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Investigating the modulation of the endocannabinoid system by probiotic *Lactiplantibacillus plantarum* IMC513 in a zebrafish model of di-*n*-hexyl phthalate exposure

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Environmental pollutants used as plasticizers in food packaging and in thousands of everyday products have become harmful for their impact on human health. Among them, phthalates, recognized as emerging endocrine disruptors (EDs) can induce toxic effects leading to different health disorders. Only few studies evaluated the effects of di-*n*-hexyl phthalate (DnHP) in in vivo models and no studies have been conducted to investigate the effect of DnHP on the endocannabinoid system (ECS), one of the majors signaling pathways involved in the microbiota–gut–brain axis. Due to the current relevance of probiotic bacteria as beneficial dietary interventions, the present study was aimed at evaluating the potential neuroprotective impact of daily administration of *Lactiplantibacillus (Lpb.) plantarum* IMC513 on zebrafish adults exposed to DnHP, with a focus on ECS modulation. Gene expression analysis revealed a promising protective role of probiotic through the restoration of ECS genes expression to the control level, in the brain of zebrafish daily exposed to DnHP. In addition, the levels of main endocannabinoids were also modulated. These findings confirm the potential ability of probiotics to interact at central level by modulating the ECS, suggesting the use of probiotics as innovative dietary strategy to counteract alterations by EDs exposure.

Keywords *Danio rerio*, Endocannabinoid system, Endocrine disruptors, Phthalates, Probiotics, *Lactiplantibacillus plantarum*

The World Health Organization (WHO) has defined endocrine disruptors (EDs) as "exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations"[1]. The daily exposure of both animals and humans to a huge variety of EDs, present in thousands of everyday products, is rapidly increasing, leading more recently to a high concern about their adverse effects on planet health[2]. Among EDs, phthalates (or phthalate esters) are synthetic diesters of phthalic acid commonly used to produce gelling and emulsifying agents, adhesives, detergents, as well as to manufacture plastic care products, kitchen plastic ware and food packaging[3]. Phthalates can easily leach into the environment from plastics goods thereby contaminating foods, water, and air by which humans can be exposed through ingestion, direct contact (i.e., skin) or inhalation[4]. It has been estimated that over 75% of the population are daily exposed to multiple phthalates that have been found in several biological fluids, such as blood, urine, sperm, and breast milk[5]. Generally, the phthalates do not accumulate in animal and human tissues because of their rapid clearance from the body[6], while their ubiquitous environmental and alimentary presence may lead to chronic exposure reflecting in different animals and humans' health disorders, even though not all the possible adverse and toxicological effects are yet elucidated[7]. Di-(2-ethylhexyl) phthalate (DEHP), the first

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and most studied member of this family, has been shown to induce developmental toxic and genotoxic effects in pre-pubertal male rats, mainly related to reproductive system as well as to induce cancer after long-term exposure in rodents[8]. Although the potential harmful effects of other phthalates, such as di-*n*-hexyl phthalate (DnHP), are not yet elucidated, several studies reported an association between phthalates exposure and oxidative stress in humans[9] strictly related to the inflammatory and chronic diseases, such as obesity, diabetes, and cancer[10]. Moreover, exposure to phthalates may lead to gut microbial dysbiosis[11,12] and EDs might directly alter microbiota composition and/or indirectly induce dysbiosis by activating host receptors that, in turn, cause inflammation[11]. In vivo studies carried out to evaluate the effects of DnHP demonstrated that this compound seriously affects embryonic development in rats with teratogenic effects[4], induces harmful effects on male reproductive system[13] as well as in pregnant female rats with intrauterine retardation of rat fetuses' development[14]. More recently, DnHP has been shown to induce ROS production, apoptosis, and inflammation in human cells[15]. Other phthalates have shown different toxic and endocrine effects in zebrafish development[16,17], but it seems that no studies have been conducted to evaluate DnHP exposure in adult zebrafish and only few studies evaluated the impact of phthalates on the ECS of zebrafish. ECS is considered a complex signaling network that includes a set of bioactive lipids with their specific receptors and enzymes that regulate their biosynthesis and degradation as well as mediate their multiple functions. Cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2) are the G-protein–coupled receptors of the two main members of ECS, anandamide (AEA) and 2-arachidononyl-glycerol (2-AG). The ECS also include the enzymes that either synthetize or degrade ECs. AEA and 2-AG are synthetized by *N*-acylphosphatidylethanolamine–specific phospholipase D (NAPE-PLD) and *sn*-1-diacylglycerol lipase α (DAGLα), respectively, while the fatty acid amide hydrolase (FAAH) and the monoacylglycerol lipase (MAGL) are the enzymes responsible for the conversion of AEA into arachidonic acid plus ethanolamine and 2-AG into arachidonic acid plus glycerol. Moreover, the endocannabinoid family may include also the so called "endocannabinoid-like" compounds, such as *N*-palmitoylethanolamine (PEA), that act independently from endocannabinoid receptors and can significantly contribute to the biological effect of 2-AG and AEA[18]. ECS plays a key role in regulating food intake and energy homeostasis[19], and in regulating the immune system and inflammation[20]. As a complex cell-signaling network, the ECS can exert a modulating action from periphery to central nervous system in a two-way direction[21], influencing also endocrine and reproductive system as well as gastrointestinal physiology, in which the gut microbiota plays a key role in many host functions[22,23]. Currently, probiotic bacteria, defined as live microorganisms that when administered in proper amounts improve host health[24], are one of the most promising strategies to positively affect human health in a variety of health disorders. Among probiotics, a large body of scientific data support the administration of many probiotic strains of *Lpb. plantarum* as dietary strategy to prevent and/or improve diverse pathological conditions[25]. Some food-borne and probiotic lactic acid bacteria, including *Lpb. plantarum* strains, have been investigated in vitro for their highly ability to detoxify and/or degrade mutagen and toxic compounds including EDs[26–28], emerging as an innovative promising therapeutic approach[29] even though their role in exerting a protective effect is still unknown. To date, scientific evidence on the use of probiotic bacteria to face increasing EDs exposure are mainly limited to in vitro studies[30], and no investigations have carried out to evaluate the potential protective effects of probiotic bacteria after phthalates exposure. Zebrafish (*Danio rerio*) has emerged as novel and promising animal models (in alternative to rodents) to study both probiotics and EDs effects[31]. Zebrafish has approximately 70% of genes and protein similar to humans, allowing its use for comprehension of human pathologies and environmental toxicity in biomedical research[32]. Moreover, by adding chemicals directly in the water, zebrafish results an ideal animal model in toxicity studies for its rapid absorption of any compound that can affect several anatomic districts, including the brain[32]. Additionally, the modulation of ECS by intestinal and probiotic bacteria has been shown also by using zebrafish as animal model[33]. First proof of the mutual ECS-microbiota interaction was highlighted in 2007[34] and since then there is a body of evidence regarding this dynamic crosstalk, including microbial metabolites, short chain fatty acids, cannabinoid receptors, and TLRs[35–38]. In this context, a molecular network through which a mixture of probiotic bacteria, such as VSL#3, activate the ECS and induce *Tlr* signalling has been previously reported[33]. In this context, based on our in vitro preliminary results showing the capability of a selected probiotic *Lpb. plantarum* IMC513 strain to inhibit the genotoxicity ofDnHP[30], the present study aimed to evaluate the impact of dietary intervention with *Lpb. plantarum* IMC513 on the physiology of zebrafish daily exposed via feed to DnHP for 6 months, with a focus on ECS modulation in the brain. In particular, we assessed the expression levels of key genes of the ECS and the levels of main endocannabinoids, 2-arachidononyl-glycerol (2-AG) and anandamide (AEA), as well as the endocannabinoid-like mediator palmitoylethanolamide (PEA) in the brain of zebrafish. The present study's findings may provide a basic reference for further research on the putative application of *Lpb. plantarum* as innovative dietary strategy to counteract toxicity related to EDs.

