244 Only the reduction in TLR9 expression was not statistically significant in the group that was
245 administered with the buttermilk-based formula. In all instances, the reduction effect elicited by
246 whey on mRNA expression levels was approximately twofold greater than that observed with
247 buttermilk administration.

248 **Effect of whey- and buttermilk-based formulas on the regulation of the central**
249 **serotoninergic system in mice with DSS-induced colitis**

250 Finally, we also investigated the impact of whey- and buttermilk-based formulas on the central
251 serotonergic system, due to its impact on neurological disorders such as depression or anxiety,
252 studying the expression of serotonin transporter (SERT), the enzyme tryptophan hydroxylase 2
253 (TPH2), and the receptors 5-HTR_{1A}, 5-HTR_{2A}, 5-HTR_{2B}, 5-HTR_3, 5-HTR_4, and 5-HTR_7 in the
254 brain. Notably, the expression of the 5-HTR_{2A} receptor was not detected in brain tissue. The
255 results of this analysis are shown in Figure 4.

DSS administration significantly upregulated the mRNA expression levels of TPH2 and 5-HT receptors, except for 5-HTR_4, which did not increase significantly. Interestingly, pre-administration of both whey and buttermilk formulas significantly mitigated the impact of DSS on the expression of 5-HTR_{1A}, 5-HTR_{2B}, 5-HTR_3, and 5-HTR_7. Moreover, pre-administration of whey also notably reduced the molecular expression of TPH2 and 5-HTR_4.

Furthermore, DSS administration led to a significant increase in SERT expression compared to the control group, with DSS causing an expression level approximately tenfold change higher than the control. This suggests a potential decrease in the availability of 5-HT at the CNS level, characteristic in depression and anxiety states. Remarkably, pre-administration of both whey and buttermilk formulas reduced the expression of serotonin transporter compared to the DSS group, with this effect reaching significance, particularly in the case of whey-based formula. Additionally, it is noteworthy that whey significantly outperformed buttermilk, reducing the expression of all analyzed components of the central serotonergic system by more than 50%.

Discussion

Our investigation contributes to the growing body of literature exploring the intricate relationship between intestinal inflammation and brain health, particularly in the context of inflammatory bowel disease (IBD), elucidating the potential therapeutic effects of whey- and buttermilk-based formulas in mitigating neuroinflammatory responses induced in intestinal inflammatory processes. By integrating our results with pertinent findings from existing research, we provide a comprehensive understanding of the molecular mechanisms underlying this complex interplay.

The DSS-induced colitis model demonstrated significant colonic damage, causing a marked reduction in colon length and increased macroscopic damage. Histopathological analysis further revealed severe inflammatory lesions, particularly in the distal colon. This substantial intestinal damage is linked to notable effects on the CNS, even in the absence of relevant microscopic lesions in the encephalon. Indeed, the significant effect of DSS-induced colitis on central serotonergic system and immune response in the brain of mice was evident in our study, reflecting

the systemic impact of DSS-induced colitis. We observed an upregulation in the expression of various elements of these systems. Specifically, the increased expression of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α, along with TLR and NOD receptors, indicates an enhanced immune activity in the brain in response to intestinal inflammation. Moreover, the upregulation of SERT transporter expression suggests an increase in serotonin reuptake, potentially affecting serotonin levels available for neuronal signaling. Concurrently, the elevation in TPH2 expression indicates enhanced neuronal synthesis of 5-HT, also further implicating alterations in serotoninergic signaling. Additionally, the increase in expression of 5-HT receptors (5-HTRs) points towards potential changes in serotonin receptor sensitivity and responsiveness within the brain. Interestingly, despite the absence of histopathological alterations in the brain tissue of any experimental group, the observed changes in the expression of serotonergic and immune system components might be associated with subclinical inflammation of the brain. These changes in the central serotonergic system could lead to disruptions in mood regulation, cognitive function, and other neurobiological processes. Additionally, the activation of central immune response might contribute to neuroinflammation, exacerbating the neurological consequences of intestinal inflammation and potentially predisposing individuals to neuropsychiatric and neurodegenerative disorders. In this sense, *in vivo* studies have shown increased permeability of the blood-brain barrier in chronic colitis models, allowing intestinal molecules to infiltrate the brain and mediate inflammatory responses (Craig et al., 2022). In this regard, we have recently shown that microbiota-derived short-chain fatty acids, which are able of crossing the blood-brain barrier, regulate intestinal serotonergic system, modulating the function and expression of SERT and 5-HTRs (Buey et al., 2023).

These findings align with previous studies that have identified inflammatory markers in regions of the hippocampus and cerebral cortex of mice with DSS-induced colitis. Elevated expression of inflammation-associated genes, such as IL-6 and TNF-α in the cortex, as well as IL-1β and TNF-α in the hippocampus, has been observed. These changes have been correlated with elevated serum levels of IL-6 and TNF-α, along with a decrease in the expression of intercellular binding

proteins like occludin and claudin-5 in brain tissue. These findings suggest an alteration in the integrity of the blood-brain barrier, facilitating the entry of inflammatory molecules into the brain (Zonis et al., 2015; Han et al., 2018). Additionally, in studies conducted in mice with colitis induced by dinitrobenzene sulfonic acid, a significant increase in the expression of TLR2, TLR4, TNF-α, IL-6, and molecular patterns associated with damage such as HMGB1 protein has been observed in the hippocampus. These enhanced or increased immune responses in the brain have been associated with depressive and anxious behavioral traits. Therefore, these investigations support the idea that neuroinflammatory changes are closely linked to alterations in animal behavior in *in vivo* models of IBD (Haj-Mirzaian et al., 2017).

