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The probiotic *Lactobacillus rhamnosus* mimics the dark-driven regulation of appetite markers and melatonin receptors' expression in zebrafish (*Danio rerio*) larvae: Understanding the role of the gut microbiome

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A R T I C L E I N F O

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A B S T R A C T

The use of probiotics has been recently considered a novel therapeutic strategy to prevent pathologies such as obesity; however, the specific mechanisms of action by which probiotics exert their beneficial effects on metabolic health remain unclear. The aim of the present study was to investigate the short-term effects of a probiotic *Lactobacillus rhamnosus* supplementation (PROB) on appetite regulation, growth-related markers, and microbiota diversity in zebrafish (*Danio rerio*) larvae, compared to a group subjected to a constant darkness photoperiod (DARK), as well as to evaluate the effects of both treatments on melatonin receptors' expression. After a 24 h treatment, both PROB and DARK conditions caused a significant increase in *leptin a* expression. Moreover, mRNA abundances of *leptin b* and *proopiomelanocortin a* were elevated in the PROB group, and DARK showed a similar tendency, supporting a negative regulation of appetite markers by the treatments. Moreover, both PROB and DARK also enhanced the abundances of melatonin receptors transcript (*melatonin receptor 1 ba* and *bb*) and protein (melatonin receptor 1) suggesting a potential involvement of melatonin in mediating these effects. Nevertheless, treatments did not exhibit a significant effect on the expression of most of the growth hormone/insulin-like growth factor axis genes evaluated. Finally, only the DARK condition significantly modulated gut microbiota diversity at such short time, altogether highlighting the rapid effects of this probiotic on modulating appetite regulatory and melatonin receptors' expression, without a concomitant variation of gut microbiota.

1. Introduction

Although the concept of probiotics began its journey over a century ago, their use in human and animal nutrition did not rise in popularity until the past few years. One of the main reasons for this new-found interest stems from the improved understanding on the composition and function of the gut microbiome. In fact, advances in computational and new generation sequencing approaches have empowered the study of microbial communities and their impact on growth, development, and host health (Arnold et al., 2016; Malla et al., 2019). According to the World Health Organization, probiotics are originally defined as live microorganisms that contribute to the intestinal microbial balance. Basically, they have the capacity to colonize and multiply in the host gut exerting relevant beneficial effects by modulating several biological processes including appetite regulation, weight gain and energy balance. Hence, manipulation of gut microbiota with probiotics is now considered a potential novel therapeutic strategy to prevent pathologies such as obesity.

Diet composition and dietary patterns are known to be major regulators of the composition and function of gut microbiota (Angelakis et al., 2016; Telle-Hansen et al., 2018). Changes in intestinal microflora appear to affect host physiological processes in many ways, including

Abbreviations: PROB, Probiotic administration; DARK, Dark photoperiod; CT, Control.

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modulation of appetite and food intake (Turnbaugh et al., 2006). Therefore, a particular change in diet, such as the use of probiotics, can potentially modulate the composition of the microbiota affecting their functional capacity, which might ultimately lead to significant changes on metabolism and food consumption regulation in the host. Studies using animal models, have shown that probiotics from the genera *Lactobacillus*, are able to downregulate food intake and improve metabolic impairment by modulating gut microbiota toward health-promoting bacteria (Bagarolli et al., 2017; Falcinelli et al., 2017). One of the main regulators of appetite is the adipocyte-derived hormone leptin. In mammals, leptin reflects fat deposition, regulating food consumption and energy expenditure according to endogenous energy availability. In healthy individuals, plasma leptin levels increase postprandially inhibiting appetite, and decrease during periods of food deprivation (Weigle et al., 1997). As in mammals, the endocrine control of food intake in fish appears to be orchestrated through an intricate interplay between multiple neuronal and peripheral factors. In brief, leptin action activates anorexigenic neurons such as the proopiomelanocortin (POMC), and inhibits orexigenic ones, including those expressing neuropeptide Y (NPY), and the agouti-related protein (AgRP), leading to changes in food intake, body weight and glucose homeostasis (Balthasar et al., 2004; Huo et al., 2009). In this regard, it has been recently demonstrated that *L. rhamnosus* significantly upregulates the expression of *leptin* and other anorexigenic genes, while decreases orexigenic genes mRNA levels in zebrafish larvae, supporting the potential role of this probiotic in the regulation of appetite (Falcinelli et al., 2016).

Appetite and food intake are also influenced by external factors such as host circadian rhythmicity (Walton et al., 2011). In fact, several studies have shown that disruption of these light-dark rhythms can affect microbiota composition, which in turn can significantly impact the host immune system and metabolism (Hieke et al., 2019; Voigt et al., 2014). Melatonin, or the "sleep hormone", is one of the main downstream coordinators of this circadian information, allowing the regulation of many functions including energy homeostasis (Delagrange and Jockers, 2003; Dragoi et al., 2017) and growth (Ostrowska et al., 2001; Vriend et al., 1990). Several studies have demonstrated that photoperiod manipulation has a significant effect on melatonin and melatonin receptors' expression, which has been linked to the regulation of hunger and food intake by leptin (Bubenik and Pang, 1994; Buonfiglio et al., 2018; López-Olmeda et al., 2006; Piccinetti et al., 2013; Piccinetti et al., 2010). Moreover, it has also been shown that melatonin regulates growth by modulating growth hormone (GH) and insulin-like growth factors' (IGFs) levels in several animal models, although such effects are highly variable. A significant increase in body weight was found in Atlantic salmon (*Salmo salar*) treated with melatonin implants (Porter et al., 1998), whereas no effect or even a weight decrease was observed after melatonin implants or injections, in other studies using Atlantic salmon and goldfish (*Carassius auratus*) (De Pedro et al., 2008; Handeland et al., 2013).

In this context, the present study investigated the administration of the probiotic *L. rhamnosus* (PROB) on zebrafish larvae at an early-life stage (72 h post-fertilization, hpf) for 24 h, focusing on the specific short-term effects of this probiotic on the transcriptional regulation of appetite and growth signals, and the possible associated changes in the gut microbiome. In addition, larvae were challenged to a concomitant photoperiod switch (24 h of continuous darkness, DARK), with the purpose of comparing this condition with the probiotic effects on melatonin receptors' expression, and appetite and growth-markers genes' regulation. Non-mammalian vertebrates, such as zebrafish, have been proposed as excellent alternative model systems to study human metabolic diseases, owing to its functional conservation in lipid metabolism and adipose tissue biology (Oka et al., 2010; Seth et al., 2013). Moreover, zebrafish larvae hatch from their chorions between 48 and 72 hpf, when their mouth is open and the digestive tract is fully functional; however, exogenous feeding does not commence until 120 hpf (5 days) (Bates et al., 2006), which is particularly important when assessing microbiota analyses, where bacteria from feed can potentially be identified and therefore affect the microbiota population analysis. To this extent, this model species and the present experimental design conferred an excellent opportunity to examine the short-term effects of the probiotic on gene expression and microbiota diversity without the confounding impact of exogenous feeding and eating frequency, which could indeed alter appetite signals due to craving or anticipatory phenomena related to feeding schedule (Weingarten, 1984).

