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Neuromodulatory and possible anxiolytic-like effects of a spice functional food ingredient in a pig model of psychosocial chronic stress

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ABSTRACT

Psychological chronic stress is associated with the development of mood disorders, and spices have shown protective properties in this context. This research investigated the effects of a supplementation with a functional food ingredient containing spice extracts in a pig model of psychosocial chronic stress. Its impact on behavior, neurophysiology, immune system and gastrointestinal tract were evaluated. Almost no significant results were found at the gut and immune levels. An increased expression of 5-HT_{1A}R and BDNF in the hippocampus and prefrontal cortex, respectively, and blood perfusion changes in several brain regions including the olfactory bulb, hippocampus, dorsolateral prefrontal cortex and dorsal anterior cingulate cortex were observed. Also, slight anxiolytic-like effects were observed in the Open-field and Novelty-Suppressed Feeding tests. These modulations of brain regions associated with the regulation of emotions and cognition as well as the potential effects on anxiety might come from the repeated stimulation of the olfactory system.

1. Introduction

1.1. Chronic stress and disease

Psychological chronic stress is of major concern nowadays, as it is highly associated with the development of mood disorders such as anxiety and depression (Caspi, 2003; Richter-Levin & Xu, 2018). Studies on rodent models have shown that chronic stress is susceptible to induce the emergence of depressive- and/or anxiety-like behaviors (Mineur, Belzung, & Crusio, 2006; Willner, 2017), in association with neurophysiological disturbances including reduced hippocampal neurogenesis, monoaminergic systems imbalances (Willner, 2017), deregulations of the hypothalamo-pituitary-adrenal axis (HPA) secretory function, and low-grade inflammation (Goshen et al., 2008; Zhang et al., 2015). Chronic stress also triggers gastrointestinal alterations, especially regarding intestinal barrier structure, function, and microbiota composition (Cryan & Dinan, 2012, 2015; Dinan, Stilling, Stanton, & Cryan, 2015; Moloney et al., 2016; Valles-Colomer et al., 2019).

1.2. Spices extracts and mood disorders

Active compounds of turmeric (curcuminoids), ginger (gingerols and shogaols), black pepper (piperines), and chili pepper (capsaicinoids) have been used for centuries in traditional medicines for their antioxidant, anti-inflammatory, and/or antimicrobial properties (Kunnumakkara et al., 2018; Kunnumakkara, Koca, Dey, Gehlot, Yodkeeree, Danda, & Aggarwal, 2009). Aside from these effects, they also have beneficial effects on mood disorders. Oral supplementation with curcumin capsules decreased depression and/or anxiety scores, potentiated the effects of antidepressant drugs in human (Al-Karawi, Al Mamoori, & Tayyar, 2016; Kanchanatawan et al., 2018; Saberi-Karimian et al., 2018), decreased circulating levels of inflammatory cytokines, cortisol, and increased neurotrophic factor BDNF levels (Al-Karawi et al., 2016; Esmaily et al., 2015; Saberi-Karimian et al., 2018; Yu, Pei, Zhang, Wen, & Yang, 2015). In rodent chronic stress models, curcumin showed antidepressant effects by reversing both behavioral despair and anhedonia (Liu et al., 2014; Mao, Huang, Zhong, Xian, & Ip, 2014b; Xu et al., 2005b; Zhang et al., 2014), also restoring the HPA axis secretory function (Xu et al., 2006) and monoaminergic balance (Bhutani, Bishnoi, & Kulkarni, 2009; Kulkarni, Bhutani, & Bishnoi, 2008; Xu et al., 2005b). It also increased hippocampal neurogenesis (Xu et al., 2007), notably by elevating BDNF expression, 5-HT_{1A} receptor density, and other neurotrophic-related factors levels (Liu et al., 2014; Xu et al., 2006). While former studies have thoroughly studied the

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impact of curcumin, piperine, another spice extract, demonstrated antidepressant capabilities (Wattanathorn, Chonpathompikunlert, Muchimapura, Priprem, & Tankamnerdthai, 2008), especially through BDNF signaling (Li et al., 2007; Mao et al., 2014b). Similarly, ginger extracts have been found to have anxiolytic-like effects on mice (Fadaki, Modaresi, & Sajjadian, 2017), while capsaicin improved the effect of a tricyclic antidepressant and antidepressant-like effects in the forced-swimming test in rats (Reyes-Mendez et al., 2018). Interactions between spice extracts and drug therapy have been described. For example, piperine is able to potentiate the effects of curcumin on depressive- and anxiety-like behaviors (Bhutani et al., 2009; Jangra et al., 2016). It is interesting to notice that curcumin and piperine have both been particularly investigated, but there is a lack of studies on other spices extracts.

1.3. Spices extracts and gastrointestinal and immune modulations

Curcumin and ginger extracts showed beneficial effects on tight junctions and intestinal permeability (Ghosh, He, Wang, Gehr, & Ghosh, 2018; Huh et al., 2018; Wang, Ghosh, & Ghosh, 2017) and on the regulation of inflammation and apoptosis (Huh et al., 2018; Montserrat-de la Paz, Garcia-Gimenez, Quilez, De la Puerta, & Fernandez-Arche, 2018). Curcumin affected the relative diversity and/or abundance of several microbiota representative families (McFadden et al., 2015; Shen, Liu, & Ji, 2017; Zam, 2018). Finally, curcumin and piperine have shown anti-insulin-resistance properties (Choi et al., 2013; Jiménez-Osorio, Monroy, & Alavez, 2016; Na et al., 2011; Shao et al., 2012; Shen, Wei, Li, Qiao, & Li, 2017), and curcumin increased ghrelin expression (Xu, Li, & Guo, 2013) and GLP-1 secretion (Kato et al., 2017). However, the links between the neurobehavioral protective effects of spices and their modulation of gastrointestinal functions have been poorly investigated in the context of mood disorders. It is likely that protective effects of spices could come from a regulation at the gastrointestinal and immune levels that would ultimately influence the brain. Indeed, many studies that showed effects of spices in human used capsules (Al-Karawi et al., 2016; Kanchanatawan et al., 2018; Saberi-Karimian et al., 2018). In rodent studies, compounds were administered *via* intraperitoneal injection (Mao, Huang, Zhong, Xian, & Ip, 2014a), by gavage (Liu et al., 2014) or diluted in oil (Xu et al., 2005a).

1.4. Our study

In the present study we investigated the effects of a functional ingredient containing a blend of *Curcuma longa L.*, *Piper nigrum L.*, *Capsicum anuum L.*, and *Zingiber officinale L.* extracts in a pig psychosocial chronic stress model based on social isolation combined with environmental impoverishment and unpredictability. This model has been previously described and validated by our team (Menneson et al., 2019). We demonstrated that it produced alterations of behavior and the HPA axis, as well as of the microbiota-gut-brain axis. The observed effects encompassed more resignation, higher levels of cortisol, decreased microbiota fermentation activity and increased intestinal permeability. There were also lower functional brain responses to a novel olfactory stimulus, lower hippocampal neuronal density and cell proliferation, and lower expression of BDNF and 5-HT_{1A} receptor.