Furthermore, alterations in serotonin availability and signaling may underlie the observed behavioral changes in animals with colitis. In this context, recent studies have also addressed the effect of intestinal inflammation on tryptophan (Trp) metabolism and its potential impact on the brain function. It has been observed that both acute and subchronic colitis induce notable changes in tryptophan metabolism in mice, characterized by a reduction in Trp and an increase in the enzyme IDO-1 in the serum. The decrease of serum Trp, a crucial precursor of 5-HT, may lead to decreased central 5-HT synthesis, thus affecting serotoninergic signaling in the brain. This decline could stem from both diminished food intake, the primary source of Trp, and the upregulation of IDO, an enzyme integral to Trp metabolism. Additionally, acute colitis has been associated with alterations in the kynurenine pathway of Trp metabolism in the brain, leading to elevated quinolinic acid levels (Zhao et al., 2023). Moreover, pharmacological magnetic resonance imaging studies have revealed that systemic inflammation can decrease brain 5-HT activity, mediated by alterations in signaling pathways, such as those involving the 5-HT2A receptor (Couch et al., 2013). These findings highlight the impact of intestinal and systemic inflammation on brain function, particularly on serotonergic signaling, commonly associated with neuropsychiatric disorders. In addition, the involvement of the intestinal microbiota in Trp metabolism is significant, as it has been associated with changes in both serum and brain metabolism in mice with colitis, suggesting a potential mechanism through which intestinal

inflammation affects central serotoninergic function (Zhao et al., 2023). In turn, the observed upregulation of SERT expression in the present study may be attributed to the direct action of certain proinflammatory cytokines, as previous studies have demonstrated an increased transporter activity in response to these molecules in various CNS cell types. Specifically, TNF-α and IL-1β have been shown to modulate SERT activity via the p38 MAPK signaling pathway (Zhu et al., 2006; Malynn et al., 2013). In this sense, the alteration in the expression of different receptors of the innate immune system would also indirectly affect the expression of SERT and serotonergic receptors, as it has previously been observed for TLR2 (Latorre et al., 2016), TLR9 (Layunta et al., 2022), or NOD2 (Layunta et al., 2018), among others. These findings underscore again the importance of inflammation in modulating serotonergic function and emphasize the relevance of the p38 MAPK signaling pathway in this process. Consequently, this could impact the amount of 5-HT available for neuronal signaling and potentially would have implications for mood regulation and other neurobiological processes.

In previous research, we demonstrated the protective effect of native bovine lactoferrin and buttermilk on oxidative stress in intestinal epithelial cells (Buey et al., 2021), and that treatment with whey or buttermilk supplemented with native bovine lactoferrin and MFGM modulated the microbiota composition and the functional pathways adversely affected by antibiotic administration (Bellés et al., 2023). In our study, both whey- and buttermilk-based formulas showed a mitigating effect on the immune and serotonergic hyperactivity associated with DSS-induced colitis in the brain, with whey showing particularly pronounced effects. This was evidenced by a significant reduction in the expression of several inflammatory markers (IL-1β, IL-6, and TNF-α), pattern recognition receptors (PRRs), and all components of the serotonergic system analyzed in animals pretreated with these dairy by-products compared to those treated only with DSS. The findings suggest a potential modulatory effect of dairy by-products on brain inflammatory response and CNS alterations secondary to intestinal inflammation.

These findings are consistent with previous research demonstrating the neuroprotective and anti-inflammatory properties of dairy components. Some studies have demonstrated that whey protein

powder could alleviate Alzheimer's disease (AD) pathology by inhibiting neuroinflammation through the peroxisome proliferator-activated receptor γ (PPARγ)–nuclear factor-κB signaling pathway in the brains of AD mice (Li et al., 2023). Additionally, supplementation with α-lactalbumin, a whey protein with high Trp content, has been shown to increase the ratio of plasma Trp to the sum of the other large neutral amino acids (Trp-LNAA ratio) and improve cognitive performance in stress-vulnerable subjects (Markus et al., 2002). These findings support the hypothesis that whey, due to its content of certain proteins, could enhance brain function by influencing Trp metabolism and 5-HT synthesis. Moreover, whey protein hydrolysate has been found to have antioxidant and anti-inflammatory effects in mice brain, reducing oxidative stress and the expression of inflammatory factors, such as TNF-α and IL-1β, which could help improve cognitive function and protect against age-related decline (Yu et al., 2021).

On the other hand, supplementation with MFGM has shown beneficial effects in attenuating the detrimental effects of obesity on brain function and memory in mice fed a high-fat diet, primarily through its anti-inflammatory role in the brain (Arnoldussen et al., 2022). Additionally, MFGM supplementation during pregnancy and lactation may promote neurocognitive development in the offspring of obese rats (Yuan et al., 2022). This supplementation has been found to modify the diversity and composition of the intestinal microbiota in the offspring, reducing the abundance of proinflammatory bacteria and increasing that of bacteria with anti-inflammatory functions. Moreover, MFGM alleviated neuroinflammation by reducing the levels of lipopolysaccharides and proinflammatory cytokines (IL-1β, IL-6, and TNF-α) in serum and brain, as well as by inhibiting the expression of the microglial activation marker Iba1. The correlation between changes in the intestinal microbiota and inflammation suggests that reducing the microbiota-mediated inflammatory response may be the mechanism by which MFGM stimulates neurological development (Yuan et al., 2022). These findings highlight the role of MFGM as an effective component in early neurological development and suggest its potential in modulating the inflammatory response, which could contribute to its beneficial effect on neurological development. Finally, clinical studies suggest that infant formulas enriched with bioactive

compounds, like MFGM, may have beneficial effects on the cognitive, behavioral and emotional development of children (Veereman-Wauters et al., 2012; Timby et al., 2014).