2. Materials and methods

2.1. Animal care and experimental design

Adult male and female zebrafish (*D. rerio*) were purchased from Acquario di Bologna (S.r.l., Italy) and maintained at the Dipartimento di Scienze della Vita e dell'Ambiente experimental facilities (Università Politecnica delle Marche, Ancona, Italy). Fish were kept in 100 L glass tanks under a 12:12 h light/dark photoperiod at 27 ± 0.5 °C. Illumination was provided by 3 ceiling fluorescent bulbs with 36 W intensity and 4000 K of color temperature (©Osram, Germany) and controlled by automatic timers. Around 7 pairs were spawned together, embryos were collected, and larvae were divided into 3 groups at hatching (72 hpf) in 1 L plastic tanks: one group was maintained at a 12:12 h light/dark photoperiod and was used as control (CT), the second group was exposed to a 12:12 h light/dark photoperiod and treated with the probiotic (PROB) *L. rhamnosus* IMC 501® (C025396A; Synbiotec, Camerino, Italy) and the third group was exposed to a 24 h dark photoperiod (DARK) (Fig. 1). The probiotic was administered in 1 L via rearing water at a concentration of 10^6 colony-forming units (CFU) according to previous studies (Gioacchini et al., 2012), as a single dose at the beginning of the 24 h-period. The experiment was set up in different tanks for each condition with 100 larvae per tank and it was repeated 3 (samples for the microbiome analysis), 4 (samples for protein expression analysis) or 5 (samples for gene expression analysis) times. After 24 h of exposure, pools of larvae from the different conditions were euthanized, at approximately the same time in the morning, using MS222 at 100 mg L^{-1} (Sigma-Aldrich) and stored at −80 °C. For gene and protein expression analyses, pooled samples of 40 larvae on each case were collected, and for the microbiome high-throughput sequence analysis, pooled samples of 100 larvae were used.

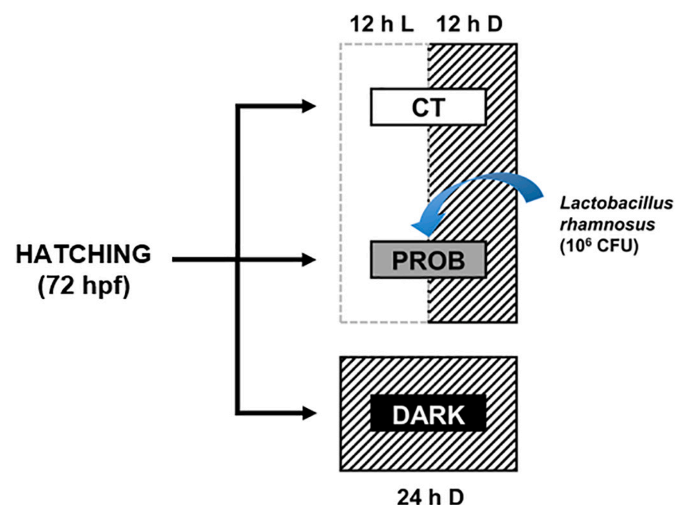

Fig. 1. Schematic representation of the experimental design. Zebrafish larvae were divided into 3 groups at hatching (72 hpf): one group was exposed to a 12:12 h light/darkness (CT, control), the second was exposed to the same photoperiod and treated with the probiotic *L. rhamnosus* (PROB), and the third one was exposed to 24 h of darkness (DARK).

2.2. RNA extraction, cDNA synthesis and gene expression analyses

Samples of larvae for quantitative real-time PCR (qPCR) analyses were homogenized with Precellys Evolution 24 homogenizer coupled to a Cryolis cooler (Bertin Instruments, Montigny-le-Bretonneux, France) and total RNA was extracted using TriReagent (Ambion, Alcobendas, Spain), according to the manufacturer's recommendations. Concentration and RNA purity were determined using a ND-2000 NanoDrop spectrophotometer (Thermo Fisher Scientific, Alcobendas, Spain) and 1000 ng of total RNA were treated with DNase I (Life Technologies, Alcobendas, Spain), following the manufacturer's protocol, to remove all genomic DNA. Afterwards, the RNA was reverse transcribed with the Transcriptor First Strand cDNA synthesis Kit (Roche, Sant Cugat del Valles, Spain) and the cDNA obtained was stored at −20 °C for gene expression analyses. The mRNA transcript levels of the appetite-related genes *lepa*; *lepb*; *lepr*; *agrp*; *npy*; *pomc*; melatonin receptors, *mtnr1aa*; *mtnr1ab*; *mtnr1al*; *mtnr1ba*; *mtnr1bb*; *mtnr1c*; growth-related genes *igf1; igf2*; *igf1ra*; *igf1rb*; *gh*; *ghra*; *ghrb*; plus two reference genes (*bactin* and *arp*) were examined with a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain).

qPCR was performed as previously described (Vélez et al., 2016). All analyses were performed in triplicate wells using 384-well plates with 2.5 μL itaq SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 250 nM forward and reverse specific primers (Table S1), and 1 μL diluted cDNA for each sample, in a final volume of 5 μL. The mRNA levels of each target gene analyzed were calculated relative to the reference genes (geometric mean of both, *bactin* and *arp*) using the Pfaffl method (Pfaffl, 2001). For primer efficiency calculations, gene expression results, and confirmation of reference genes stability, the implemented BioRad CFX manager 3.1. software was used.

2.3. Protein extraction and Western blot analysis

Samples for protein expression analyses were homogenized in radioimmunoprecipitation assay (RIPA) buffer with Precellys Evolution 24 homogenizer coupled to a Cryolis cooler (Bertin Instruments, Montigny-le-Bretonneux, France) and protein extraction, quantification and Western blot analysis were performed using the protocol previously described (Vélez et al., 2019). Briefly, 15 μg of protein were subjected to SDS-PAGE electrophoresis on 10% polyacrylamide gels, transferred overnight to a PVDF-FL membrane, and the total protein amount was tested with Revert Total Protein Stain solution (Cat. No. 926–11,011, Odyssey reagents, Servicios Hospitalarios, Barcelona, Spain). The membranes were blocked (Cat. No. 927–40,000, Servicios Hospitalarios, Barcelona, Spain), incubated overnight at 4 °C with the goat primary antibody MTNR1 (ab87639, Abcam, Cambridge, United Kingdom) diluted in the same blocking buffer at a concentration of 1:500, washed, and then incubated for 1 h at room temperature with a donkey anti-goat fluorescence-conjugated secondary antibody at 1:10000 dilution (Cat. No 925–68074, Servicios Hospitalarios). The membranes were rewashed, and the bands signal detected at 700 nm with the Odyssey® FC Imaging System (Li-Cor, Alcobendas, Spain), and quantified by the Odissey software Image Studio ver. 5.2.5. Results from the densitometry analysis of each specific band were normalized by the densitometry values of the two most abundant bands (∼ 37 KDa) after staining with Revert Total Protein staining as previously reported (Kirshner and Gibbs, 2018).