Results

Morphometric results

Morphometric measures are shown in Fig. 1. No fish, belonging to treated or control group, died during the 6 months treatment period. Body and brain weight and the total length were like that of control group. Interestingly, no significance differences have been found comparing body weight of male and female animals (Fig. 1).

Probiotic administration restores the DnHP alteration of central CB1 and CB2

The expression of genes encoding for the two main cannabinoid receptors and for the enzymes involved in the synthesis and degradation of the ECS were analyzed in the brain of zebrafish. As shown in Fig. 2a, the exposure to DnHP significantly upregulated the mRNA levels of *cnr1* ($p < 0.05$) and *cnr2* in the brain, whilst *cnr1* and *cnr2* mRNA levels were downregulated in the brain of zebrafish fed with probiotic *Lpb. plantarum* IMC513 and DnHP (Fig. 2a). Regarding the mRNA levels of *nape-pld* and *abdh4*, the main enzymes involved in the AEA synthesis,

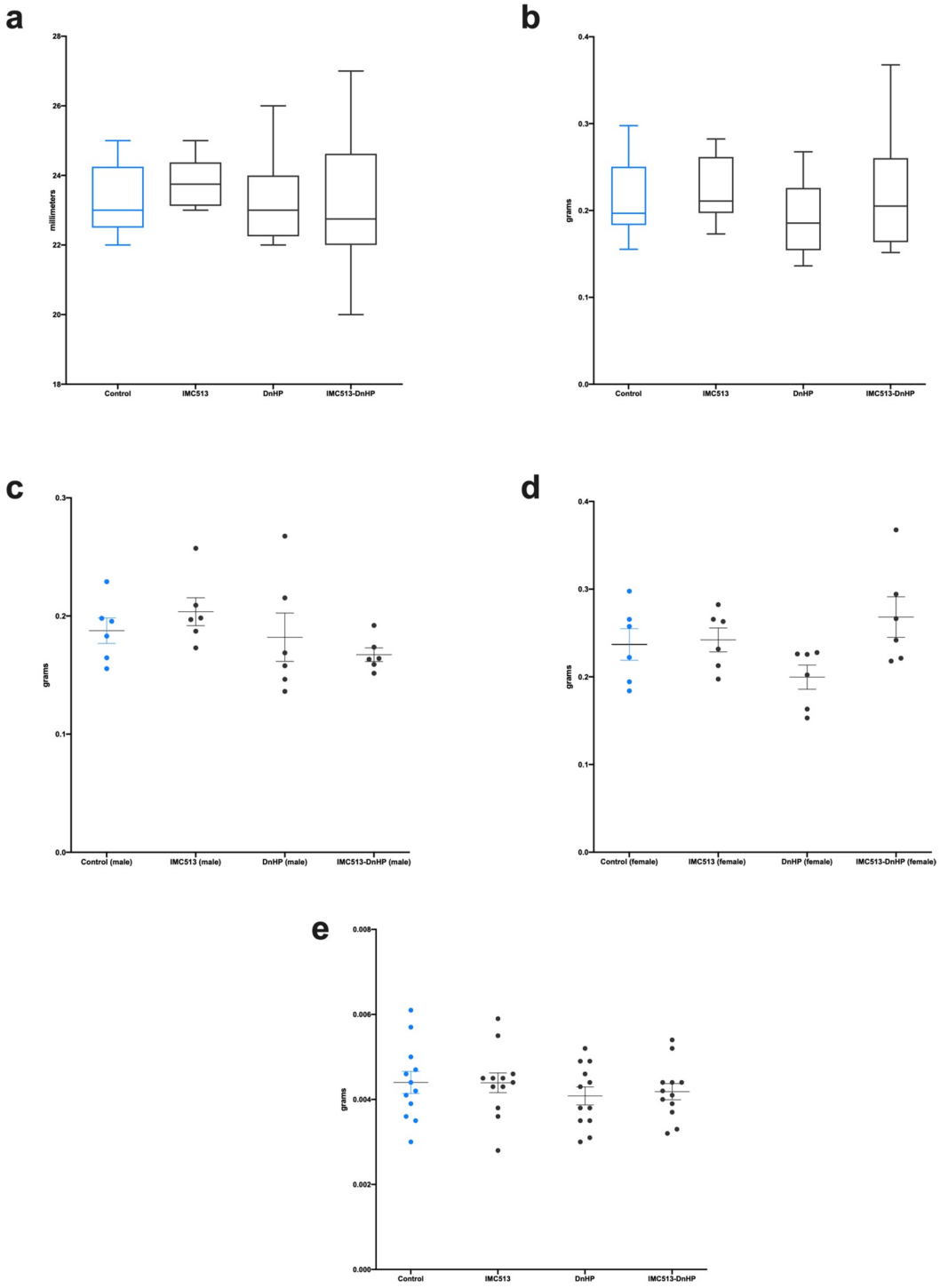

Figure 1. Morphometric results of (**a**) body length of all animals; (**b**) body weight of all animals; (**c**) body weight of male zebrafish; (**d**) body weight of female zebrafish and (**e**) brain weight of all animals. Values are expressed as mean ± SEM (n = 12) for each treatment group. No significant differences were found by one-way ANOVA analysis ($p > 0.05$).