Coinciding with our results, research carried out in *C. elegans* confirms the role of lactoferrin in the regulation of the serotonergic system by modulating the biosynthesis of 5-HT (Martorell *et al.*, 2016). In this context, studies in rats show that a diet enriched with lactoferrin could also indirectly regulate central serotonergic signaling by modulating the microbiota, increasing the gene expression of certain serotonin receptors, such as 5-HTR_{1A} and 5-HTR_{2C}, in brain regions such as the prefrontal cortex, which could have implications in brain function (Mika et al., 2018). On the other hand, another study revealed that lactoferrin can suppress the inflammatory response induced by Epstein-Barr virus by interfering with the activation of TLR2 and TLR9 (Zheng et al., 2014).

In summary, our study underscores the potential of the dairy by-products whey and buttermilk, to modulate CNS inflammatory responses and promote brain health in the context of intestinal inflammation. The observed effects on serotoninergic function and immune response modulation further emphasize the therapeutic potential of these dairy components in mitigating neuroinflammation, mood dysregulation, cognitive impairments, and other neurobiological alterations commonly associated with neurodegenerative and neuropsychiatric disorders. Furthermore, although our primary focus was to study the impact on brain health, the positive outcomes observed suggest that these dairy by-products might also have beneficial effects on intestinal inflammation. Future research should explore this potential, while continuing to investigate the mechanistic insights and clinical implications of dairy-based interventions, focusing on both preserving brain health and addressing the neurological consequences of intestinal inflammation in IBD patients.

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Competing interest statement

The authors declare there are no competing interests.

Author contribution statement

B.B.: data curation, formal analysis, investigation, methodology, software, writing – original draft; E.L: formal analysis, investigation, methodology, writing – review & editing; M.C.: investigation, methodology; M.S.V.: investigation, methodology; M.A.P.: conceptualization, formal analysis, investigation, methodology, supervision, writing – review & editing; M.P.A.: formal analysis, writing – review & editing; I.A.: investigation, methodology; A.R.: investigation, methodology; L.S.: conceptualization, funding acquisition, project administration, visualization, writing-review and editing; J.E.M.: conceptualization, data curation, formal analysis, investigation, methodology, supervision, visualization, writing – review & editing.

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Data availability statement

Data available upon request.

References

Abad, I., Serrano, L., Graikini, D., Pérez, M.D., Grasa, L. and Sánchez, L. 2023. Effect of in vitro gastrointestinal digestion on the antibacterial activity of bioactive dairy formulas supplemented with lactoferrin against *Cronobacter sakazakii*. *BioMetals*, 36(3): 667–681. doi: 10.1007/s10534-022-00459-5.

Akesolo, O., Buey, B., Beltrán-Visiedo, M., Giraldos, D., Marzo, I. and Latorre, E. 2022. Toll-like receptors: New targets for multiple myeloma treatment?. *Biochemical Pharmacology*, 199: 114992. doi: 10.1016/j.bcp.2022.114992.

Ananthakrishnan, A.N. 2015. Epidemiology and risk factors for IBD. *Nature Reviews Gastroenterology and Hepatology*, 12(4), 205–217. doi: 10.1038/nrgastro.2015.34.

Anto, L., Warykas, S.W., Torres-Gonzalez, M. and Blesso, C.N. 2020. Milk polar lipids: Underappreciated lipids with emerging health benefits. *Nutrients*, 12(4): 1001. doi: 10.3390/nu12041001.

Arnoldussen, I.A.C., Morrison, M.C., Wiesmann, M., van Diepen, J.A., Worms, N., Voskuilen, M., Verweij, V., Geenen, B., Gualdo, N.P., van der Logt, L., Gross, G., Kleemann, R. and Kiliaan, A. 2022. Milk fat globule membrane attenuates high fat diet-induced neuropathological changes in obese Ldlr-/-.Leiden mice. *International Journal of Obesity*, 46(2): 342–349. doi: 10.1038/s41366-021-00998-w.

Barukčić, I., Jakopović, K.L. and Božanić, R. 2019a. Valorisation of whey and buttermilk for production of functional beverages - An overview of current possibilities. *Food Technology and Biotechnology*, 57(4): 448–460. doi: 10.17113/ftb.57.04.19.6460.

Barukčić, I., Jakopović, K.L. and Božanić, R. 2019b. Whey and Buttermilk—Neglected Sources of Valuable Beverages. *Natural Beverages*, 13: 209–242. doi: 10.1016/B978-0-12-816689-5.00008-0.

Bellés, A., Abad, I., Sánchez, L. and Grasa, L. 2023. Whey and Buttermilk-Based Formulas Modulate Gut Microbiota in Mice with Antibiotic-Induced Dysbiosis', *Molecular Nutrition and*

Food Research, 67(20): 2300248. doi: 10.1002/mnfr.202300248.

Buey, B., Bellés, A., Latorre, E., Abad, I., Pérez, M.D., Grasa, L., Mesonero, J.E. and Sánchez, L. 2021. Comparative effect of bovine buttermilk, whey, and lactoferrin on the innate immunity receptors and oxidative status of intestinal epithelial cells. *Biochemistry and Cell Biology*, 99(1): 54–60. doi: 10.1139/bcb-2020-0121.