2.4. High-throughput microbiome sequencing, reads pre-processing and OTU assignments

Microbiome sequencing analyses were performed as previously described (Basili et al., 2019). Briefly, total DNA was extracted and V4 and V3 variable regions of the 16S rRNA were amplified using Illumina adapted primer 341F (CCTACGGGNGGCWGCAG) and Illumina adapted barcoded 805R primers (GACTACHVGGGTATCTAATCC) followed by 16S Metagenomic Sequencing Library Preparation protocol (llumina, San Diego, CA). Libraries were quantified and quality tested, and amplicons were sequenced on the Illumina MiSeq instrument run in paired-end mode with 300-bp read length by IGA Technology Services (www.igatechnology.com). Demultiplexing and raw sequence reads were processed with CASAVA v. 1.8 and Python package Cutadapt (Martin, 2011) v1.4.2, respectively. Quality trimming of paired-end reads were performed using the erne-filter command (Erne v1.4.6, default parameters except -min-size = 200) (Del Fabbro et al., 2013) and reads with a minimum length of 200 bp were retained and analyzed with QIIME v1. The USEARCH (v8.1.1756, 32-bit) quality filter pipeline was employed to filter chimeric reads, group replicate sequences and to identify OTUs. A minimum pairwise identity threshold of 97% was applied for OTU picking. Taxonomic assignment was attained by selecting the most abundant sequence in each OTU and using the Greengenes database (v 2013_5) coupled with the RDP classifier (v2.2), clustering the sequences at 97% similarity with a 0.50 confidence threshold. Outliers were identified and removed according to a principal component analysis (PCoA) before running downstream analysis.

2.5. Statistical analyses

qPCR and Western blot data were analyzed using SPSS Statistics v.22 (IBM, Armonk, USA) and GraphPad Prism 8 (La Jolla, USA, www.graphpad.com) and presented as mean ± SEM. Data normality and homoscedasticity were assessed using Shapiro-Wilk and Levene's tests, respectively. Statistical significance was assessed by one-way analysis of variance (one-way ANOVA), followed by Tukey HSD post-hoc test. For the microbiota analyses, alpha and beta diversity estimates along with rarefaction were performed using the R package Phyloseq (McMurdie and Holmes, 2013). Community composition was analyzed using the ADONIS function based on Bray-Curtis distances (R vegan package) (Oksanen et al., 2019). Statistical differences were considered significant for all analyses when p-value <0.05. Differential analysis in pairwise test adjusted for multiple comparisons was performed using raw counts as input into DESeq2 (Love et al., 2014) and considering a 1% FDR threshold (multiple testing correction applied using the Benjamini-Hochberg method).

3. Results

3.1. PROB and DARK increase the mRNA levels of inhibitory regulators of appetite

The first step in the present study was to elucidate whether the short-term probiotic treatment could modulate the expression of several appetite-related genes as in the larvae exposed to constant darkness. Results showed that both PROB and DARK conditions caused a very similar effect compared to the control (CT), increasing the mRNA levels of the appetite inhibitors leptin a (*lepa*), leptin b (*lepb*), leptin receptor (*lepr*) and *pomca* in the larvae (Fig. 2A-C and F). Specifically, PROB treatment significantly enhanced *lepa*, *lepb* and *pomca* expression whereas fish exposed to a DARK photoperiod showed a significant increase in *lepa* and *lepr* mRNA levels. No effect on the mRNA levels of the inducers of appetite, *npy* or *agrp* was recorded upon any of the treatments (Fig. 2D and E).

3.2. PROB treatment induces similar changes as DARK on melatonin receptors' expression

Transcriptional regulation, as well as protein expression levels of melatonin receptors in response to probiotic administration or photoperiod manipulation, were analyzed. Both PROB and DARK conditions showed a tendency for higher mRNA values of all the melatonin receptors genes tested than in the CT condition (Fig. 3). Particularly, both treatments significantly enhanced *mtnr1ba* and *mtnr1bb* mRNA levels