it was found a similar trend, although not statistically significant, showing that probiotic administration restores the *nape-pld* and *abhd4* gene expression, that are upregulated following DnHP treatment, to the control levels (Fig. S1a). Interestingly, similar results were obtained by the analysis of mRNA levels of *faah*, the enzyme involved in the conversion of AEA into arachidonic acid and ethanolamine, and of transient receptor potential vanilloid type 1 (*trpv1*) channel, able to bind ECs (Fig. S1b). In order to ascertain the effectiveness of the administration of the probiotic *Lpb. plantarum* IMC513, zebrafish intestine has been used to evaluate the levels of gene encoding

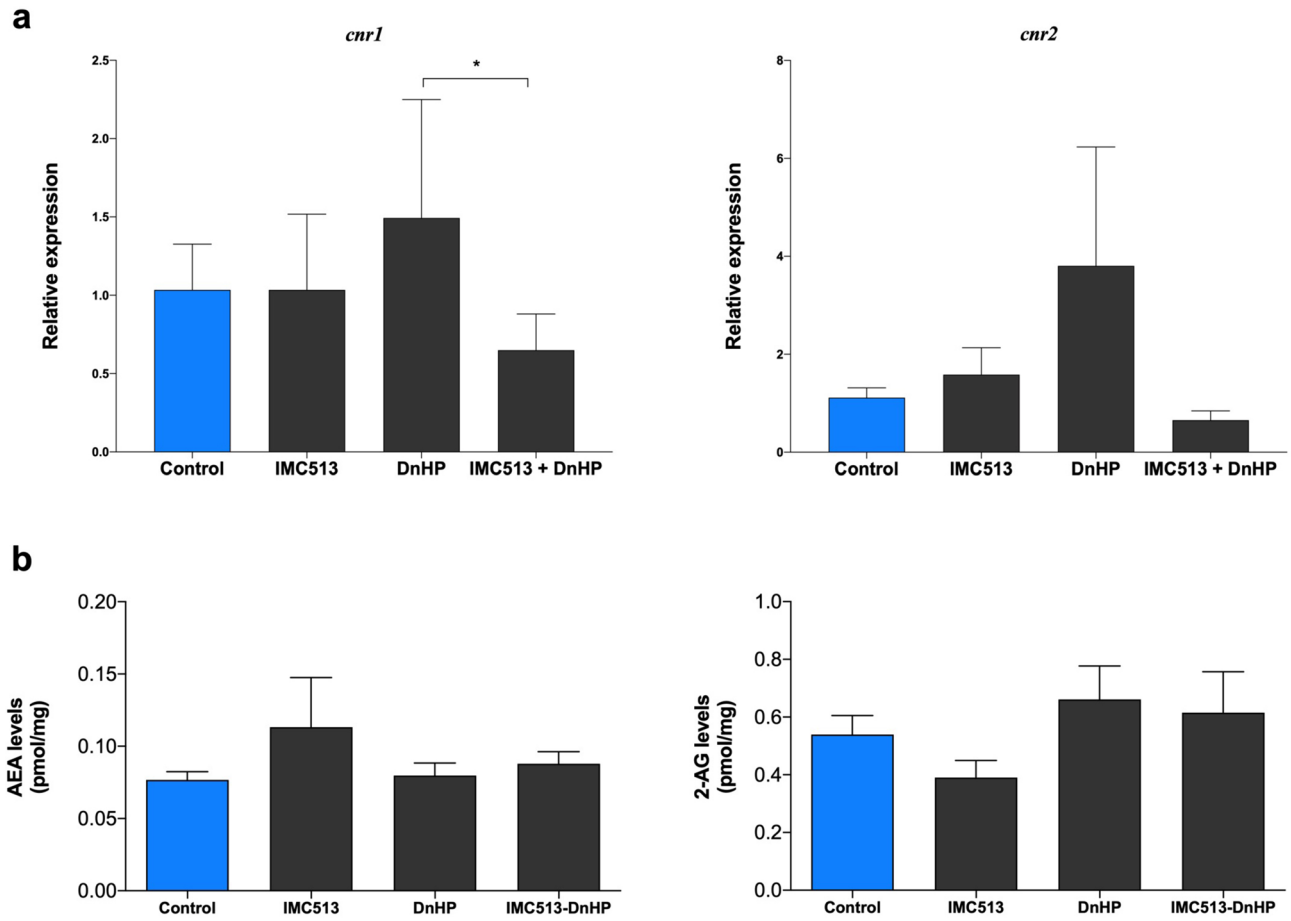

Figure 2. Impact of DnHP and probiotic on cannabinoid receptors gene expression and endocannabinoids levels in zebrafish brain. (**a**) Relative mRNA expression of *cnr1* and *cnr2* (**b**) AEA and 2-AG levels in zebrafish brain after 6 months treatment with DnHP and the probiotic *Lpb. plantarum* IMC513. Data are expressed as mean ± SEM (n = 6) and were analyzed for statistical differences by one-way ANOVA followed by Bonferroni post hoc test (*$p < 0.05$).

for TLR9, a key molecule involved in recognition of microbes in the gut. As shown in Fig. S1c, mRNA levels of TLR9 were increased in the treated group, due to the 6 months administration of the probiotic *Lpb. plantarum* IMC513 (Fig. S1c) confirming the host-microbe interaction[39].