Buey, B., Forcén, A., Grasa, L., Layunta, E., Mesonero, J.E. and Latorre, E. 2023. Gut Microbiota-Derived Short-Chain Fatty Acids: Novel Regulators of Intestinal Serotonin Transporter. *Life*, 13(5): 1085. doi: 10.3390/life13051085.

Conway, V., Gauthier, S.F. and Pouliot, Y. 2014. Buttermilk: much more than a source of milk phospholipids. *Animal Frontiers*, 4(2): 44–51. doi: 10.2527/af.2014-0014.

Couch, Y., Martin, C.J., Howarth, C., Raley, J., Khrapitchev, A.A., Stratford, M., Sharp, T., Sibson, N.R. and Anthony, D.C. 2013. Systemic inflammation alters central 5-HT function as determined by pharmacological MRI. *NeuroImage*, 75: 177–186. doi: 10.1016/j.neuroimage.2013.02.046.

Craig, C.F., Filippone, R.T., Stavely, R., Bornstein, J.C., Apostolopoulos, V. and Nurgali, K. 2022. Neuroinflammation as an etiological trigger for depression comorbid with inflammatory bowel disease. *Journal of Neuroinflammation*, 19(1): 4. doi: 10.1186/s12974-021-02354-1.

Ge, L., Liu, S., Li, S., Yang, J., Hu, G., Xu, C. and Song, W. 2022. Psychological stress in inflammatory bowel disease: Psychoneuroimmunological insights into bidirectional gut–brain communications. *Frontiers in Immunology*, 13: 1016578. doi: 10.3389/fimmu.2022.1016578.

Haj-Mirzaian, Arya, Amiri, S., Amini-Khoei, H., Hosseini, M.J., Haj-Mirzaian, Arvin, Momeny, M., Rahimi-Balaei, M. and Dehpour, A.R. 2017. Anxiety- and Depressive-Like Behaviors are Associated with Altered Hippocampal Energy and Inflammatory Status in a Mouse Model of Crohn's Disease. *Neuroscience*, 366: 124–137. doi: 10.1016/j.neuroscience.2017.10.023.

Han, Y., Zhao, T., Cheng, X., Zhao, M., Gong, S.H., Zhao, Y.Q., Wu, H.T., Fan, M. and Zhu,

493 L.L. 2018. Cortical Inflammation is Increased in a DSS-Induced Colitis Mouse Model.
494 *Neuroscience Bulletin*, 34(6): 1058–1066. doi: 10.1007/s12264-018-0288-5.

495 Hsieh, C.C., Hernández-Ledesma, B., Fernández-Tomé, S., Weinborn, V., Barile, D. and De
496 Moura Bell, J.M.L.N. 2015. Milk proteins, peptides, and oligosaccharides: Effects against the
497 21st century disorders. *BioMed Research International*, 2015: 146840. doi:
498 10.1155/2015/146840.

499 Hu, S., Chen, Yiping, Chen, Yan and Wang, C. 2021. Depression and Anxiety Disorders in
500 Patients With Inflammatory Bowel Disease. *Frontiers in Psychiatry*, 12: 714057. doi:
501 10.3389/fpsyt.2021.714057.

502 Jones, L.A., Sun, E.W., Martin, A.M. and Keating, D.J. 2020. The ever-changing roles of
503 serotonin. *International Journal of Biochemistry and Cell Biology*, 125: 105776. doi:
504 10.1016/j.biocel.2020.105776.

505 Kim, G.H., Lee, Y.C., Kim, T.J., Kim, E.R., Hong, S.N., Chang, D.K. and Kim, Y.H. 2022.
506 Risk of Neurodegenerative Diseases in Patients with Inflammatory Bowel Disease: A
507 Nationwide Population-based Cohort Study. *Journal of Crohn's & Colitis*, 16(3): 436–443. doi:
508 10.1093/ecco-jcc/jjab162.

509 Kip, E. and Parr-Brownlie, L.C. 2023. Healthy lifestyles and wellbeing reduce
510 neuroinflammation and prevent neurodegenerative and psychiatric disorders. *Frontiers in*
511 *Neuroscience*, 17: 1092537. doi: 10.3389/fnins.2023.1092537.

512 Latorre, E., Layunta, E., Grasa, L., Castro, M., Pardo, J., Gomollón, F., Alcalde, A.I. and
513 Mesonero, J.E. 2016. Intestinal serotonin transporter inhibition by toll-like receptor 2 activation.
514 A feedback modulation. *PLoS ONE*, 11(12): e0169303. doi: 10.1371/journal.pone.0169303.

515 Layunta, E., Buey, B., Mesonero, J.E. and Latorre, E. 2021. Crosstalk Between Intestinal
516 Serotonergic System and Pattern Recognition Receptors on the Microbiota–Gut–Brain Axis.
517 *Frontiers in Endocrinology*, 12: 748254. doi: 10.3389/fendo.2021.748254.

518 Layunta, E., Latorre, E., Forcén, R., Grasa, L., Castro, M., Arias, M.A., Alcalde, A.I. and

519 Mesonero, J.E. 2018. NOD2 Modulates Serotonin Transporter and Interacts with TLR2 and
520 TLR4 in Intestinal Epithelial Cells. *Cellular Physiology and Biochemistry*, 47(3): 1217–1229.
521 doi: 10.1159/000490218.

522 Layunta, E., Latorre, E., Grasa, L., Arruebo, M.P., Buey, B., Alcalde, A.I. and Mesonero, J.E.
523 2022. Intestinal serotonergic system is modulated by Toll-like receptor 9. *Journal of Physiology*
524 *and Biochemistry*, 78(3), pp. 689–701. doi: 10.1007/s13105-022-00897-2.