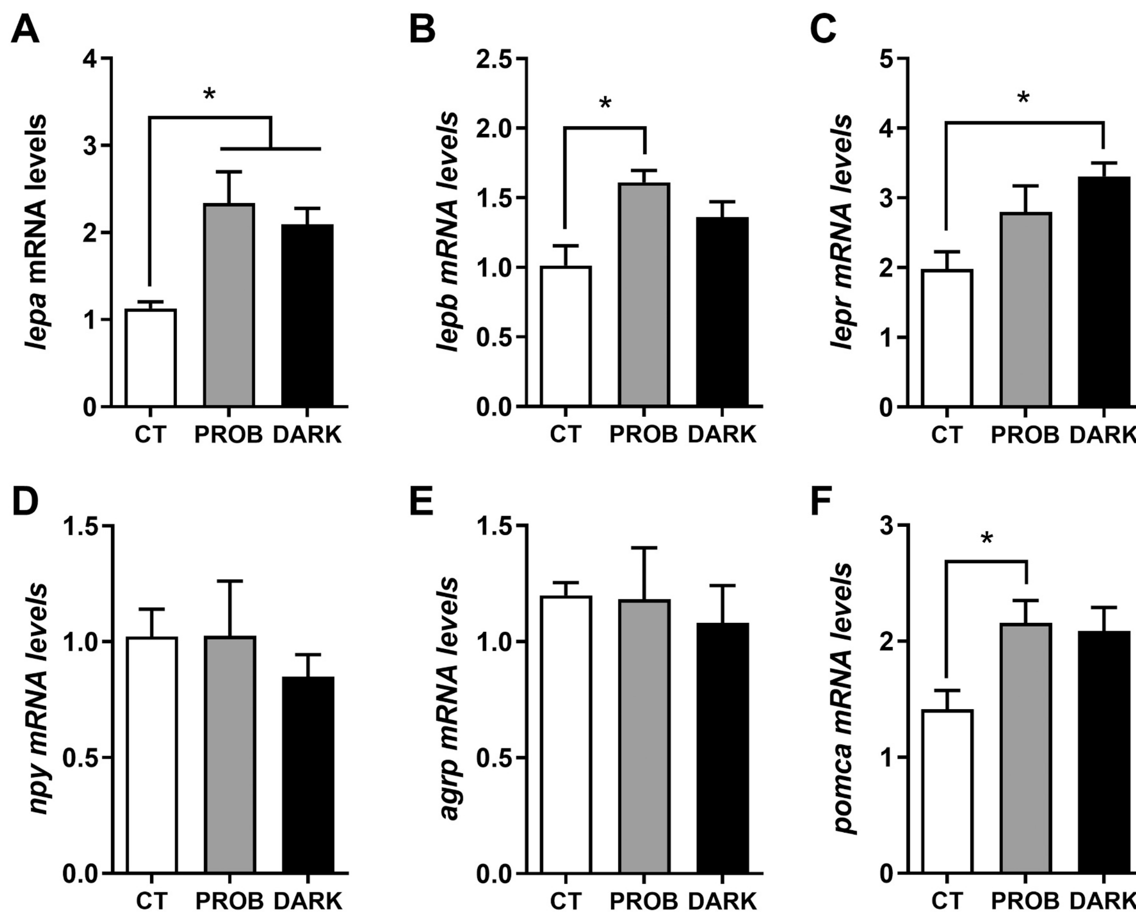

Fig. 2. Gene expression levels of appetite-related markers. Leptin a (*lepa*, **A**), leptin b (*lepb*, **B**), leptin receptor (*lepr*, **C**), neuropeptide Y (*npy*, **D**), agouti-related protein (*agrp*, **E**) and proopiomelanocortin (*pomca*, **F**) mRNA levels of zebrafish larvae exposed to the following conditions: 12:12 h light/darkness (CT, control), the same photoperiod while treated with the probiotic Lactobacillus rhamnosus (PROB), or 24 h of darkness (DARK). Relative expression levels were normalized to the geometric mean of two reference genes, beta actin (*bactin*) and acidic ribosomal protein (arp). Data are shown as mean+SEM (n = 4-5). Significant differences (p < 0.05) are indicated by asterisks and, were determined using a one-way ANOVA followed by Tukey's post hoc test.

(Fig. 3D and E), while only the DARK treatment induced a significant increase in *mtnr1aa* expression compared to the CT (Fig. 3A). Significant changes were not found for *mtnr1ab*, *mtnr1al* and *mtnr1c* (Fig. 3B, C and F). In addition, the PROB condition induced a significant increase of MTNR1 protein levels compared to the CT treatment (Fig. 4).

3.3. Effects of PROB and DARK on the mRNA levels of gh and igf-related genes

Significant effects upon the different treatments were not observed for most of the GH/IGF axis genes evaluated, specifically igf 1 (*igf1*), igf 2 (*igf2*), igf 1 receptor a (*igf1ra*), igf 1 receptor b (*igf1rb*) and gh receptor a (*ghra*) (Fig. 5A-D and F). On the other hand, *gh* mRNA levels showed a tendency to increase when larvae were treated with PROB and significantly increased when they were subjected to a DARK photoperiod compared to the CT animals (Fig. 4E). Moreover, PROB significantly induced the expression of gh receptor b (*ghrb*) (Fig. 5G).

3.4. PROB and DARK effects on gut microbiota

In light of the results obtained, we wanted to elucidate whether the aforementioned changes in both gene and protein expression caused by PROB and DARK could be accompanied by variations in gut microbiota diversity. Hence, a high-throughput sequence analysis of bacterial 16S rRNA (V3 and V4 regions) was conducted. The alpha rarefaction plot of observed species reached a plateau at approximately 60 thousand sequences indicating that adequate sequence coverage was obtained (data not shown). Species richness was evaluated computing alpha diversity by the number of observed operation taxonomic units (OTUs), Shannon, and Simpson indexes. Particularly, the DARK condition showed an increased number of observed OTUs, and higher Shannon (significant) and Simpson indexes, both compared to CT and PROB-treated larvae. Nevertheless, significant differences were not observed between CT and PROB conditions in any of the alpha diversity methods tested (Table 1).

Moreover, in order to evaluate relationships among samples and the ability of both DARK and PROB to modulate bacterial composition, a PCoA based on Bray-Curtis distance was performed (Fig. S2). DARK samples clustered away from both, the CT and PROB samples suggesting that darkness represent a key factor in shaping microbial communities. In addition, a single administration of probiotic in a time window of 24 h seemed unable to induce significant changes in the microbiota composition. This finding was also statistically supported by a PERMANOVA analysis ($p < 0.02$).

A multivariate differential abundance analysis considering all groups together was performed; however, no differentially expressed taxa was observed in the PROB condition compared to CT at any taxonomic level (data not shown), in accordance with the alpha and beta diversity results (Table 1). Therefore, in order to display a more detailed analysis and capture any potential difference present, a pairwise test adjusted for multiple comparisons was implemented. At the phylum level, abundance of Proteobacteria was significantly reduced in the DARK compared to the PROB condition (Fig. 6A). In addition, despite the other phyla only accounted for a small fraction of the total composition, we detected Bacteroidetes to be significantly less abundant in the CT