Impact of DnHP and probiotic on AEA and 2-AG levels in zebrafish brain

In these experiments, the levels of AEA and 2-AG were detected in zebrafish brain samples after 6 months exposure to DnHP with and without simultaneous administration of the probiotic *Lpb. plantarum* IMC513. As reported in Fig. 2b, no statistically significant differences were found in AEA and 2-AG levels in the brain, although a slight increase only of 2-AG content was detected in zebrafish exposed to DnHP compared to control (0.66 ± 0.11 pmol/mg versus 0.54 ± 0.06 pmol/mg). In addition, we observed that DnHP induced a higher release of PEA (16.03 ± 1.55 pmol/mg versus 10.19 ± 1.05 pmol/mg) respect to control group, as reported in Fig. S2. Regarding the effect of probiotic, our data suggest that the daily intake of *Lpb. plantarum* IMC513 in healthy animals might alter the AEA, 2-AG and PEA content at the central level (Fig. 2b and Fig. S2) by increasing (as for AEA and PEA) or reducing (as for 2-AG) their levels. Interestingly, the administration of the probiotic in presence of DnHP did not affect neither AEA and 2-AG nor PEA levels (Fig. 2B).

Discussion

The present study was aimed at investigating whether the dietary intervention with the probiotic *Lpb. plantarum* IMC513 on the physiology of zebrafish exposed to the endocrine-disrupting DnHP are mediated in the brain via the ECS. In the last decade probiotics have been shown to have multiple positive effects on the host by influencing the immune system, brain development and potentially acting as biotherapeutic agent in many diseases[40,41]. Recently, our group reported the beneficial role of the probiotic strain *Lpb. plantarum* IMC513 to inhibit the genotoxicity of some compounds including DnHP in vitro[26,30] as well as to ameliorate inflammation in a DSS-induced colitis mice model[42]. In this study, molecular analyses revealed that the ECS was altered in the brain of zebrafish exposed for 6 months to a commercial diet mixed with DnHP. Among the genes regulated by the DnHP, *cnr1* and *cnr2* resulted up-regulated compared to the control group, while in the group treated with commercial

diet mixed with DnHP and probiotics there was a significant down-regulation, suggesting that the DnHP toxicity interfering with the ECS seems based on transcriptome regulation. In the literature there are currently no scientific studies on the toxicity of DnHP in zebrafish, but previous papers demonstrated that other endocrine interferents belonging to the category of phthalates, such as Bisphenol A and di-isononyl phthalate, derange the ECS[6,43–45]. In particular, Forner–Piquer and colleagues observed that DnHP exposure causes an up-regulation of *cnr1* and *cnr2* in the zebrafish brain, despite an unchanged brain concentration of endocannabinoids levels[6]. More recently, in silico studies demonstrated that activation of the CB1 receptor might be one of the possible mechanisms by which bisphenols induce obesity in zebrafish[46]. ECS has been recently chosen as a new target for the activity of some EDs, since it represents a complex lipid signaling network essential for the well-being of the organisms. ECS plays a role in normal adipogenesis and interferes with orexigenic and anorexigenic circuits, being one of the main systems regulating appetite and body weight and, thus, would be a perfect candidate for testing the obesogenic capacity of pollutants, as alterations induced by environmental contaminants can affect the ECS regarding the regulation of food intake and energy homeostasis[6]. Some phthalates as DEHP in mice were identified as obesogen by increasing food intake, body weight, fat mass, serum leptin and decreasing serum adiponectin[46].

Currently, the inclusion of probiotic bacteria in the diet seems to represent a promising strategy in positively influencing human health in a series of health disorders, in fact the protective capacity given by the administration of probiotics into the diet for the improvement of pathological conditions in humans is well confirmed[29,47]. In agreement with other studies that demonstrated in other species that probiotics interact with the host by communicating via toll-like receptors (TLRs) activation[48], we have found in this study that also in zebrafish *Lpb. plantarum* IMC513 up-regulated TLR9, one of the most conserved TLR receptor among zebrafish and human[49]. TLR9 as part of the innate immune system, specifically recognize unmethylated CpG motifs prevalent in bacterial genomic DNA[49] and its role in maintaining gut homeostasis has been demonstrated, since TLR9 is considered as one of the fundamental mechanisms of action of probiotics in ameliorating the inflammatory response in the host[49]. Additionally, previous findings indicate that oral supplementation of *Lactobacillus acidophilus* probiotics can positively influence the mRNA expression of *cnr1* receptor[50] as well as it has been observed that gut barrier integrity is modulated through a CB1-dependent mechanism following probiotic administration[51,52]. Therefore, the perturbation of the composition of gut microbiota may consequently result in a change of production of various metabolites (i.e. short chain fatty acids) that, translocating throughout the blood–brain barrier, might impact the ECS.

In the present study, the group fed with a diet based on DnHP and IMC513, compared to zebrafish fed with only DnHP, showed a down-regulation of gene expression of CB1 and CB2 that result increased by exposure to DnHP (Fig. 3). Taken together, these findings highlight the possible role of ECS as a *trait d'union* in the microbiota-gut-brain axis[53] and shed light on the potential ability of probiotics to modulate at the level of the central nervous system some ECS components in zebrafish, suggesting the use of probiotics as innovative dietary strategy to counteract the emerging health risk of EDs, which is worth to be further investigated.

Data literature suggest that EDs are responsible for the dysregulation of the central ECS in zebrafish and our previous research has found that *Lpb. plantarum* probiotic strain IMC513 shows appreciable ability to inhibit the biological activity of genotoxic compounds[30]. Collectively, the present study's findings prove that 6 months exposure to DnHP impairs endocannabinoid signaling by upregulating both cannabinoid receptors and that the combined administration of DnHP and *Lpb. plantarum* probiotic strain IMC513 reverses the ECS modulation (Fig. 3), suggesting the suitability of *Lpb. plantarum* strains as a putative bio-protective tool to counteract genotoxic and mutagenic agents by assessing the involvement of the ECS in the microbiota-gut-brain axis.