The originality of our study is that we studied the impact of a complex functional food ingredient in an animal model with a particularly good face validity, especially regarding brain, microbiota and gut physiology, and performed repeated behavioral and physiological explorations in combination with specific *in vivo* and *post mortem* explorations at the gut and brain levels. Our hypothesis was that the functional ingredient would alleviate several criteria associated with chronic stress, especially depressive- and anxiety-like behaviors.

2. Materials and methods

2.1. Ethics statement

Experiments were conducted in accordance with the current ethical standards of the European Community (Directive 2010/63/EU), Agreement No. C35-275-32 and Authorization No. 35-88. The Regional Ethics Committee in Animal Experiment of Brittany has validated the entire procedure described in this paper (project n°2017080511347475) and the French Ministry of Research approved it.

2.2. Animals, housing and experimental diets

Experiments were carried out from October to December 2017 at the INRA experimental research station of Saint Gilles (France) on twenty-four Piétrain × (Large White/Landrace) pigs. Tails were cut, teeth were clipped, and males were castrated a few days after birth, in accordance with usual practices in commercial pig facilities. Piglets were housed in conventional farrowing crates until weaning at 28 days and then mixed in larger groups. When 63-day-old, they were moved to the experimental building and separated into two rooms according to their group. They got used to their novel environment for one week. They were then subjected to a multifactorial psychosocial chronic stress characterized by social isolation, environmental impoverishment and unpredictability (Menneson et al., 2019). They were housed in individual pens (0.85 × 2.65 m) with an empty pen between each to avoid physical and eye contact, without enrichment and with unpredictable sounds (sirens, metallic noises, gunshots, *etc.*) and lights randomly diffused (every 10 min ± 30% during the day, every 120 min ± 30% during the night). After habituation, animals of the standard group (SC, n = 12) were still fed with the standard food, and animals of the functional ingredient group (SF, n = 12) received food supplemented with a functional ingredient provided by Phodé (Terssac, France). Animals received a daily feed ration adapted to their weight/age (from 1 to 1.8 kg/day along the experimental period, equivalent to a metabolizable energy of 3020–5440 kcal/day). The feed was composed of 32% wheat, 15% corn, 25% barley, 5% wheat bran, 7% rapeseed, 11% soya meal, 1% vegetal oil, 1.5% calcium carbonate, 0.1% bi-calcic phosphate, 0.5% salt, 0.5% vitamin and mineral premix complement. The functional ingredient contained extracts of spices (1%) and was formulated as an extended release type solid galenic form with a vehicle composed of surfactant (1%), silica (5%), water (9%), monoglycerides (39%) and cellulose (45%). It was incorporated into food at 1% w/w, corresponding to 0.13 mg/kg of live weight/day of each of the four main families of active compounds: capsaicinoids, piperines, curcuminoids and gingerols. Capsaïcinoids consisted in 65 ± 5% of capsaicin and 35 ± 5% of dihydrocapsaicin; piperines isomers contained piperine, isopiperine and chavicine; gingerols were composed of 65 ± 5% of 6-gingerol, 10 ± 2% of 8-gingerol, 17.5 ± 2.5% of 10-gingerol; curcuminoids included 75 ± 5% of curcumin, 21 ± 4% of desmethoxycurcumin, and 4 ± 1% of bismethoxycurcumin. The vehicle without spice extracts was incorporated to the control feed. Both groups were composed of 6 males and 6 females. Eight additional females per group were necessary for the *in vivo* brain imaging sessions, leading to a total of 20 animals in both groups, originating from 13 different litters and randomly distributed. Females only were subjected to imaging session to limit variability, as there is an important brain functioning sexual dimorphism. Animals had unlimited access to water, were regularly provided with straw, and were subjected to a natural day/night cycle (with a light period progressively decreasing from 12 h to around 8 h30).

2.3. Experimental design (Fig. 1)

2.3.1. Behavioral observations

2.3.1.1. Behavior in the home pens. Pigs' postures, behaviors, and vocalizations were recorded 6 times in the home pens with a one-minute scan sampling (1 scan every 10 s). Data were analyzed *via* a Principal Component Analysis (PCA) on the FactoMineR plugin of R commander. Behaviors were first compared for each day, and then a second comparison was made on the average of the 6 observations.

2.3.1.2. Open-Field (OF) test. The test was conducted to study locomotion in a non-familiar environment as described in (Menneson et al., 2019). Briefly, animals were observed for 5 min in a 3.5 × 3.5 m arena and locomotion, vocalizations and number of flight escapes were recorded using The Observer XT 10 *(Noldus, Wageningen, The Netherlands)*.

2.3.1.3. Novelty-Suppressed feeding (NSF) test. The test was conducted to study anxiety-like behavior (Blasco-Serra, González-Soler, Cervera-Ferri, Teruel-Martí, & Valverde-Navarro, 2017; Bodnoff, Suranyi-Cadotte, Aitken, Quirion, & Meaney, 1988) the day following the OF test. The usual food of each pig was presented in the center of the OF arena and surmounted by a new object (yellow balloon) suspended 60 cm above the trough. The latency to eat was recorded.

2.3.1.4. Restraint test. This test was adapted from the forced-swimming and tail suspension tests used in rodents to study resignation as a depression-like symptom (David et al., 2009; Porsolt, Bertin, & Jalfre, 1977; Steru, Chermat, Thierry, & Simon, 1985), and processed as detailed in (Menneson et al., 2019). Pigs were equipped with two suspension harnesses and elevated with an electric-hydraulic system until the animals' feet rose off the ground. The total duration of mobility, number of attempts to escape, and of vocalizations were recorded for 5 min. A perseverance index was determined as the average duration of one escape attempt.