525 Li, Y., Zhang, Z.H., Huang, S.L., Yue, Z.B., Yin, X.S., Feng, Z.Q., Zhang, X.G. and Song, G.L.
526 2023. Whey protein powder with milk fat globule membrane attenuates Alzheimer's disease
527 pathology in 3×Tg-AD mice by modulating neuroinflammation through the peroxisome
528 proliferator-activated receptor γ signaling pathway. *Journal of Dairy Science*, 106(8): 5253–
529 5265. doi: 10.3168/jds.2023-23254.

530 Malynn, S., Campos-Torres, A., Moynagh, P. and Haase, J. 2013. The pro-inflammatory
531 cytokine TNF-α regulates the activity and expression of the serotonin transporter (SERT) in
532 astrocytes. *Neurochemical Research*, 38(4): 694–704. doi: 10.1007/s11064-012-0967-y.

533 Markus, C.R., Olivier, B. and De Haan, E.H.F. 2002. Whey protein rich in α-lactalbumin
534 increases the ratio of plasma tryptophan to the sum of the other large neutral amino acids and
535 improves cognitive performance in stress-vulnerable subjects. *American Journal of Clinical*
536 *Nutrition*, 75(6): 1051–1056. doi: 10.1093/ajcn/75.6.1051.

537 Martorell, P., Llopis, S., Gonzalez, N., Ramón, D., Serrano, G., Torrens, A., Serrano, J.M.,
538 Navarro, M. and Genovés, S. 2016. A nutritional supplement containing lactoferrin stimulates
539 the immune system, extends lifespan, and reduces amyloid β peptide toxicity in *Caenorhabditis*
540 *elegans*. *Food Science and Nutrition*, 5(2): 255–265. doi: 10.1002/fsn3.388.

541 Masanetz, R.K., Winkler, J., Winner, B., Günther, C. and Süss, P. 2022. The Gut–Immune–
542 Brain Axis: An Important Route for Neuropsychiatric Morbidity in Inflammatory Bowel
543 Disease. *International Journal of Molecular Sciences*, 23(19): 11111. doi:
544 10.3390/ijms231911111.

545 Mika, A., Gaffney, M., Roller, R., Hills, A., Bouchet, C.A., Hulen, K.A., Thompson, R.S.,
546 Chichlowski, M., Berg, B.M. and Fleshner, M. 2018. Feeding the developing brain: Juvenile
547 rats fed diet rich in prebiotics and bioactive milk fractions exhibit reduced anxiety-related
548 behavior and modified gene expression in emotion circuits. *Neuroscience Letters*, 677: 103–
549 109. doi: 10.1016/j.neulet.2018.01.052.

550 Mitchell, J., Kim, S.J., Howe, C., Lee, S., Her, J.Y., Patel, M., Kim, G., Lee, J., Im, E. and
551 Rhee, S.H. 2022. Chronic Intestinal Inflammation Suppresses Brain Activity by Inducing
552 Neuroinflammation in Mice. *American Journal of Pathology*, 192(1): 72–86. doi:
553 10.1016/j.ajpath.2021.09.006.

554 Osorio-González, C.S., Gómez-Falcon, N., Brar, S.K. and Ramírez, A.A. 2022. Cheese Whey
555 as a Potential Feedstock for Producing Renewable Biofuels: A Review. *Energies*, 15(18): 6828.
556 doi: 10.3390/en15186828.

557 Timby, N., Domellöf, E., Hernell, O., Lönnerdal, B. and Domellöf, M. 2014.
558 Neurodevelopment, nutrition, and growth until 12 mo of age in infants fed a low-energy, low-
559 proteinformula supplemented with bovine milk fat globule membranes: A randomized
560 controlled trial. *American Journal of Clinical Nutrition*, 99(4): 860–868. doi:
561 10.3945/ajcn.113.064295.

562 Valero, M.S., González, M., Ramón-Gimenez, M., Andrade, P.B., Moreo, E., Les, F.,
563 Fernandes, F., Gómez-Rincón, C., Berzosa, C., García de Jalón, J.A., Arruebo, M.P., Plaza,
564 M.Á., Köhler, R., López, V., Valentão, P. and Castro, M. 2020. Jasonia glutinosa (L.) DC., a
565 traditional herbal medicine, reduces inflammation, oxidative stress and protects the intestinal
566 barrier in a murine model of colitis. *Inflammopharmacology*, 28(6): 1717–1734. doi:
567 10.1007/s10787-019-00626-0.

568 Veereman-Wauters, G., Staelens, S., Rombaut, R., Dewettinck, K., Deboutte, D., Brummer,
569 R.J., Boone, M. and Le Ruyet, P. 2012. Milk fat globule membrane (INPULSE) enriched
570 formula milk decreases febrile episodes and may improve behavioral regulation in young

children. *Nutrition*, 28(7–8): 749–752. doi: 10.1016/j.nut.2011.10.011.

Yu, X.C., Li, Z., Liu, X.R., Hu, J.N., Liu, R., Zhu, N. and Li, Y. 2021. The antioxidant effects of whey protein peptide on learning and memory improvement in aging mice models. *Nutrients*, 13(6): 2100. doi: 10.3390/nu13062100.

Yuan, Q., Gong, H., Du, M., Li, T. and Mao, X. 2022. Milk fat globule membrane supplementation to obese rats during pregnancy and lactation promotes neurodevelopment in offspring via modulating gut microbiota. *Frontiers in Nutrition*, 9: 945052. doi: 10.3389/fnut.2022.945052.