Methods

Materials

All reagents used were of analytical grade. Healthy AB adult wild-type zebrafish were obtained from breeding facility at University of Teramo. Dry GEMMA Micro 300 feed were obtained from Skretting (US). Di-*n*-hexylphtalate (49617) Tricaine pharmaq (PHARMAQ AS, Norway) were purchased from Sigma–Aldrich (Milano, Italy). Isolate II RNA mini kit was obtained from Bioline, Medical Supply Company Ltd, (Dublin, Ireland) and NZY first-strand cDNA synthesis kit from Nzytech (Lisbon, Portugal). Nuclease-free water and primers were obtained from Sigma-Aldrich (Milano, Italy). iTaq™ Universal SYBR® Green Supermix was purchased from Biorad Laboratories srl (Milano, Italy). Homogenization of brain samples was performed by using Precellys® bead-beating technology in homogenization tubes (Precellys ceramic kit) from Bertin Technologies. HPLC-grade water, methanol and chloroform were purchased from Sigma–Aldrich (Milano, Italy). Deuterated internal standards (AEA-d_8, 2-AG-d_8 and PEA-d_4) were obtained from Vinci Biochem (Vinci, FI, Italy). The micro-solid phase extraction (μSPE) was performed by using OMIX C18 tips from Agilent Technologies (Santa Clara, CA, USA).

Probiotic used as dietary intervention

In this study a selected probiotic strain, *Lpb. plantarum* IMC513 (kindly provided by Synbiotec srl, Camerino, Italy) was used as dietary intervention. This strain was previously characterized for several properties, including the antigenotoxic activity, the ability to survive the gastrointestinal conditions[26], to interact with intestinal cell models[54,55] and to ameliorate DSS-induced colitis in mice[42]. In addition, this strain was selected for this experimental trial, based on its ability to inhibit the genotoxicity of DnHP in a preliminary in vitro study[30]. *Lpb. plantarum* IMC513 was administrated as described below.

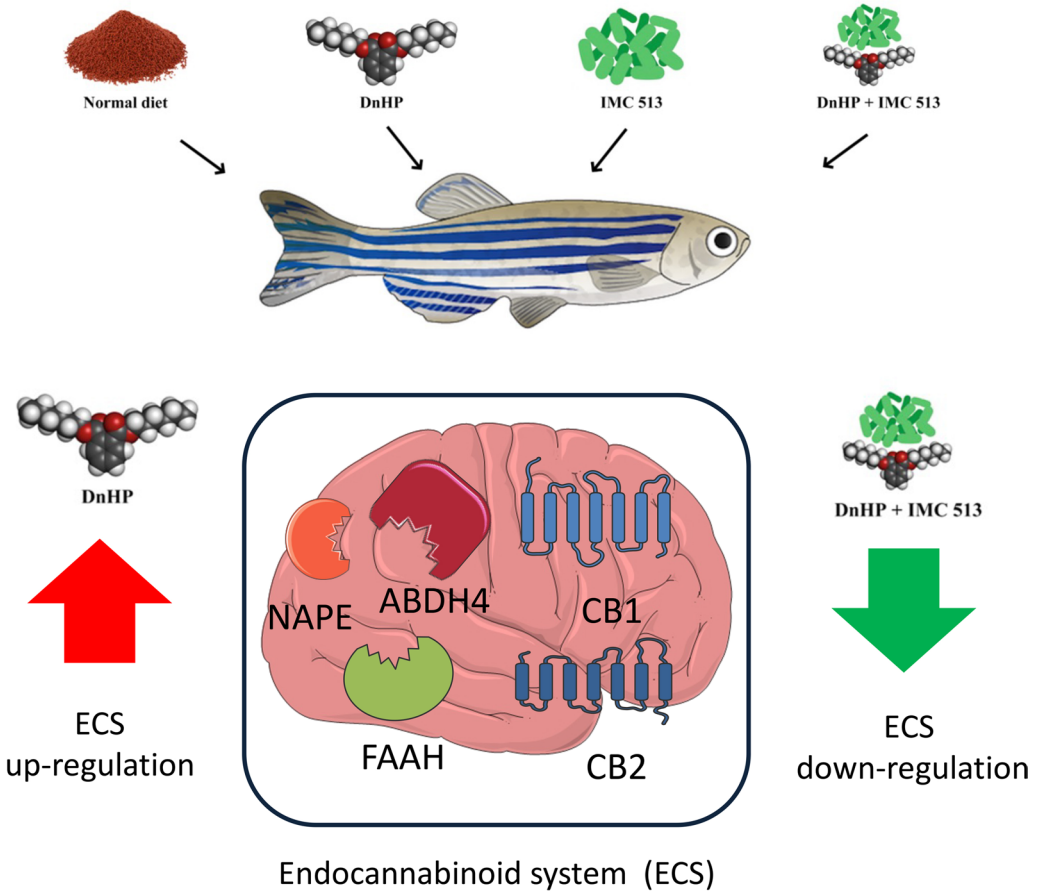

Figure 3. Schematic representation of the main findings of the study. Six-months exposure to DnHP impairs endocannabinoid signaling by upregulating both cannabinoid receptors while the combined administration of DnHP and *Lpb. plantarum* probiotic strain IMC513 reverses the ECS modulation. Graphical illustrations were created by using some graphical elements from Servier Medical Art by Servier, available on https://smart.servier.com/ under a Creative Commons Attribution 3.0 Unported License.

Animals and probiotic administration

Adults wild-type zebrafish pairs (90 day post fertilization (dpf)) were kept in 68 L glass tanks (pure aquarium kit L, Askoll, Italy) under controlled conditions. The temperature was maintained at 28 °C, the pH at 6.9 ± 0.2, the conductivity at 499 µS/cm and the dissolved O_2 at 6.1 mg/L. The photoperiod was 14 h light and 10 h dark and chemical parameters were kept as follows: ammonia 0.01 mg/L, nitrite 0.01 mg/L, nitrate 15.6 mg/L. Animals were fed twice a day with dry feed (GEMMA Micro 300, Skretting, US). Experiments were performed on four groups: a control group (n = 12) that were fed only on commercial diet, one treated group (n = 12) that were fed on commercial diet mixed with lyophilized probiotic, another (n = 12) fed with DnHP contaminated feed and the last one (n = 12) that were fed on commercial diet mixed with lyophilized probiotic and DnHP. The administration by food was chosen both because the low aqueous solubility of DnHP and because it is expected as the relevant route of exposure to these chemicals for wildlife. *Lpb. plantarum* IMC513 was administrated by directly adding to the water freeze-dried cells together with the standard diet at a final concentration of 10^9 CFU/g, already used as dietary administration in previous in vivo study[36]. The DnHP was supplied in a final concentration in the dry feed of 1 mg/mL, based on previous in vitro tested concentration[30]. The contaminated diet was delivered for 6 months, until the fish reached 270 dpf. At the end of experimental period, both male and female zebrafish were euthanized using an anesthetic overdose (Tricaine pharmaq at 0.04%) and after recording the total length and the wet weight of each zebrafish, brain and intestine from each animal were collected for quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) analysis (n = 6) and for quantification of endocannabinoid levels (n = 6).

Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from zebrafish brain (n = 6) by using an Isolate II RNA mini kit (Bioline, Medical Supply Company Ltd, Dublin, Ireland). mRNA was eluted in RNase-free water and checked for concentration and purity using a Nanodrop spectrophotometer system (ND-1000 3.3 Nanodrop Technologies, USA). Only samples with a ratio between 1.8 and 2.1 were used for gene expression assays. For each sample, cDNA was obtained from RNA (1000 ng) following reverse transcription with NZY first-strand cDNA synthesis kit (Nzytech, Lisbon, Portugal)

Gene	Gene ID	Forward primer	Reverse primer
b-act	57934	CGAGCTGTCTTCCCATCCA	TCACCAACGTAGCTGTCTTTCTG
rpl13a	378961	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG
cnr1	404209	GCGCGAACTATGTGAAGGTT	CCGTCCAGGACAGATTTAACA
cnr2	795246	TGCATCAACAGCAAAACACA	TCTTGTTCCAGTTTGTTCTCCA
trpv1	561195	TCGCAGATTCCTTGTTGTGT	TTCCTGGTCAAGGAGAGTCAC
abhd4	550276	GAAGAGCAGTTTGTTTCCTCCATAG	ACTCACTCTTTCTGGGTATTGGAT
faah	569067	CATTCTCGGACCAGCCTTCA	CCTTCACTGCCTGTTGGAAGA
nape-pld	568061	CTCAAGGACATGCACTCA	GAGCACAATCTTCAAGACAAT
tlr9	403128	TCCTCAGTTTTATCCGTGTGAA	GTGCTGGCAGTCCACATTTA

Table 1. Primer sequences used for RT-qPCR. *b-act* ß-actin, *rpl13a* ribosomal protein L13a, *cnr1* cannabinoid receptor type 1, *cnr2* cannabinoid receptor type 2, *trpv1* vanilloid receptor 1, *abhd4* alfa/beta hydrolase domain 4, *faah* fatty acid amide hydrolase, *nape-pld* N-acylphosphatidylethanolamine–specific phospholipase D, *tlr9* toll-like receptor 9.

according to the manufacturer's instructions and, then stored at −20 °C until use. The RTq-PCR experiments were performed by using the iTaq™ Universal SYBR® Green Supermix (Biorad Laboratories srl, Milano, Italy) according to the manufacturer's instructions. Transcriptional levels of target genes (Table 1) were normalized using the average CT (cycle threshold) value derived from ß-actin (*b-act*) and ribosomal protein L13 (*rpl13*A) housekeeping genes, to minimize variation in cDNA and mRNA quality and quantity. mRNA from intestinal tissue was used to evaluate the expression of the gene encoding for Toll-like Receptor 9 (*tlr*9). The relative quantification of gene expression was calculated by using the $2^{-\Delta\Delta T}$ method. The primers used for RT-qPCR with their corresponding sequences and gene ID code are listed in Table 1.

Quantification of endocannabinoid levels in zebrafish brain

A solution of methanol containing AEA-d_8, 2-AG-d_8 and PEA-d_4 as internal standards was added to each brain sample (n = 6) into 2 ml homogenization tubes (Precellys ceramic kit, Bertin Technologies). Then, each sample was homogenized by using Precellys® bead-beating technology. For each sample 1 ml of chloroform was added, followed by 1 ml of water. The solution obtained was vortex-mixed and centrifuge at 4000 rpm for 5 min at 4 °C. The lipid-containing organic phases were recovered and dried under nitrogen stream. The organic phases were resuspended in 50:50 acidified water/methanol solution, sonicated for 6 min and centrifuged at 4000 rpm for 12 min at 4 °C to allow protein precipitation. The resulting supernatants underwent a micro-solid phase extraction (µSPE) by using OMIX C18 tips from Agilent Technologies (Santa Clara, CA, USA) according to Fanti and colleagues[56]. The analytes were detected through UHPLC/MS–MS analysis using Sciex QTrap 4500 mass spectrometer (Sciex, Toronto, Ontario, Canada) coupled to Shimadzu Nexera LC-20AD (Shimadzu, Tokyo, Japan); Kinetex C18-XB 1.7 µm 100 × 2.1 mm (Phenomenex, Torrance, California, USA) was used to separate the target molecules. UHPLC-MS/MS data were elaborated by Analyst 1.7.3 software; MultiQuant 3.0.3 software was used for peak integration and quantification; target analytes quantification was performed based on calibration curve response and normalized by internal standards[56]. The levels of AEA, 2-AG and PEA were then calculated based on their area ratios with the internal deuterated standard signal areas, and their amounts in pmols were normalized per mg of wet sample weight.

Statistical analysis

Data are reported as mean ± SEM for all experiments. Data were analyzed by means of Prism 8.3 program (GraphPad Software Inc., La Jolla, CA, United States) using the One-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. A level of $p < 0.05$ was considered statistically significant.

Ethics declarations

The work was carried out following the Italian law for the protection of research animals D.L. n. 26, 4 March 2014, the European regulation directive 2010/63/U and ARRIVE guidelines. The protocol was approved by the Italian Ministry of Health (auth. n. 529/2018-PR). License for fish maintenance and breeding at the University of Teramo is n. 02/2016-UT.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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References

1. Bergman, A., Heindel, J. J., Jobling, S., Kidd, K. A. & Zoeller, R. T. State of the science of endocrine disrupting chemicals, 2012. *Toxicol. Lett.* **211**, S3 (2012).
2. Darbre, P. D. The history of endocrine-disrupting chemicals. *Curr. Opin. Endocr. Metab. Res.* **7**, 26–33 (2019).