2.3.2. Single-Photon Emission Computed Tomography (SPECT)

Brain perfusion was quantified using SPECT imaging as previously described (Malbert, Genissel, Divoux, & Henry, 2019). The imaging sessions were performed between Weeks 7 and 10. Briefly, overnight-fasted anesthetized animals were injected with 370 MBq IV-^{99m}Technetium labelled with hexamethyl-propylene-amine-oxime (HMPAO, *Ceretec, GE Healthcare, Velizy, France)*. Anesthesia was achieved with isoflurane (1.8 minimal alveolar concentration) delivered by a mechanical ventilator. Eyes and ears were concealed to avoid external stimulations. Fifteen minutes after the administration of ^{99m}Tc-HMPAO, while the radioactive label has been incorporated by neurons and circulating fraction has been washed-out, 180-degree acquisitions were performed (3° Step, 45 s per frame, 128 * 128 pixels per frame) using a double head gamma camera (Millenium VG, GE). The corresponding projections were reconstructed using OSEM algorithm with Xeleris 2.1 software (GE *Healthcare, Velizy, France*) and filtered afterward with a non-local means filter. Attenuation was calculated and removed using Chang correction. Scatter compensation was also achieved using the Jaszczak subtraction method (Jaszczak, Floyd, & Coleman, 1985). Images were manually reoriented and coregistered to a SPECT template obtained in the laboratory and already validated on pigs of the same age and weight (Sauleau, Lapouble, Val-Laillet, & Malbert, 2009). A "without a priori" analysis was performed using SPM 12 on the whole brain to identify regions differentially activated between groups. A two-sample *t*-test was performed to compare the SPECT brain images between SF (n = 8) and SC (n = 7) animals. Indeed, one SC animal was diagnosed with a circle of Willis anomaly during the imaging session, leading to possible bias in blood perfusion and was thus excluded. The number of days of stress application was used as a covariate, and the threshold was set at $p = 0.05$ uncorrected. A small-volume correction (SVC) analysis was performed afterwards on thirty regions of interest (ROIs), corresponding to fifteen bilateral brain structures previously identified in studies with functional food ingredients (Val-Laillet, Meurice, & Clouard, 2016) and chronic stress (Menneson et al., 2019). These regions encompass the olfactory bulb, prepyriform area, insular cortex, hippocampus, amygdala, anterior and dorsolateral PFC, orbitofrontal cortex, ventral and dorsal anterior and posterior cingulate cortex, putamen, caudate, and accumbens nuclei. Those brain ROIs were identified according to the three-dimensional digital pig brain atlas developed in our laboratory (Saikali et al., 2010). All the cerebral ROIs used for the SVC analysis were constructed upon the anatomical volumes created for this atlas. The threshold was set at $p = 0.05$, corresponding to $p = 0.0016$ after Bonferroni correction.

2.3.3. Biological sampling and quantitative analyses

All samples were collected in the morning, and all samples except feces were collected on overnight-fasted animals.

2.3.3.1. Salivary samples. For one minute, pigs were allowed to chew cotton buds *(*Salivette®, *Sarstedt, Germany)* that were then rapidly centrifuged (2,500 g, 10 min, 4 °C). Supernatants were stored at −20 °C until cortisol quantification with a luminescence immunoassay kit *(LIA, Germany). The sensitivity was 0.004 μg/dl and the intra-assay CV was < 5%.*

2.3.3.2. Feces. Samples were manually collected by rectal stimulation and processed as described in (Lemaire et al., 2018) for quantification of short chain fatty acids (SCFAs) until gas chromatography analysis (Jouany et al., 1981). A part of feces was immediately frozen in liquid nitrogen for later 16S RNA sequencing. The DNA extraction and sequencing was performed as described in (Lemaire et al., 2018). The analysis of α- and β-diversity was also performed as mentioned in this publication (corresponding respectively to the intra- and inter-group variability). For differential abundance analyses, the negative binomial model (DESeq2 analysis) was fitted at phyla level, after aggregation at the corresponding taxonomic rank.

2.3.3.3. Blood samples. Samples were collected at 8:30 am (at dawn or just after) from the jugular vein into EDTA (1.8 mg EDTA/mL of blood), heparin *(Vacutest Kima, Italy)* or aprotinin *(BD Life Science, France)* vacutainers, depending on the assay. Samples were kept in ice until centrifugation (2500*g*, 10 min, 4 °C) and plasma was stored at −20 °C or −80 °C until the quantification of glucose, haptoglobin, insulin, LPS, GLP-1, PYY, ghrelin and leptin.

2.3.3.4. Plasmatic quantitative analyses. Blood from one of the EDTA vacutainers was rapidly transferred into a tube containing anti-DPP IV (10 μl/ml of blood; *Merck, Germany)*. Glucose and haptoglobin levels were assessed by an automated spectrophotometric method (*Konelab 20i, Thermo Fisher Scientific, France*) using respectively *Thermo Fisher Scientific* and *Eurobio* kits. The sensitivity was respectively of 0.1 mmol/l and 0.005 mg/ml, and the intra-assay CV < 5% and < 7%. Insulin, ghrelin and leptin levels were assayed using RIA kits (respectively *EMD Millipore, MA, USA, sensitivity: 0.05 μU/ml), Phoenix France S.A.S., Strasbourg, France (sensitivity: 84.7 pg/ml), and Immunodiagnostic system, Paris, France (sensitivity: 0.801 ng/ml)*. LPS, GLP-1 and PYY levels were measured using ELISA kits (*Mybiosource, CA, USA (sensitivity: 5 ng/ml); Millipore Corporation, USA (sensitivity: 2 pM); Phoenix Pharmaceuticals inc (sensitivity: 0.13 ng/ml)*.

2.3.3.5. Post mortem sampling. Pigs were euthanized in the morning of Weeks 7–9 in the experimental slaughterhouse by electrical stunning immediately followed by exsanguination. Blood was collected in sterile BD vacutainer® CPT™ tubes *(BD Biosciences, France)* for isolation of peripheral blood mononuclear cells (PBMC) and cytokines quantification (see supplementary materials and results). Left