Zandona, E., Blažić, M. and Režek Jambrak, A. 2021. Whey utilisation: Sustainable uses and environmental approach', *Food Technology and Biotechnology*, 59(2): 147–161. doi: 10.17113/ftb.59.02.21.6968.

Zhao, L.P., Wu, J., Quan, W., Zhou, Y., Hong, H., Niu, G.Y., Ting-Li, Huang, S.B., Qiao, C.M., Zhao, W.J., Cui, C. and Shen, Y.Q. 2023. DSS-induced acute colitis causes dysregulated tryptophan metabolism in brain: an involvement of gut microbiota. *Journal of Nutritional Biochemistry*, 115: 109282. doi: 10.1016/j.jnutbio.2023.109282.

Zheng, Y., Qin, Z., Ye, Q., Chen, P., Wang, Z., Yan, Q., Luo, Z., Liu, X., Zhou, Y., Xiong, W., Ma, J. and Li, G. 2014. Lactoferrin suppresses the Epstein-Barr virus-induced inflammatory response by interfering with pattern recognition of TLR2 and TLR9. *Laboratory Investigation*, 94(11): 1188–1199. doi: 10.1038/labinvest.2014.105.

Zhu, C. Bin, Blakely, R.D. and Hewlett, W.A. 2006. The proinflammatory cytokines interleukin-1beta and tumor necrosis factor-alpha activate serotonin transporters. *Neuropsychopharmacology*, 31(10): 2121–2131. doi: 10.1038/sj.npp.1301029.

Zonis, S., Pechnick, R.N., Ljubimov, V.A., Mahgerefteh, M., Wawrowsky, K., Michelsen, K.S. and Chesnokova, V. 2015. Chronic intestinal inflammation alters hippocampal neurogenesis. *Journal of Neuroinflammation*, 12: 65. doi: 10.1186/s12974-015-0281-0.

Figure captions

Fig. 1. Assessment of DSS-induced damage in the colon. For experimental induction of colitis mice were daily treated for 5 days with 2.5% (w/v) solution of DSS. **A**: Colon length. **B**: Macroscopic damage score calculated for each animal according to a standard scoring system. Data are presented as the mean ± SEM of four animals (n=4). **p<0.01 compared to control group. **C-D**: Images of hematoxylin-eosin staining of distal colon from control (C) and DSS (D) groups. Arrows: infiltration of inflammatory cells; asterisk: edema. Scale bar: 100 μm.

Fig. 2. Analysis of the inflammatory state of the brain of mice treated with DSS. **A**: Histopathological images of brain sections corresponding to mice from the control (a) and DSS (b) groups. Scale bar: 2 mm. Detail of the frontal cortex corresponding to mice from the control (c) and DSS (d) groups. Scale bar: 300 μm. No evident lesions were observed in the cerebral cortex, cerebellum, or hypothalamus in any of the groups. Sections were stained with hematoxylin-eosin. **B**: Effect of whey and buttermilk on mRNA expression levels of IL-1β, IL-6, and TNF-α in the brain of mice treated with DSS. mRNA levels were determined using qPCR and expressed in arbitrary units (control = 1) and presented as the mean ± SEM of four animals (n=4). **p<0.01 and ***p<0.001 compared to control. ##p<0.01 and ###p<0.001 compared to the DSS group.

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623 **Fig. 4.** Effect of whey- and buttermilk-based formulas on the serotonergic system in the brain of
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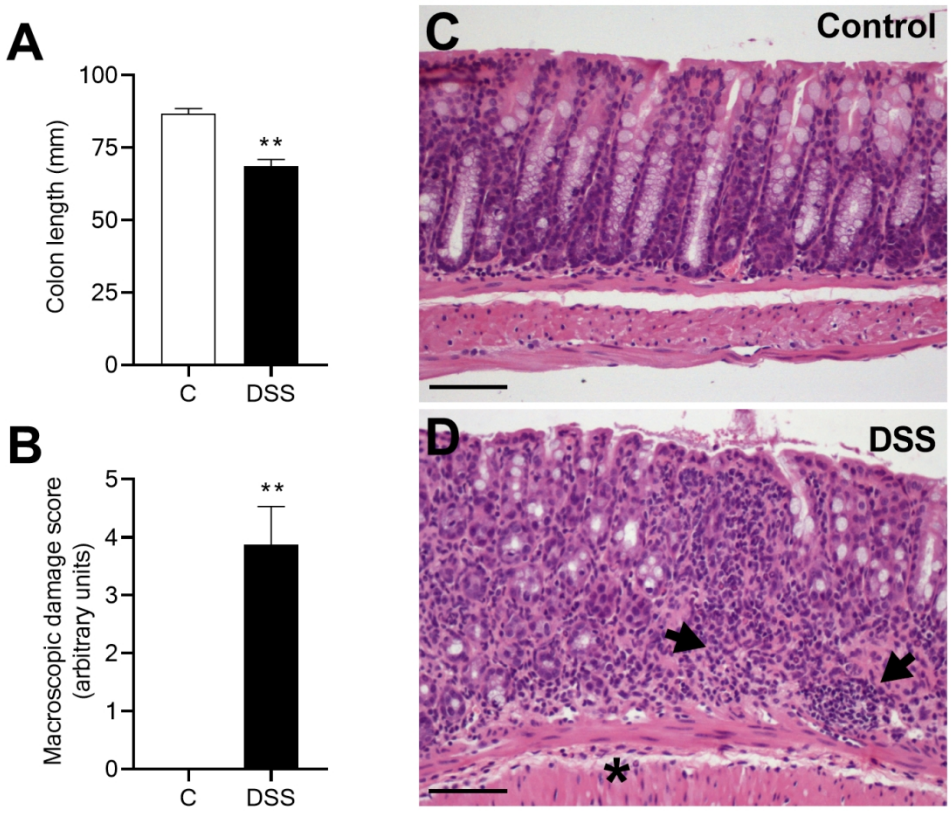
630

Table 1. Concentration of product and total protein present in the whey, buttermilk, and MFGM fractions used in whey- and buttermilk-based formulas.