3. Zarean, M. *et al.* A systematic review on the adverse health effects of di-2-ethylhexyl phthalate. *Environ. Sci. Pollut. Res. Int.* **23**(24), 24642–24693 (2016).
4. Saillenfait, A. M., Gallissot, F. & Sabaté, J. P. Differential developmental toxicities of di-n-hexyl phthalate and dicyclohexyl phthalate administered orally to rats. *J. Appl. Toxicol.* **29**(6), 510–521 (2009).
5. Wang, Y. & Qian, H. Phthalates and their impacts on human health. *Healthcare* **9**(5), 603 (2021).
6. Forner-Piquer, I. *et al.* Dose-specific effects of di-isononyl phthalate on the endocannabinoid system and on liver of female zebrafish. *Endocrinology* **158**(10), 3462–3476 (2017).
7. Katsikantami, I. *et al.* A global assessment of phthalates burden and related links to health effects. *Environ. Int.* **97**, 212–236 (2016).
8. Karabulut, G. & Barlas, N. Genotoxic, histologic, immunohistochemical, morphometric and hormonal effects of di-(2-ethylhexyl)-phthalate (DEHP) on reproductive systems in pre-pubertal male rats. *Toxicol. Res.* **7**(5), 859–873 (2018).
9. Ferguson, K. K., McElrath, T. F., Chen, Y. H., Mukherjee, B. & Meeker, J. D. Urinary phthalate metabolites and biomarkers of oxidative stress in pregnant women: A repeated measures analysis. *Environ. Health Perspect.* **123**(3), 210–216 (2015).
10. Yaghjyan, L., Sites, S., Ruan, Y. & Chang, S. H. Associations of urinary phthalates with body mass index, waist circumference and serum lipids among females: National Health and Nutrition Examination Survey 1999–2004. *Int. J. Obes.* **39**(6), 994–1000 (2015).
11. Chiu, K., Warner, G., Nowak, R. A., Flaws, J. A. & Mei, W. The impact of environmental chemicals on the gut microbiome. *Toxicol. Sci.* **176**(2), 253–284 (2020).
12. Yang, Y. N. *et al.* Phthalate exposure alters gut microbiota composition and IgM vaccine response in human newborns. *Food Chem. Toxicol.* **132**, 110700 (2019).
13. Saillenfait, A. M. Adverse effects of diisooctyl phthalate on the male rat reproductive development following prenatal exposure. *Reprod. Toxicol.* **42**, 192–202 (2013).
14. Ahbab, M. A. *et al.* Comparative developmental toxicity evaluation of di- n-hexyl phthalate and dicyclohexyl phthalate in rats. *Toxicol. Ind. Health* **33**(9), 696–716 (2017).
15. Song, P. Ecotoxicological evaluation of zebrafish liver (*Danio rerio*) induced by dibutyl phthalate. *J. Hazard Mater.* **425**, 128027 (2022).
16. Qian, Y., Shao, H., Ying, X., Huang, W. & Hua, Y. The endocrine disruption of prenatal phthalate exposure in mother and offspring. *Front. Public Health* **8**, 366 (2020).
17. Mu, X. *et al.* A multi-omics approach reveals molecular mechanisms by which phthalates induce cardiac defects in zebrafish (*Danio rerio*). *Environ. Pollut.* **265**, 113876 (2020).
18. Battista, N. & Maccarrone, M. Basic mechanism of synthesis and hydrolysis of major endocannabinoids. In *The Endocannabinoid System* (ed. Murillo-Rodriguez, E.) 1–23 (Academic Press, 2017).
19. Silvestri, C. & Di Marzo, V. The endocannabinoid system in energy homeostasis and the etiopathology of metabolic disorders. *Cell. Metab.* **17**(4), 475–490 (2013).
20. Chiurchiù, V., Leuti, A. & Maccarrone, M. Cannabinoid signaling and neuroinflammatory diseases: A melting pot for the regulation of brain immune responses. *J. Neuroimmune Pharmacol.* **10**(2), 268–280 (2015).
21. Oltrabella, F., Melgoza, A., Nguyen, B. & Guo, S. Role of the endocannabinoid system in vertebrates: Emphasis on the zebrafish model. *Dev. Growth Differ.* **59**(4), 194–210 (2017).
22. Rapino, C., Battista, N., Bari, M. & Maccarrone, M. Endocannabinoids as biomarkers of human reproduction. *Hum. Reprod. Update* **20**(4), 501–516 (2014).
23. Crowe, M. S., Nass, S. R., Gabella, K. M. & Kinsey, S. G. The endocannabinoid system modulates stress, emotionality, and inflammation. *Brain Behav. Immun.* **42**, 1–5 (2014).
24. Hill, C. *et al.* The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* **11**(8), 506–514 (2014).
25. Garcia-Gonzalez, N., Battista, N., Prete, R. & Corsetti, A. Health-promoting role of *Lactiplantibacillus plantarum* isolated from fermented foods. *Microorganisms* **9**(2), 349. https://doi.org/10.3390/microorganisms9020349 (2021).
26. Prete, R. *et al.* Food-associated *Lactobacillus plantarum* and yeasts inhibit the genotoxic effect of 4-nitroquinoline-1-oxide. *Front. Microbiol.* **8**, 2349 (2017).
27. Baralić, K. *et al.* Multi-strain probiotic ameliorated toxic effects of phthalates and bisphenol A mixture in Wistar rats. *Food Chem. Toxicol.* **143**, 111540 (2020).
28. Średnicka, P., Juszczuk-Kubiak, E., Wójcicki, M., Akimowicz, M. & Roszko, M. Ł. Probiotics as a biological detoxification tool of food chemical contamination: A review. *Food Chem. Toxicol.* **153**, 112306 (2021).
29. Pan, I. & Umapathy, S. Probiotics an emerging therapeutic approach towards gut- brain-axis oriented chronic health issues induced by microplastics: A comprehensive review. *Heliyon* **10**, e32004 (2024).
30. Garcia-Gonzalez, N. *et al.* Probiotic antigenotoxic activity as a DNA bioprotective tool: A minireview with focus on endocrine disruptors. *FEMS Microbiol. Lett.* https://doi.org/10.1093/femsle/fnaa041 (2020).
31. Perugini, M. Sublethal exposure to propylparaben leads to lipid metabolism impairment in zebrafish early-life stages. *J. Appl. Toxicol.* **40**(4), 493–503 (2020).
32. Linney, E., Upchurch, L. & Donerly, S. Zebrafish as a neurotoxicological model. *Neurotoxicol. Teratol.* **26**(6), 709–718 (2004).
33. Gioacchini, G., Rossi, G. & Carnevali, O. Host-probiotic interaction: New insight into the role of the endocannabinoid system by in vivo and ex vivo approaches. *Sci. Rep.* **7**(1), 1261. https://doi.org/10.1038/s41598-017-01322-1 (2017).
34. Rousseaux, C. *et al. Lactobacillus acidophilus* modulates intestinal pain and induces opioid and cannabinoid receptors. *Nat. Med.* **13**(1), 35–37. https://doi.org/10.1038/nm1521 (2007).
35. Guida, F. *et al.* Antibiotic-induced microbiota perturbation causes gut endocannabinoidome changes, hippocampal neuroglial reorganization and depression in mice. *Brain Behav. Immun.* **67**, 230–245 (2018).
36. Everard, A. *et al.* Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. *Proc. Natl. Acad. Sci. U. S. A.* **110**(22), 9066–9071. https://doi.org/10.1073/pnas.1219451110 (2013).
37. Jansma, J., Brinkman, F., van Hemert, S. & El Aidy, S. Targeting the endocannabinoid system with microbial interventions to improve gut integrity. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **106**, 110169 (2021).
38. Wiacek, J. *et al.* Evaluating the impact of probiotic therapy on the endocannabinoid system, pain, sleep and fatigue: A randomized, double-blind, placebo-controlled trial in dancers. *Int. J. Mol. Sci.* **25**, 5611 (2024).
39. Palti, Y. Toll-like receptors in bony fish: From genomics to function. *Dev. Comp. Immunol.* **35**(12), 1263–1272 (2011).
40. Mazziotta, C., Tognon, M., Martini, F., Torreggiani, E. & Rotondo, J. C. Probiotics mechanism of action on immune cells and beneficial effects on human health. *Cells* **12**(1), 184. https://doi.org/10.3390/cells12010184 (2023).
41. Diamanti, T., Prete, R., Battista, N., Corsetti, A. & De Jaco, A. Exposure to antibiotics and neurodevelopmental disorders: Could probiotics modulate the gut-brain axis?. *Antibiotics* **11**(12), 1767. https://doi.org/10.3390/antibiotics11121767 (2022).
42. Vetuschi, A. *et al.* The antiinflammatory and antifibrotic effect of olive phenols and *Lactiplantibacillus plantarum* IMC513 in dextran sodium sulfate-induced chronic colitis. *Nutrition* **94**, 111511 (2022).
43. Martella, A. *et al.* Bisphenol A induces fatty liver by an endocannabinoid-mediated positive feedback loop. *Endocrinology* **157**(5), 1751–1763 (2016).
44. Ernst, J., Grabiec, U., Falk, K., Dehghani, F. & Schaedlich, K. The endocrine disruptor DEHP and the ECS: Analysis of a possible crosstalk. *Endocr. Connect.* **9**(2), 101–110 (2020).