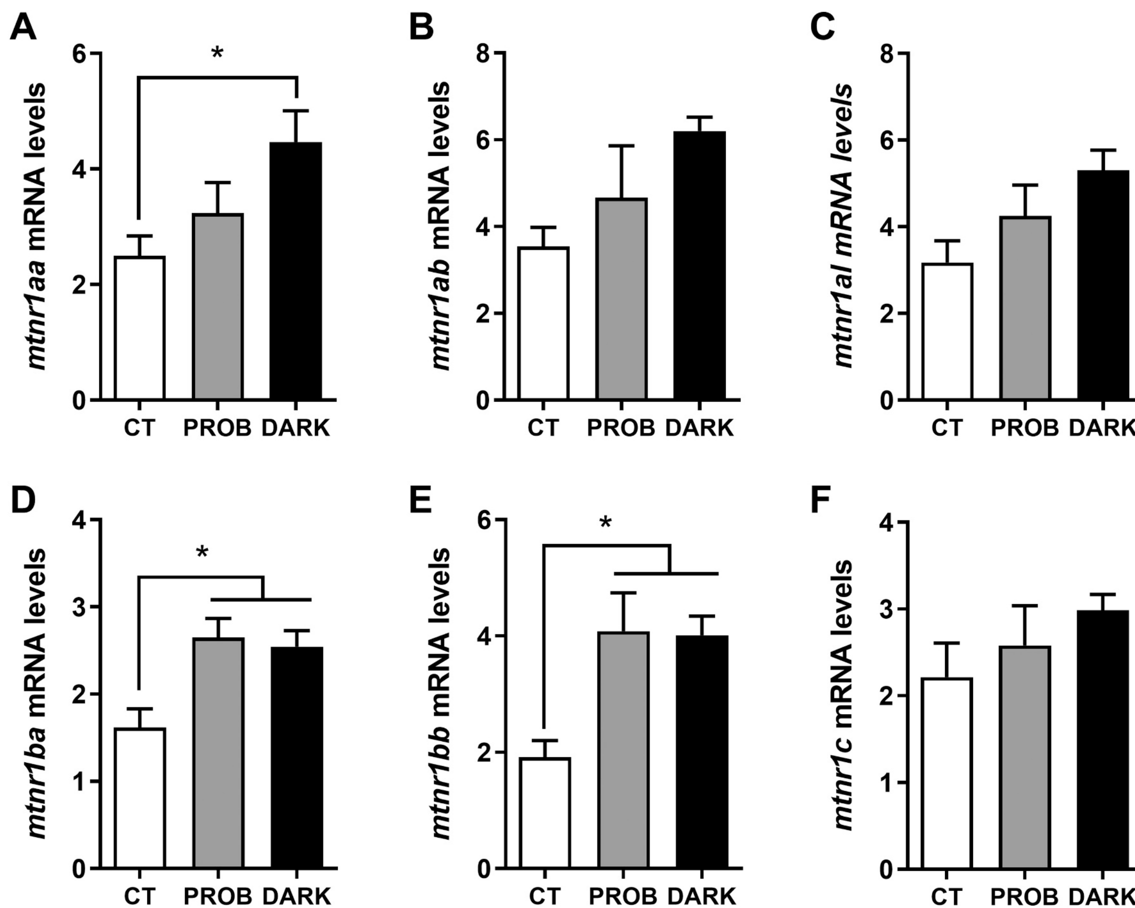

Fig. 3. Gene expression levels of melatonin receptors. Melatonin receptor (mtnr) 1Aa (*mtnr1aa*, **A**), mtnr 1Ab (*mtnr1ab*, **B**), mtnr type 1A like (*mtnr1al*, **C**), mtnr 1Ba (*mtnr1ba*, **D**), mtnr 1Bb (*mtnr1bb*, **E**) and mtnr 1C (mtnr1c, F) mRNA levels of zebrafish larvae exposed to the following conditions: 12:12 h light/darkness (CT, control), the same photoperiod while treated with the probiotic *Lactobacillus rhamnosus* (PROB), or 24 h of darkness (DARK). Relative expression levels were normalized to the geometric mean of two reference genes, beta actin (bactin) and acidic ribosomal protein (arp). Data are shown as mean+SEM (n = 4-5). Significant differences ($p < 0.05$) are indicated by asterisks and, were determined using a one-way ANOVA followed by Tukey's post hoc test.

compared to the PROB condition, and the Cyanobacteria compared to DARK.

At the class level, within the phylum of the Proteobacteria, the abundance of Betaproteobacteria was significantly reduced in the DARK condition compared to CT and PROB-treated zebrafish, whereas Alphaproteobacteria abundance was found to be significantly lower in the CT compared to the DARK and in the CT compared to the PROB (Fig. 6B). Moreover, we found abundance of Sphingobacteriia and Nostocophycidae to be significantly higher in the PROB compared to the CT and in the DARK compared with the CT fish, respectively. At order and family levels, the DARK condition also showed a more diversified microbial community compared to both CT and PROB. More specifically, at the order level, the abundance of both Burkholderiales and Enterobacteriales showed a significant reduction in the DARK compared to both CT and PROB, while at the family level, the abundance of Comamonadaceae, the most abundant family found, was significantly reduced (Fig. S3 A and B). At the genera level, around 200 genera were successfully identified. Among them, 64 genera were specific for DARK, which corresponded to 4 times more genera compared to the ones resolved for either CT (13) and PROB (14), thus again, the DARK condition showing a higher richness diversity (Fig. 6C). Particularly, OTUs belonging to the genera *Cetobacterium* and *Shinella* were significantly more abundant in the larvae exposed to the DARK photoperiod compared to both PROB and CT conditions (abundance greater than 1%).

On the other hand, OTUs belonging to the genera *Azohydromonas*, *Plesiomonas*, *Rubrivivax*, and *Variovorax* were significantly less abundant in the DARK condition compared to the other two groups. The genera *Acidovorax*, *Pelomonas*, *Pseudomonas*, *Rheinheimera* and *Rhodobacter* were significantly more abundant in the PROB condition compared to the CT, whereas *Plesiomonas* and *Hydrocarboniphaga* appeared to be less abundant in the former (Fig. 6D). At the species level (Table 2), again the main differences were observed when comparing the DARK to the other two conditions, either CT or PROB, with more than double of differentially abundant identified species in the PROB condition compared to DARK, and showing differences around 10 to 20-fold (log2FC). For instance, OTUs belonging to the species *N. subterraneum*, *D. invisus*, *P. simplex*, *P. amoebophila* and *M. organophilum* were significantly more abundant in the DARK condition compared to CT and PROB, while *V. paradoxus*, *H. daqingensis*, *S. oneidensis*, *S. aquatica* and *S. putrefaciens* showed reduced levels in the DARK condition. Interestingly, even though both DARK and PROB showed the presence of the *Lactobacillus* genera (Fig. 6D), only in the DARK condition the species *L. rhamnosus* could be identified (more than 1% abundance) and appeared to be significantly higher compared to its levels on the CT fish (Table 2).

4. Discussion

In recent years, there has been growing interest in understanding the potential beneficial health effects of probiotics and their implications in host metabolism and energy balance (Brusaferro et al., 2018; Molinaro et al., 2012). However, despite many studies support the beneficial properties of probiotics in this matter, the specific mechanisms underlying their health effects are still poorly understood (Cerdó et al., 2019;