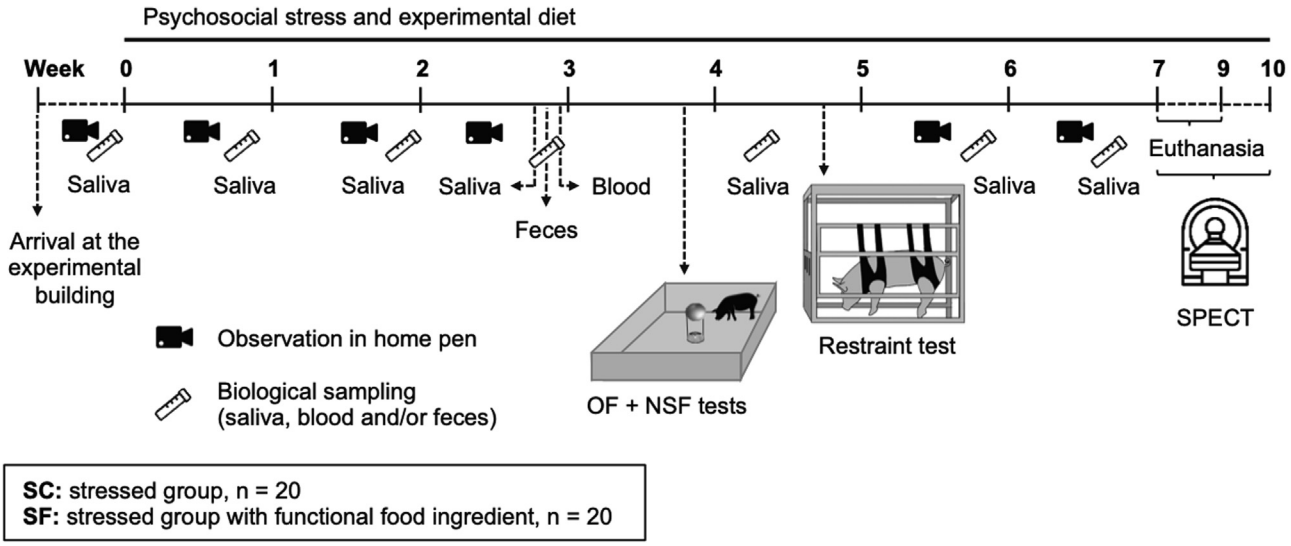


Fig. 1. Experimental paradigm. At their arrival at the experimental building, pigs had one week to get used to their new environment and food and were then subjected to the psychosocial stress consisting in social isolation combined with environmental impoverishment and unpredictability, and to the experimental food. Behavioral observations were performed in home pens during the habituation week, and in Weeks 0, 1, 2, 5 and 6, on 12 pigs per group. Anxiety- and depression-like behaviors were also assessed during the Open-field (OF), Novelty-suppressed feeding (NSF), and restraint tests in Weeks 3 and 4, on 12 pigs per group. Saliva samples were collected 7 times, and feces and blood were collected in Week 2. The 12 pigs per group subjected to behavioral observations and physiological samplings were euthanized between Weeks 7 and 9, and samples were collected for further analyses of brain, gut and immune cells. Single-Photon Emission Computed Tomography (SPECT) imaging was conducted on 8 animals per group between Weeks 7 and 10, to investigate the effects of the functional food ingredient on brain basal activity.

hippocampus, PFC and striatum were dissected and immediately frozen in liquid nitrogen for molecular biology analysis. Segments of jejunum and colon were collected and stored in cold DMEM *(Thermo Fisher Scientific, USA)* for Ussing chamber measurements, or fixed in 4% PFA (72 h, 4 °C) and stored (70% ethanol, 4 °C) until histological analyses.

2.4. Brain molecular biology

Total RNA was extracted from 100 mg of frozen tissue with phenol/chloroform treatment followed by silica membrane purification *(Qiagen, Germany)* and quantified by a microspectrophotometer *(Denovix, DE)*. Two µg of RNA were converted to cDNA using a High Capacity Complementary DNA Reverse Transcription Kit (Applied Biosystems, CA), and RT-PCR was performed with the StepOnePlus real-time PCR machine using Fast SyberGreen master mix *(Applied Biosystems)* for detection. Expression of BDNF, 5-HT_{1A}R, 5-HT_{2B}R, DRD2 was studied. ACTB, YWHAZ and B2M genes were selected as housekeeping genes according to their stability among experimental groups. Geometric mean of these housekeeping genes was calculated for each sample and used to determine the relative expression of each target gene with the $2^{-\Delta\Delta Ct}$ method. For primer sequences, see Table S1.

2.5. Intestinal analyses

2.5.1. Permeability. Colonic and jejunal tissues processing and measurements in Ussing chamber were performed as already described (Hamilton, Boudry, Lemay, & Raybould, 2015). Paracellular and transcellular permeability were respectively measured as the flux of FITC-4000 (FD-4; *Sigma-Aldrich*), and horseradish peroxidase (HRP Type VI-a; *Sigma Aldrich*).

2.5.2. Morphometry. Samples were embedded in paraffin, cut in sections of 7 µm, and stained with alcian blue *(Sigma)* and periodic acid Schiff *(VWR, France)*. They were examined under a white-light microscope *(ApoTome 2, Zeiss, Germany)*. Crypts characteristics were assessed in colon and jejunum, as well as villi characteristics in jejunum. The analysis was performed on 10 crypts and villi per animal. The number of goblet cells was also counted.

2.6. Statistical analysis

Blind statistical analyses were performed using SPSS software version 25 (IBM Corp). Comparisons were made with a two-way ANOVA (group × sex × group * sex), as there is a high variability in the way to respond to a similar stressor and to antidepressant drugs (Kokras & Dalla, 2017; Sapolsky, 1994; Willner, 2017). For cortisol data, a two-way repeated-measures ANOVA was also performed to investigate the effect of time (time × group). Residuals were tested for normality with the Shapiro-Wilk test. If they failed to pass normality test, data were log-transformed and the same analysis was performed on transformed data. Data are expressed as mean ± SEM. Differences were considered significant at $p < 0.05$ and as a trend when $0.05 < p < 0.1$.

3. Results

The effect of sex has been tested but was never found significant and thus will not be discussed later.

3.1. Body weight and food intake

Body weight the day of arrival did not differ between groups and was respectively 25.0 ± 0.7 kg for SC and 25.0 ± 0.9 kg for SF animals ($p = 0.96$). Animals were weighed weekly and no difference appeared during the entire experimental period, leading to a final weight of 63.7 ± 1.3 for SC and 61.7 ± 1.1 kg for SF animals ($p = 0.25$). All pigs ate daily their entire dedicated ration, and no difference was observed between groups (no food refusal and no behavioral differences at feeding).

3.2. Behavioral observations

3.2.1. Behavior in home pens

There was no behavioral difference detected between SC and SF animals in home pens.

3.2.2. OF test

Animals did not show difference in locomotion in the non-familiar environment measured as total duration ($F(1,20) = 0.341$, $p = 0.57$),

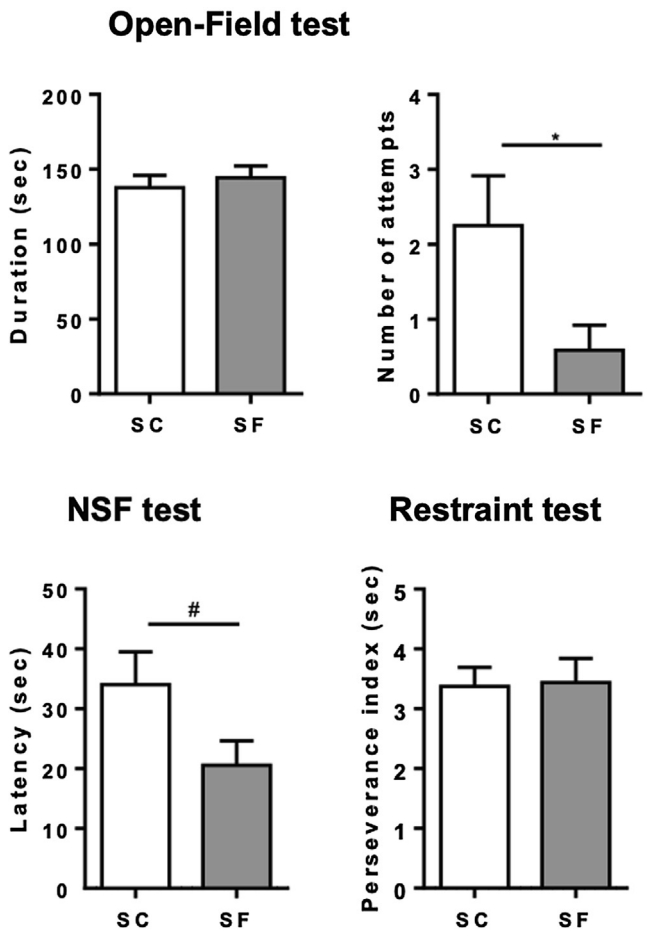


Fig. 2. Behavioral observations. Animals from the standard group (SC) and from the functional ingredient group (SF) exhibited the same level of locomotor activity in the Open-field test, but SF animals had a lower number of attempts to escape from the test arena. During the Novelty-suppressed feeding test, SF animals showed a trend for a lower latency to eat. The perseverance index characterized by the averaged duration of one attempt to escape did not differ between groups in the restraint test. N = 11–12/group, mean ± SEM, two-way ANOVA, # $p < 0.1$, * $p < 0.05$.

but SF animals had a lower number of attempts to escape than SC animals ($F(1,20) = 5.181$, $p = 0.03$) (Fig. 2). There was no difference in the number of vocalizations.