45. Tian, S. *et al.* New insights into bisphenols induced obesity in zebrafish (*Danio rerio*): Activation of cannabinoid receptor CB1. *J. Hazard. Mater.* **418**, 126100 (2021).
46. Lv, Z. *et al.* DEHP induces obesity and hypothyroidism through both central and peripheral pathways in C3H/He mice. *Obesity* **24**(2), 368–378 (2016).
47. Sanders, M. E., Merenstein, D., Merrifield, C. A. & Hutkins, R. Probiotics for human use. *Nutr. Bull.* **43**, 212–225 (2018).
48. Good, M. *et al. Lactobacillus rhamnosus* HN001 decreases the severity of necrotizing enterocolitis in neonatal mice and preterm piglets: Evidence in mice for a role of TLR9. *Am. J. Physiol. Gastrointest. Liver Physiol.* **306**(11), 1021–1032 (2014).
49. Kant, R., de Vos, W. M., Palva, A. & Satokari, R. Immunostimulatory CpG motifs in the genomes of gut bacteria and their role in human health and disease. *J. Med. Microbiol.* **63**, 293–308 (2014).
50. Dothel, G. *et al.* m-opioid receptor, b-endorphin, and cannabinoid receptor-2 are increased in the colonic mucosa of irritable bowel syndrome patients. *Neurogastroenterol. Motil.* **31**(11), e13688 (2019).
51. Chen, M. *et al.* Resveratrol attenuates high-fat diet induced non-alcoholic steatohepatitis by maintaining gut, barrier integrity and inhibiting gut inflammation through regulation of the endocannabinoid system. *Clin. Nutr.* **39**, 1264–1275 (2020).
52. Cani, P. D. *et al.* Endocannabinoids: At the crossroads between the gut microbiota and host metabolism. *Nat. Rev. Endocrinol.* **12**, 133–143 (2016).
53. Passani, M. B., Provensi, G. & Piomelli, D. Editorial: The paracannabinoid system: Endocannabinoid-like lipids and their functions. *Front. Endocrinol.* **14**, 1263924 (2023).
54. Garcia-Gonzalez, N., Prete, R., Battista, N. & Corsetti, A. Adhesion properties of food-associated *Lactobacillus plantarum* strains on human intestinal epithelial cells and modulation of IL-8 release. *Front. Microbiol.* **9**, 2392 (2018).
55. Prete, R., Garcia-Gonzalez, N., Di Mattia, C. D., Corsetti, A. & Battista, N. Food-borne *Lactiplantibacillus plantarum* protect normal intestinal cells against inflammation by modulating reactive oxygen species and IL-23/IL-17 axis. *Sci. Rep.* **10**(1), 16340. https://doi.org/10.1038/s41598-020-73201-1 (2020).
56. Fanti, F. *et al.* Determination of endocannabinoids and their conjugated congeners in the brain by means of μSPE combined with UHPLC-MS/MS. *Talanta* **257**, 124392 (2023).

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Author contributions

N.B, M.P., and A.C. conceived the experiments, validated data, supported manuscript writing, supervised the overall study and provide financial resources. M.S. validated data and supervised chemical analysis. R.P., C.M., N.G.G., F.F. and G.A. performed the experiments. R.P. analyzed the results, prepared figures, drafted the manuscript and discussed the data with N.B., M.P. and A.C. All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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