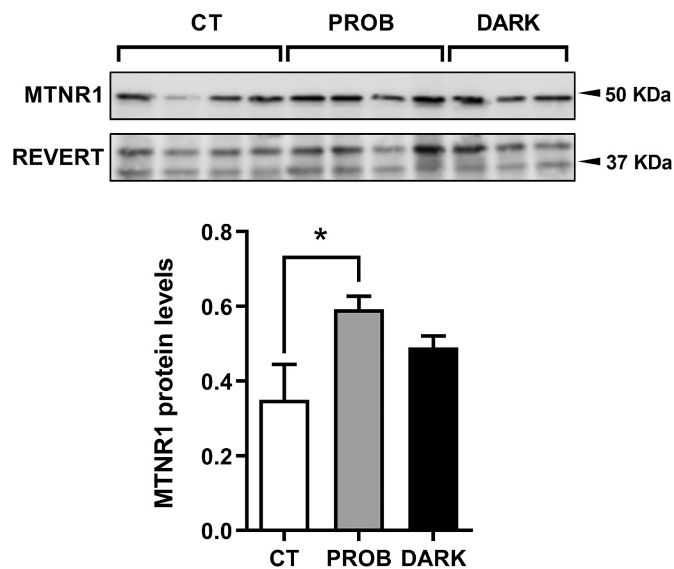

Fig. 4. Protein expression levels of melatonin receptor 1. Representative immunoreactive bands and quantification of melatonin receptor 1 (MTNR1) protein expression of zebrafish larvae exposed to the following conditions: 12:12 h light/darkness (CT, control), the same photoperiod while treated with the probiotic *Lactobacillus rhamnosus* (PROB), or 24 h of darkness (DARK). Densitometry levels of each specific MTNR1 band (~50 KDa) were normalized by the densitometry values of the 2 most abundant bands of Revert Total Protein staining (~ 37 KDa). Data are shown as mean+SEM ($n = 3$-4). Significant differences ($p < 0.05$) are indicated by asterisks and, were determined using a one-way ANOVA followed by Tukey's post-hoc test.

Plaza-Diaz et al., 2019). In addition, some results differ depending on experimental details, including the probiotic strain and the amount or duration of the probiotic exposure, often pointing toward opposing conclusions, suggesting that extended information is needed (Suez et al., 2019). In this regard, the present study has demonstrated that a short-term administration of the probiotic *L. rhamnosus* regulates some orexigenic markers in zebrafish (*D. rerio*) larvae in a similar way than exposure to constant darkness but without concomitant changes in microbiota diversity, highlighting for the first time the effects of this probiotic on melatonin receptors' expression.

4.1. Short-term probiotic administration and constant darkness effects on the transcriptional regulation of selected appetite markers

A lipostatic model for appetite and food intake regulation has been proposed for fish (Johansen et al., 2002; Johnson et al., 2000). However, leptin's role in this group of vertebrates is still in some cases under debate. In this regard, some studies have shown no effects of leptin or even a negative correlation between its expression and food intake or appetite markers (van de Pol et al., 2017), while others have supported a similar regulatory function of leptin on appetite in fish as it is in mammals (Gorissen and Flik, 2014). Hence, results should be interpreted carefully, and specific protocols for different species and experimental design conditions must be always considered. In this regard, our results showed an increase in satiety signals such a significant upregulation of *lepa*, *lepb, pomca* and a slight increase in *lepr* mRNA levels when larvae were treated with PROB, in accordance with the appetite suppressing role of leptin in mammals, which has been also previously described in zebrafish (Ahi et al., 2019; Falcinelli et al., 2016). Appetite regulation has been traditionally defined as the homeostatic balance that corresponds to energy/nutrient deficit or excess. However, extensive scientific evidence also suggested the existence of a non-homeostatic process involving feeding, driven by environmental factors such as the alternation of light and dark (Cheung et al., 2016). In fact, in many living organisms, physiological, metabolic, and behavioral processes are subjected to circadian rhythms adjusted by light-dark cycles. Moreover, it has been demonstrated that numerous endocrine signals, including leptin, modulate food intake by acting in both, classic homeostatic and non-homeostatic ways, which in some cases result in similar feeding-related behavioral outcomes (Liu and Kanoski, 2018). In our work, the previously mentioned effects of the PROB condition on appetite-related genes expression' were also accompanied by a comparable up-regulatory effect in the DARK group, indicating that both treatments have similar effects on these satiety markers.

4.2. Potential role of melatonin in regulating the appetite suppressing signals induced by the probiotic treatment

Melatonin has been proposed as one of the main hormonal mediators of photoperiod information, influencing several physiological processes including energy homeostasis, adiposity, reproduction, osmoregulation, cell proliferation, and growth, among others. This hormone is produced rhythmically showing increased levels at night and low levels during the day in species with regular light/dark circadian rhythms, regardless of whether the animal is diurnally or nocturnally active. Particularly, its effects on appetite regulation have been extensively reported in different species (Montalbano et al., 2018; Sanchez-Mateos et al., 2007). In fact, a study performed on zebrafish demonstrated that administration of melatonin for a period of 10-days reduced food intake by stimulating the expression of molecules involved in appetite inhibition, including leptin and the melanocortin 4 receptor (*mc4r*) (Piccinetti et al., 2013). Moreover, melatonin supplementation reduced food intake in response to a high-fat diet (HFD) in rats (Puchalski et al., 2003). Several studies using mammalian white adipocytes in culture demonstrated that melatonin is critically involved in leptin synthesis and release (Alonso-Vale et al., 2008; Alonso-Vale et al., 2005; Cardoso Alonso-Vale et al., 2006). Furthermore, a study performed in adult rats revealed that absence of melatonin leads to long-term leptin resistance and overweight (Buonfiglio et al., 2018), altogether suggesting that melatonin may play a key role in the regulation of energy metabolism and appetite by acting on the leptin pathway. Therefore, understanding the interaction between melatonin and leptin, and their potential effects on appetite and energy homeostasis is critical to improving strategies to prevent obesity.

Most of the chronobiotic effects of melatonin are mediated through its 3 receptors (MTNR1A, MTNR1B and MTNR1C) (Li et al., 2013). In the present study, both PROB and DARK conditions modulated melatonin receptors' expression. Although significant differences compared to the CT condition were only found for *mtnr1ba*, *mtnr1bb* (DARK and PROB), *mtnr1aa* (DARK) mRNA levels and MNTR1 protein expression (PROB), a similar tendency to increase *mtnr1ab*, *mtnr1al* and *mtnr1c* mRNA levels was also observed in both conditions. These results showed for the first time the effects of *L. rhamnosus* on melatonin receptors expression, suggesting that this hormone might be involved in the probiotic effects on appetite control in zebrafish larvae maybe as a rapid trigger signal. Nevertheless, further studies are required in order to elucidate whether or not melatonin could be a potential mediator of the changes on appetite signals produced by the probiotic. In any case, the present study is a novel contribution since to our knowledge, only another study has explored the role of melatonin as a potential mechanism of action of probiotics (Wong et al., 2014). Those authors demonstrated that this hormone is not only implicated in the control of the sleep-wake cycle, but also in the modulation of bowel function in humans. Indeed, that study demonstrated the correlation of morning melatonin to improve irritable bowel syndrome symptoms, pointing toward the existence of a potential link between probiotics and this hormone (Wong et al., 2014).