3.2.3. NSF test

There was a trend for a decreased latency to eat in SF compared to SC animals ($F(1,20) = 0.261$, $p = 0.07$) (Fig. 2).

3.2.4. Restraint test

The total duration (SC: 23.64 ± 3.37 sec and SF: 25.17 ± 4.06 sec, $F(1,19) = 0.068$, $p = 0.80$), number of attempts to escape (SC: 7.45 ± 0.90 and SF: 7.08 ± 0.81, $F(1,19) = 0.113$, $p = 0.74$), number of vocalizations (SC: 139.00 ± 14.21 and SF: 147.17 ± 15.60, $F(1,19) = 0.117$, $p = 0.75$) and perseverance index ($F(1,19) = 0.597$, $p = 0.74$) did not differ significantly between groups (Fig. 2).

3.3. Corticotropic axis (Fig. 3)

The cortisol level in saliva did not differ between groups at any moment of the experiment. However, it significantly decreased over time in both groups ($p < 0.0001$).

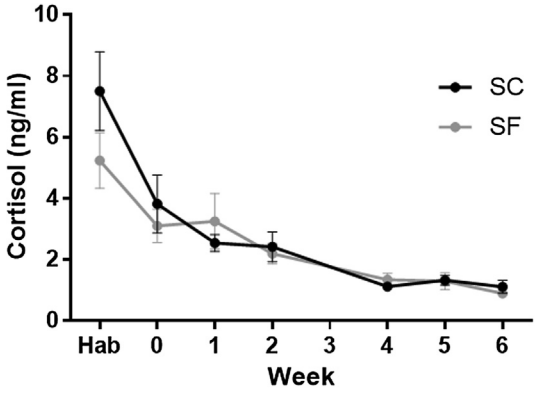


Fig. 3. Salivary cortisol evolution. No difference was detected in cortisol levels along time between the standard group (SC) and supplemented (SF) groups. However, cortisol significantly decreased over time in both groups ($p < 0.0001$). N = 11–12/group, mean +/- SEM, two-way ANOVA.

3.4. Brain systems and basal metabolism

3.4.1. Basal metabolism (Fig. 4)

The brain basal metabolism was significantly different between SC and SF animals. The whole-brain analysis showed that there was a higher metabolism in the olfactory bulb of SF animals, whereas the activity of thalamus, dorsolateral prefrontal cortex (dlPFC), dorsal anterior cingulate cortex (daCC), and hippocampus notably was higher in SC animals. The small volume correction second-level analysis performed on *a priori* ROIs highlighted a reduced brain metabolism in the left caudate nucleus and left daCC of SC animals.

3.4.2. BDNF and monoaminergic systems (Fig. 5)

The expression levels of 5-HT_{1A}R in the hippocampus and of BDNF in the PFC were higher in SF compared to SC animals ($p = 0.02$ and $p = 0.05$ respectively). No difference between groups was detected in the striatum.

3.5. Glucose metabolism and gut hormones (Table 1)

There was no significant difference of glucose and insulin levels between groups ($p = 0.61$ and $p = 0.47$ respectively). The analysis of plasma hormones did not show any difference in leptin ($p = 0.20$) and PYY ($p = 0.32$) levels, but there was a trend for a lower level of ghrelin and GLP-1 in SF compared to SC ($p = 0.09$ and $p = 0.06$).

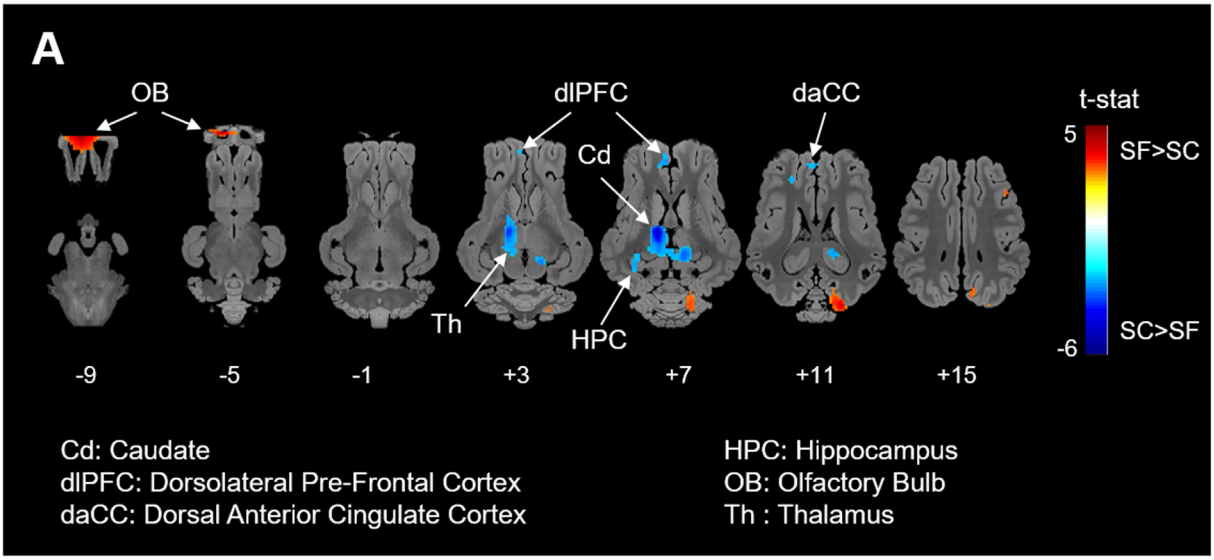
3.6. Microbiota fermentation activity and composition

The level of fermentation activity assessed by the quantification of SCFAs in feces did not differ in terms of total concentration level ($p = 0.33$). However, the level of caproate was lower ($p = 0.03$) and valerate tended to be lower ($p = 0.09$) in SF compared to SC. Results are summarized in Table 1. The analysis of α- and β-diversity did not highlight differences between supplemented and control animals. The composition of fecal microbiota did not differ at the phyla level.