Fractions	% dry matter	mg protein/mL	mg protein/g product
Whey	6.8	10.46	153.79
Buttermilk	8.7	11.89	136.72
MFGM	10	9.77	97.72

Table 2. Primer sequences used for real-time PCR analysis of brain mice samples.

Gen	Forward (5'-3') and reverse (3'-5') primers
SERT	GGCAACATCTGGCGTTTTCC // ATTTCGGTGGTACTGGCCCA
TPH2	GAGTTGCTCCACGCTTTGC // ACACTCAGTCTACATCCATCCC
5-HTR_{1A}	TCTGTGAGAGCAGTTGCCACAT // AGCGGCAGAACTTGCACTTGAT
5-HTR_{2A}	TGCCGTCTGGATTTACCTGGATGT // TACGGATATGGCAGTCCACACCAT
5-HTR_{2B}	AGGAAATGAAGCAGACTGTGGAGG // CAGTGCAACAGCCAGAATCACAAG
5-HTR_{3}	TCTTGCTGCCCAGTATCTTCCTCA // TTATGCACCAGCCGCACAATGAAG
5-HTR_{4}	AATGCAAGGCTGGAACAACATCGG // TGTATCTGCTGGGCATGCTCCTTA
5-HTR_{7}	TCTTCGGATGGGCTCAGAATGT // AACTTGTGTTTGGCTGCGCT
NOD1	CACAGCGCTCTTCACTTTTG // GTTAGCCAGCAGGACCAGAG
NOD2	CTTCATTTGGCTCATCCGTAG // CTGGAGATGTTGCAGTACAAAG
TLR1	TCTTCGGCACGTTAGCACTG // CCAAACCGATCGTAGTGCTGA
TLR2	GCCACCATTTCCACGGACT // GGCTTCCTCTTGGCCTGG
TLR3	GGTCCCCAGCCTTCAAAGAC // ACGAAGAGGGCGGAAAGGT
TLR4	AGAAATTCCTGCAGTGGGTCA // TCTCTACAGGTGTTGCACATGTCA
TLR5	ATGGCATGTCAACTTGACTT // GATCCTAAGATTGGGCAGGT
TLR6	TCATCTCAGCAAACACCGAGTATAGC // CAACCTTATTGAATGTGACCCTCCAGC
TLR7	GTACCAAGAGGCTGCAGATTAGAC // AGCCTCAAGGCTCAGAAGATG
TLR8	GAAGCATTTCGAGCATCTCC // GAAGACGATTTCGCCAAGAG
TLR9	ACTTCGTCCACCTGTCCAA // AGGAAGGTTCTGGGCTCAAT
TLR11	TCCTTCCTCTGATTAGCTGTCCTAA // TCCACATAATTTCCACCAACAAGT
IL-6	TCCTACCCCAATTTCCAATGC // TGAATTGGATGGTCTTGGTCCT
IL-1β	CTCGTGCTGTCGGACCCAT // CAGGCTTGTGCTCTGCTTGTGA
TNF-α	AAATGGGCTTTCCGAATTCA // CAGGGAAGAATCTGGAAAGGT
HPRT1	CTGGTGAAAAGGACCTCTCGAA // CTGAAGTACTCATTATAGTCAAGGGCAT
GAPDH	AACGACCCCTTCATTGAC // TCCACGACATACTCAGCAC



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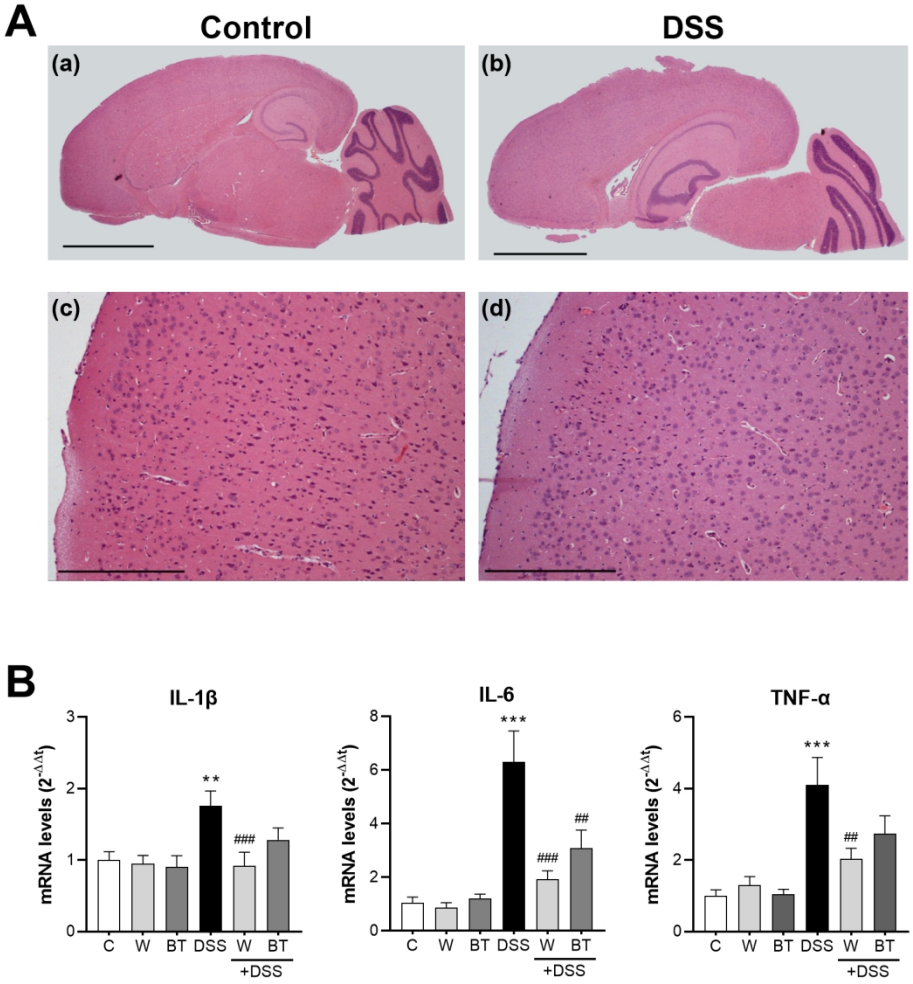