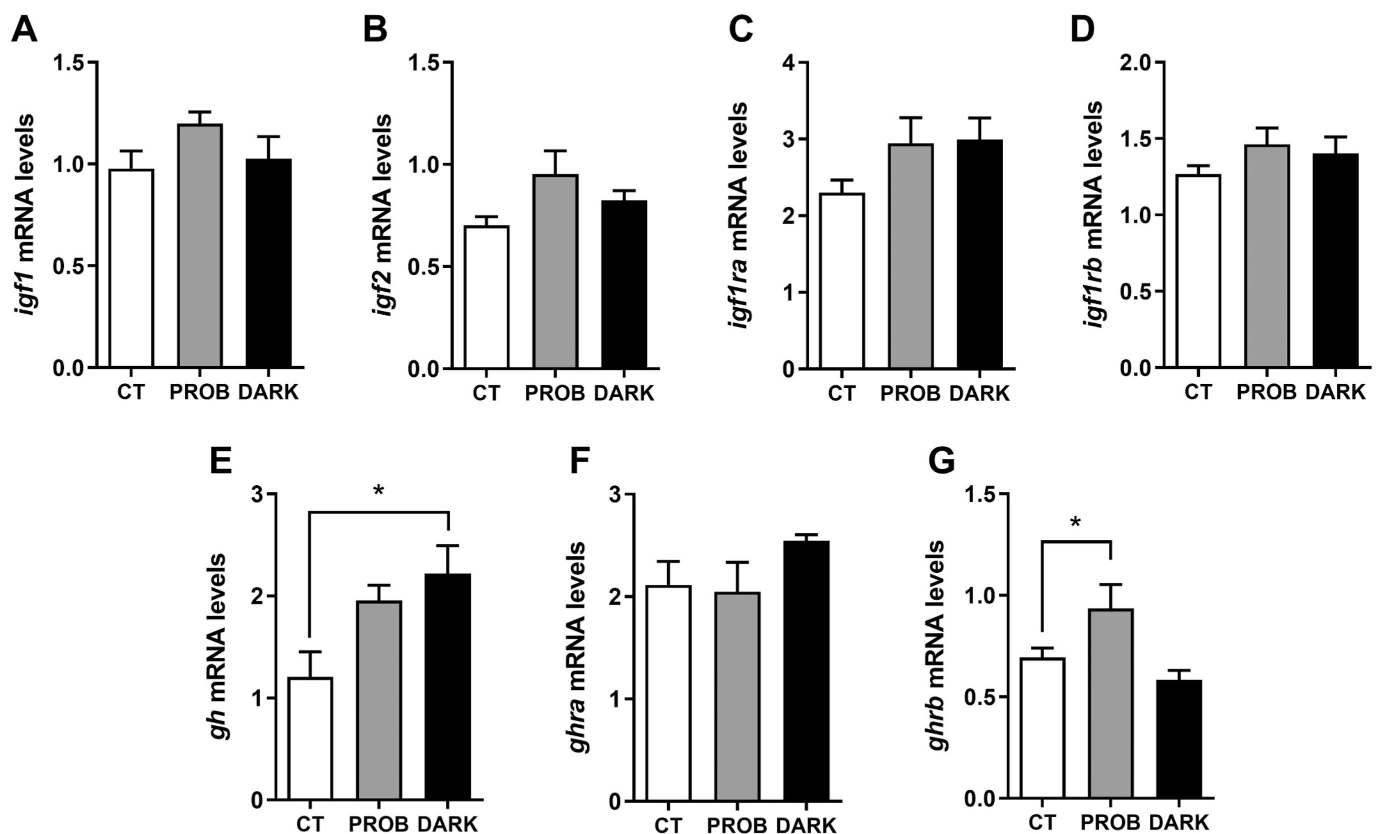

Fig. 5. Gene expression levels of insulin-like growth factors and growth hormone-related markers. Insulin-like growth factor (igf) 1 (*igf1*, **A**), igf 2 (*igf2*, **B**), igf receptor a (*igf1ra*, **C**), igf receptor b (*igf1rb*, **D**), growth hormone (*gh*, **E**), growth hormone receptor a (*ghra*, **F**) and growth hormone receptor b (*ghrb*, **G**) mRNA levels of zebrafish larvae exposed to the following conditions: 12:12 h light/darkness (CT, control), the same photoperiod while treated with the probiotic *Lactobacillus rhamnosus* (PROB), or 24 h of darkness (DARK). Relative expression levels were normalized to the geometric mean of two reference genes, beta actin (bactin) and acidic ribosomal protein (arp). Data are shown as mean+SEM (n = 4-5). Significant differences ($p < 0.05$) are indicated by asterisks and, were determined using a one-way ANOVA followed by Tukey's post hoc test.

Table 1
Alpha diversity values of observed OTUs, Shannon index and Simpson index.

	CT	PROB	DARK
Observed OTU's	340.0 ± 17.4	385.8 ± 33.2	458.9 ± 83.4
Shannon index	2.10 ± 0.19[a]	2.16 ± 0.36[a]	3.65 ± 0.58[b]
Simpson index	0.73 ± 0.03	0.72 ± 0.08	0.90 ± 0.08

Data are shown as mean ± SEM (n = 2–3). Significant differences ($p < 0.05$) are indicated by different letters and, were determined using a one-way ANOVA followed by Tukey's post hoc test.

4.3. Short-term probiotic administration and constant darkness effects on the transcriptional regulation of growth hormone/insulin-like growth factor axis-related genes

Melatonin has been traditionally implicated in the hormonal regulation of growth. A number of studies in mammalian and fish models demonstrated that melatonin administration stimulates growth, upregulating GH and IGFs levels. Indeed, melatonin injections and short-day conditions (10 h:14 h light/dark) increased the expression of GH in Olive flounder (*Paralichthys olivaceus*), promoting growth and weight gain (Kim et al., 2019). Similarly, several studies have also reported that some probiotic strains increase growth through modulation of these hormones. For instance, the probiotics *Bacillus amyloliquefaciens* and *B. subtilis* exhibited a growth potential effect by up-regulating GH and IGF-I levels, and improving the intestinal and rumen development in growth-retarded beef calves (Du et al., 2018). In addition, *L. rhamnosus* and *L. plantarum* appeared to increase *igf1* transcript abundance in zebrafish larvae (Avella et al., 2012; Carnevali et al., 2006) and broiler chickens liver (Kareem et al., 2016), respectively. In this regard, our results showed a slight tendency to increase the expression of most of the GH/IGF axis genes evaluated. Nevertheless, only *gh* mRNA levels showed a significant upregulation when larvae were subjected to a DARK photoperiod compared to CT animals, whereas PROB significantly induced the expression of *ghrb*. The lack of effect on the expression of growth transcriptional regulators could be explained by the length of the experimental design, being 24 h of treatment not sufficient to trigger a significant change on these growth markers. Thus, an extended experiment would be needed in order to fully elucidate the potential relation of *L. rhamnosus* upon GH/IGF axis regulation in zebrafish.