3.7. Intestinal barrier function and immunity

The functional food ingredient did not impact the intestinal paracellular and transcellular permeability in jejunum and colon. There was also no difference in crypts and villis morphology as well as in the number of Goblet cells in these two tissues (Table 1).

Plasma levels of LPS tended to be lower in SF compared to SC animals (p = 0.09) but the haptoglobin levels did not differ (p = 0.64) (Table 1). The analysis of the peripheral immune system secretory activity did not highlight differences except for the IL-1β cytokine level



B

Anatomical area		Statistics		
ROI	Hemisphere	*t-value*	P_{FWE}	P_{Uncorr}
Caudate Nucleus	L	4.13	ns	0.0007
Dorsal Anterior cingulate Cortex	L	3.66	ns	0.0016

SVC-corrected treshold at $p < 0.0016$, ROI number = 30

Fig. 4. Basal brain metabolism. (Panel A) indicates the cerebral blood flow (CBF) significant differences between the standard group (SC) and supplemented group (SF) for the whole-brain analysis, where hot colors indicate a significantly higher CBF in SF animals, and cold colors a significantly higher CBF in SC animals. Notably, SC animals showed a higher basal CBF compared with SF animals in the thalamus, hippocampus, caudate nucleus, dorsolateral prefrontal cortex (dlPFC), and dorsal anterior cingulate cortex (daCC), whereas SF animals showed a higher basal CBF in the olfactory bulb. The *y* coordinates in the CA-CP (*commissura anterior-commissura posterior*) plane are indicated below the images. The significant threshold was set at $p = 0.05$ uncorrected. (Panel B) indicates the significant clusters highlighted with a small volume correction (SVC) analysis performed on a restricted number of regions of interest (*i.e.* bilateral hippocampus, amygdala, anterior, dorsolateral PFC, orbitofrontal cortex, insular cortex, ventral and dorsal anterior and posterior cingulate cortex, olfactory bulb, putamen, accumbens nuclei, pre-pyriform area and caudate nuclei). Only significant cluster at $p = 0.0016$ (Bonferroni correction) were indicated. N = 7–8/group.

that tended to be higher in SF animals when cells were stimulated with ConA (SC: 111.27 ± 17.85 pg/ml, SF: 188.16 ± 39.76 pg/ml, $p = 0,07$) (Table S2 in supplemental information).

4. Discussion

We comprehensively investigated the effects of a prolonged oral supplementation with a spice functional food ingredient in a validated pig model of psychosocial chronic stress. We demonstrated a modulation of blood perfusion of several brain regions as well as a modulation of BDNF and 5-HT_{1A} receptor expression, associated with slight behavioral anxiolytic-like effects. On the contrary, we failed to identify any significant effect of these compounds on gut, metabolic and immune functions.

4.1. Potential anxiolytic-like effects of spices

Our ethological observations did not emphasize behavioral differences in home pens, suggesting that the functional ingredient did not modulate the pigs' behavior in deteriorated housing conditions. However, it is important to argue here that the scan sampling observations were short. Longer observations might be more appropriate. Our chronic stress model was associated with the onset of resignation behavior in the restraint test (Menneson et al., 2019), which was not affected by the functional ingredient. This is surprising as spices and especially curcumin and piperine have shown antidepressant properties in rodent models (Bhutani et al., 2009; Liu et al., 2014; Mao et al., 2014b; Xu et al., 2005b; Zhang et al., 2014) and in depressive patients (Al-Karawi et al., 2016; Yu et al., 2015). However, the lower number of attempts to escape from the OF arena and the trend for a lower latency to eat in the NSF test are suggesting a potential anxiolytic effect of the ingredient. This last indicator is particularly used in the literature to study anxious behavior in rodents (Garcia-Garcia et al., 2018; Qin et al., 2015). Even though it has been less investigated, curcumin especially also reduced the Beck Anxiety Inventory score of patients with oral supplementation (Esmaily et al., 2015; Saberi-Karimian et al., 2018). Ginger extracts and capsaicin also showed anxiolytic effects in rodent models (Terzian, Aguiar, Guimarães, & Moreira, 2009; Vishwakarma, Pal, Kasture, & Kasture, 2002).

4.2. Brain and HPA axis modulations

The functional ingredient was found to regulate the serotoninergic system and neurotrophic factor expression in the hippocampus and prefrontal cortex, two regions altered in most mood disorders (Goto, Yang, & Otani, 2010; Kempermann, Krebs, & Fabel, 2008; Sapolsky, 2000). Similar changes have been reported with curcumin (Huang et al., 2011; Lian et al., 2018; Liu et al., 2014; Xu et al., 2006) and piperine (Mao et al., 2014b, 2014a). Moreover, one study also reported that ginger contains 5-HT_{1A}R agonist (Nievergelt, Huonker, Schoop,