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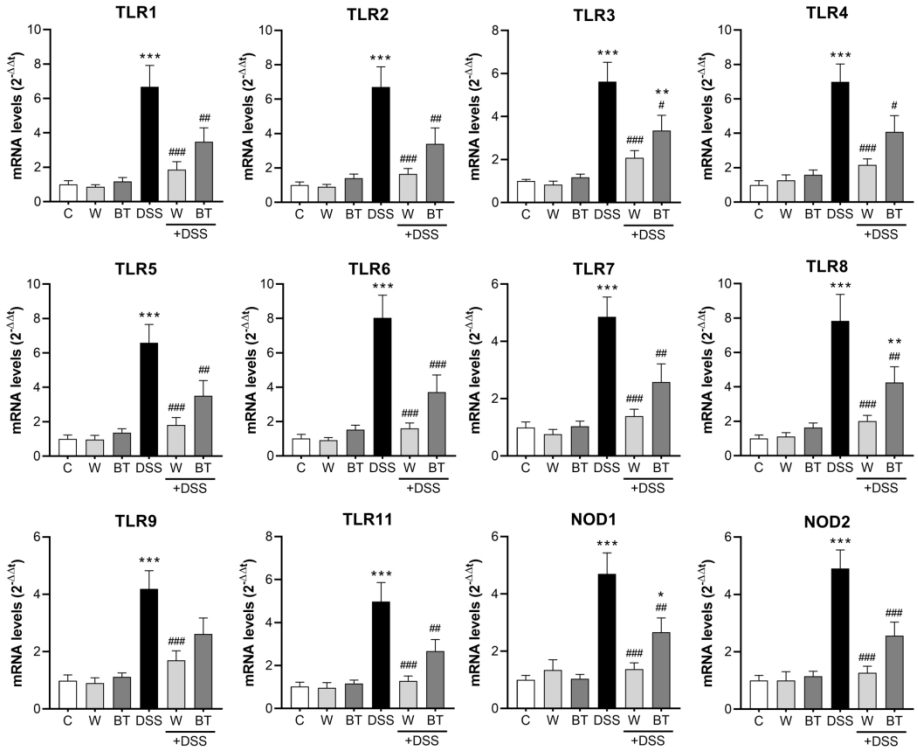

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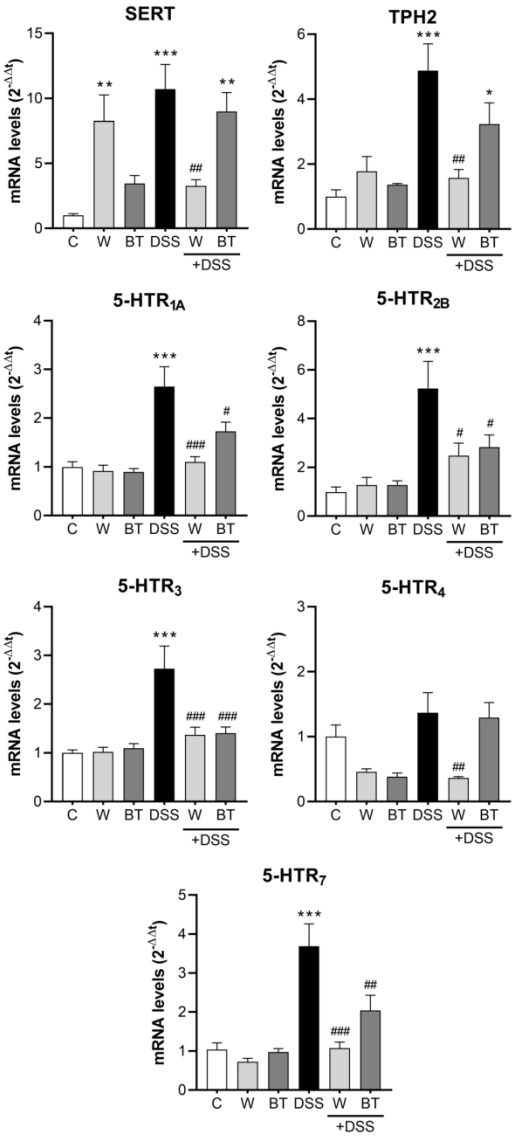

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106x208mm (300 x 300 DPI)