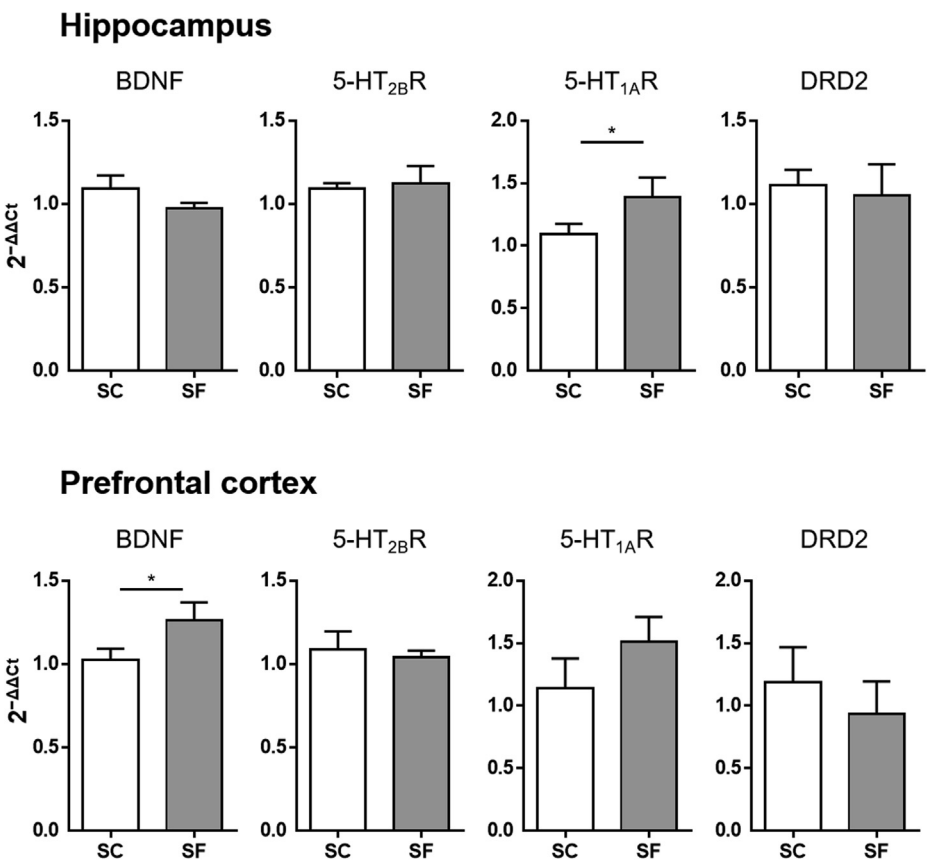


Fig. 5. Genes expression in the hippocampus and prefrontal cortex. In the hippocampus there was no difference between the standard (SC) and supplemented (SF) groups in the expression of brain-derived neurotrophic factor (BDNF), 5-HT_{2B} receptor and DRD2 receptor. However, there was a significantly higher level of 5-HT_{1A} receptor in SF than SC animals. In prefrontal cortex, the level of BDNF was significantly higher in SF than SC animals, but no difference was found in the level of 5-HT_{2B}, 5-HT_{1A} and DRD2 receptors. N = 11–12/group, mean ± SEM, two-way ANOVA, * $p < 0.05$.

Altmann, & Gertsch, 2010). Further 5-HT_{1A}R immunohistochemistry in the hippocampus would provide more information on the location and density of these receptors. *In vivo* SPECT imaging highlighted differences of basal perfusion, reflecting metabolic changes and activation in several brain regions. First, the olfactory bulbs were more activated in animals supplemented with the ingredient, presumably due to a stronger stimulation of their olfactory system during the experimental period. Indeed, exposure to an enriched olfactory environment or training can enhance newborn neurons survival and connectivity in the olfactory bulb (Alonso et al., 2006; Rochefort & Lledo, 2005; Valle-Leija, 2015; Valle-Leija, Blanco-Hernández, Drucker-Colín, Gutiérrez-Ospina, & Vidaltamayo, 2012), and thus presumably modify its activity. Moreover, the olfactory bulb volume is generally reduced in depressive patients, and the level of anosmia is correlated to the depression severity (Yuan & Slotnick, 2014). There is also obviously a strong functional relationship between the olfactory system and hippocampus, as bulbectomy induces depressive-like states and decreases hippocampal neurogenesis (Jaako-Movits, Zharkovsky, Pedersen, & Zharkovsky, 2006; Morales-Medina, Iannitti, Freeman, & Caldwell, 2017). The potential higher neurogenesis suggested by the higher expression of BDNF and 5-HT_{1A}R in our supplemented animals might be linked to this increased solicitation and activity of the olfactory system, although this assumption is purely speculative at this moment. Further investigations are necessary to test whether hippocampal neurogenesis is enhanced. Other structures including the thalamus, hippocampus, caudate nucleus, dlPFC and daCC were less activated in the supplemented group. The thalamus is an important relay structure for multisensory integration (Tyll, Budinger, & Noesselt, 2011), the hippocampus contributes to cognitive and limbic functions, the anterior cingulate cortex is involved in cognitive control, reward anticipation, and decision-making, while the frontostriatal circuit plays a major role in pleasure and motivation (Berridge & Kringelbach, 2015; Clemmensen et al., 2017). It is reported that, in the context of depression, different therapies including antidepressant drugs and cognitive behavioral therapy, are associated with different patterns of modulation of cerebral activity, especially of the limbic and cortical regions (Goldapple et al., 2004). Modulation of this complex network was thus expected, but it is difficult to precisely interpret why the basal metabolism of these structures was decreased. Moreover, sequential imaging throughout the experimental period might have resulted in different results since it is probable that brain activation has changed over time and along increased duration of supplementation. It might also have been interesting to investigate the cerebral responses to an olfactory stimulation with the ingredient, as previously performed in fMRI with other odorants (Coquery et al., 2019). Curcumin is also known to regulate the HPA axis (Kaufmann, Gazal, Bastos, Kaster, & Ghisleni, 2016; Xu et al., 2006), which is generally deregulated in depressive patients (Taylor & Fink, 2008) and so was in our model (Menneson et al., 2019). However, the ingredient did not impact the levels of salivary cortisol at any moment, suggesting the absence of impact on the HPA axis.

At this point of the study, it is not possible yet to establish a direct link between the effects observed at the behavioral and brain levels. Nevertheless, it could be considered in further studies.

4.3. Glucose homeostasis, gastrointestinal and immune investigations

A previous study of our chronic stress model had shown an increased insulin resistance in stressed animals (Menneson et al., 2019). Curcumin and piperine have anti-insulin-resistance properties (Choi et al., 2013; Jiménez-Osorio et al., 2016; Na et al., 2011; Shao et al., 2012; Shen et al., 2017), nevertheless the functional ingredient did not impact glycaemia nor insulinemia in our study. It had also very few effects on gut hormones, while several effects on their secretion have been reported (Kato et al., 2017; Xu et al., 2013). Our chronic stress was also associated with an increased intestinal permeability that remained unaffected by the functional ingredient, even though spices are often

Table 1
Physiological data in standard animals (SC) and animals supplemented with the warm spices functional food ingredient (SF). The effects of the supplementation on the gut sphere were very slight. Considering the plasmatic quantifications, there was a trend for a lower level of ghrelin, GLP-1, and LPS in SF compared to SC animals. The production of valerate and caproate also were or tended lower in this group. There was no effect of the functional ingredient on the intestinal permeability and morphometry. Bold values indicate a significant difference between groups or a statistical trend.

		SC (mean ± SEM)	SF (mean ± SEM)	N SC,SF	Statistics	*P-value*
Plasma levels	Glucose (mmol/ml)	5.617 ± 0.118	5.533 ± 0.129	12,12	F(1,20) = 0.27	ns
	Insulin (μIU/ml)	2.218 ± 0.316	2.675 ± 0.484	11,12	F(1,19) = 0.56	ns
	Homa-IR	0.560 ± 0.087	0.683 ± 0.135	11,12	F(1,19) = 0.536	ns
	Ghrelin (pg/ml)	578.196 ± 64.405	445.167 ± 36.639	10,11	F(1,17) = 3.337	**0.09**
	GLP-1 (pmol/l)	29.077 ± 2.248	22.508 ± 2.486	11,12	F(1,19) = 3.908	**0.06**
	Leptin (ng/ml)	3.630 ± 0.509	2.741 ± 0.406	12,12	F(1,20) = 1.757	ns
	PYY (ng/ml)	2.610 ± 0.192	2.310 ± 0.218	12,1	F(1,18) = 1.042	ns
	LPS (ng/ml)	36.653 ± 4.387	23.601 ± 4.152	10,12	F(1,18) = 3.995	**0.06**
	Haptoglobin (mg/ml)	0.897 ± 0.147	0.811 ± 0.109	12,12	F(1,20) = 0.222	ns
Feces levels of SCFAs	Total SCFAs (mmol/kg)	147.25 ± 8.636	135.457 ± 7.31	12,12	F(1,20) = 0.993	ns
	Acetate (mmol/kg)	84.084 ± 4.71	80.624 ± 4.219	12,12	F(1,20) = 0.276	ns
	Propionate (mmol/kg)	30.709 ± 1.674	27.355 ± 1.719	12,12	F(1,20) = 1.814	ns
	Butyrate (mmol/kg)	17.972 ± 1.284	15.745 ± 1.103	12,12	F(1,20) = 1.597	ns
	Isobutyrate (mmol/kg)	3.492 ± 0.286	3.149 ± 0.197	12,12	F(1,20) = 1.012	ns
	Valerate (mmol/kg)	6.146 ± 0.671	4.710 ± 0.421	12,12	F(1,20) = 3.137	**0.09**
	Isovalerate (mmol/kg)	4.288 ± 0.461	3.605 ± 0.276	12,12	F(1,20) = 1.720	ns
	Caproate (mmol/kg)	0.560 ± 0.122	0.269 ± 0.047	12,12	F(1,20) = 5.519	< **0.05**
Intestinal permeability	Colonic FD4 flux (ng/cm^2/h)	194.338 ± 34.631	196.424 ± 34.226	12,11	F(1,19) = 0.007	ns
	Jejunal FD4 flux (ng/cm^2/h)	98.816 ± 19.324	108.102 ± 23.266	12,11	F(1,19) = 0.083	ns
	Colonic HRP flux (ng/cm^2/h)	29.700 ± 8.274	31.028 ± 9.904	12,1	F(1,18) = 0.018	ns
	Jejunal HRP flux (ng/cm^2/h)	21.3421 ± 7.629	26.614 ± 8.105	12,11	F(1,19) = 0.184	ns
Intestinal morphometry	Crypts depth (colon) (μm)	462.091 ± 11.872	491.964 ± 23.814	8,8	F(1,12) = 1.084	ns
	Crypts width (colon) (μm)	58.489 ± 1.459	58.561 ± 2.426	8,8	F(1,12) = 0.001	ns
	Crypts surface (colon) (μm)	28322.627 ± 1102.004	29872.583 ± 2404.914	8,8	F(1,12) = 0.298	ns
	Crypts perimeter (colon) (μm)	1043.809 ± 28.513	1095.888 ± 52.748	8,8	F(1,12) = 0.649	ns
	Number of Goblet cells per crypt (colon)	95.100 ± 4.111	101.638 ± 6.674	8,8	F(1,12) = 0.879	ns
	Villis length (jejunum) (μm)	452.403 ± 22.195	439.794 ± 17.392	8,8	F(1,12) = 0.190	ns
	Villis width (jejunum) (μm)	176.126 ± 6.951	173.164 ± 5.151	8,8	F(1,12) = 0.101	ns
	Villis surface (jejunum) (μm)	68631.306 ± 3592.084	67408.732 ± 4112.519	8,8	F(1,12) = 0.048	ns
	Villis perimeter (jejunum) (μm)	1252.812 ± 43.321	1235.224 ± 43.089	8,8	F(1,12) = 0.076	ns
	Number of Goblet cells per villi (jejunum)	47.717 ± 3.325	46.142 ± 3.823	8,8	F(1,12) = 0.090	ns
	Crypts depth (jejunum) (μm)	499.919 ± 24.593	528.400 ± 18.61	8,8	F(1,12) = 0.762	ns
	Crypts width (jejunum) (μm)	47.004 ± 1.420	46.904 ± 1.341	8,8	F(1,12) = 0.003	ns
	Crypts surface (jejunum) (μm)	22601.221 ± 1540.515	24526.461 ± 1378.872	8,8	F(1,12) = 0.919	ns
	Crypts perimeter (jejunum) (μm)	1064.632 ± 49.363	1123.595 ± 38.378	8,8	F(1,12) = 0.802	ns
	Number of Goblet cells per crypt (jejunum)	54.188 ± 4.288	57.075 ± 3.774	8,8	F(1,12) = 0.269	ns

reported to regulate the tight junctions of the intestinal epithelium (Ghosh et al., 2018; Huh et al., 2018; Wang et al., 2017). Microbiota diversity and composition were not impacted by the ingredient at the phyla level, and its effects on fermentation activity were low. Though, our chronic stress resulted in a massive decrease in microbiota fermentation activity. Ginger notably has demonstrated protective effects against a large range of gastrointestinal disorders (Haniadka et al., 2013), which makes surprising the absence of effects in our study. Numerous papers reported a major impact of spices on the immune system (Aggarwal & Harikumar, 2009; Wang et al., 2017; Yu et al., 2015), however the effects at the peripheral level observed in our study were negligible. Whether the dose and/or the formulation used in this study were adequate to trigger significant modulations at the gastrointestinal and immune levels is a question that remains unanswered yet and would require further investigations at different concentrations or with different vectors.

4.4. Limitations and conclusions

The general physiological and behavioral effects of the functional food ingredient were low in this study. A first hypothesis is that the dose might have not been sufficient to induce significant differences, which legitimates to further investigate the impact of various doses. Indeed, when orally administered alone, curcumin dose for example is generally between 5 and 20 mg/kg of live weight/day in rodents (Xu et al., 2007, 2006). A study also investigated the effects of a 8 mg/kg of live weight/day dose of curcumin in pigs and showed a decreased cortisolemia and increased levels of BDNF (Wei, Xu, Xia, & Zhao, 2010). As we used a complex blend, we chose to administer a lower dose of each compound. Secondly, even if the absorption of curcumin is highly increased in the presence of piperine (Shoba et al., 1998), and the ingredient has been formulated to ensure a better absorption, it is possible that it was not sufficient. Another hypothesis would be that, at the opposite, compounds were absorbed earlier in the digestion process, and did not reach distal gut and microbiota. The poor bioavailability of curcumin has already been reported because of poor absorption, rapid metabolism and elimination (Anand, Kunnumakkara, Newman, & Aggarwal, 2007; Kulkarni et al., 2008; Lopresti, 2018). Moreover, it is also possible that, as we studied a complex blend of spice extracts, several effects were opposite instead of synergetic, and thus led to poor observable effects. Nevertheless, it is possible to hypothesize that in our situation the active molecules contained in the functional ingredient have acted *via* sensory inputs during eating, through olfaction and gustation notably, to produce these modulations of brain regions involved in limbic, hedonic, and cognitive functions as well as the potential effects on anxiety.

Ethics statement

Experiments were conducted in accordance with the current ethical standards of the European Community (Directive 2010/63/EU), Agreement No. C35-275-32 and Authorization No. 35-88. The Regional

Ethics Committee in Animal Experiment of Brittany has validated the entire procedure described in this paper (project n°2017080511347475) and the French Ministry of Research approved it.

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Declaration of Competing Interest

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103599.

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