4.4. Short-term probiotic effects are independent of significant changes in the gut microbiome

Many authors have highlighted the existence of a gut microbiota and brain "crosstalk", recognized as a bidirectional connection that sends signals to influence appetite, secretions, and permeability of the gut (Backhed et al., 2004; Clemmensen et al., 2017; Turnbaugh et al., 2006). Hence, not only the brain can affect gut functionality, but the gut can also modulate numerous processes that were thought to be controlled by the central nervous system (CNS) (Carabotti et al., 2015; Heijtz et al., 2011). The effects of probiotics on the regulation of metabolism and appetite are normally linked to changes in gut microbiota composition. Nevertheless, as already stated, the specific mechanisms by which the probiotics exert their beneficial metabolic effects are still not fully elucidated. In fact, it remains unclear if the modulation of the gut

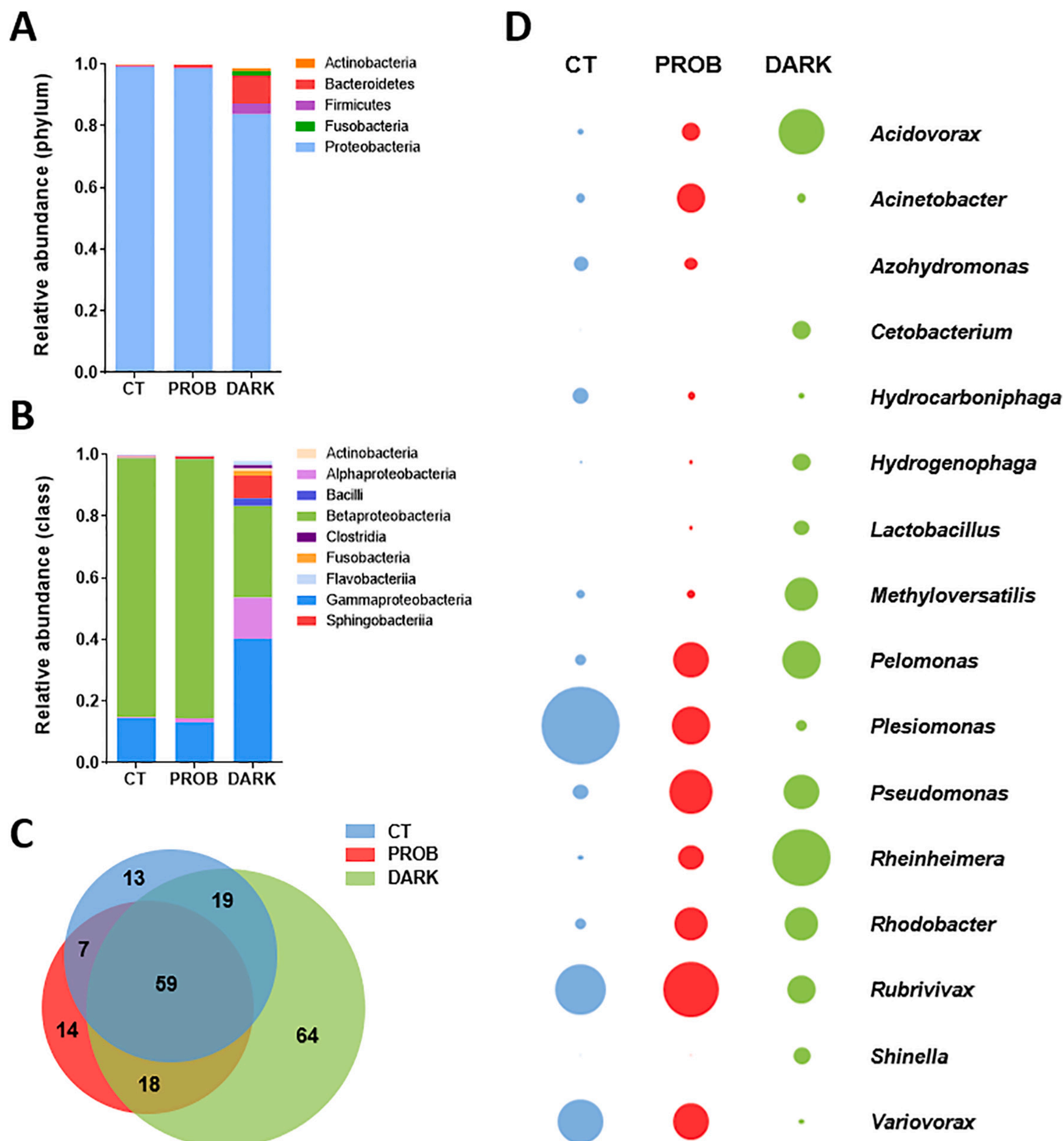

Fig. 6. Gastrointestinal bacterial community analysis. Zebrafish larvae were exposed to the following conditions: 12:12 h light/darkness (CT, control), the same photoperiod while treated with the probiotic *Lactobacillus rhamnosus* (PROB), or 24 h of darkness (DARK). Stacked bar chart representing the relative abundance of bacterial phylum (**A**) and class (**B**) (only taxa contributing to at least 1% of the total composition are displayed). Venn diagram showing the distribution of OTUs at genera level (**C**). Bubble chart showing genera differentially abundant upon the different conditions (abundance greater than 1%), determined by a pairwise test adjusted for multiple comparisons (Benjamini-Hochberg) (n = 2-3). Bubble size indicates higher (big) or lower (small) relative abundance of bacterial genera (D).

microbiota is responsible for these positive outcomes or, on the other hand, might be a consequence of the direct effects produced by the probiotic treatment. But indeed, most of the studies concluded that these effects are in fact a consequence of gut microbiota changes. For example, *L. paracasei*, *L. rhamnosus* and *B. animalis* administration was shown to attenuate obesity comorbidities, including weight gain and improved liver steatosis, in part through specific impacts on gut microbiota in a HFD-fed mice (Wang et al., 2015). Moreover, studies performed in larvae and adult zebrafish, claimed that the administration of *L. rhamnosus* was able to produce significant changes in lipid metabolism and appetite-related genes expression, showing a decrease in the mRNA levels of genes involved in cholesterol transport and triglyceride synthesis, as well as orexigenic genes, while causing an upregulation of anorexigenic ones, through changes in microbiota composition (Falcinelli et al., 2017; Falcinelli et al., 2016). Although these changes in appetite markers and microbiota diversity are allegedly linked and directly correlated in relatively long-term treatments (ranging from few days to several months), very little information is available regarding the effects of probiotics in shorter-term trials. Thus, from our perspective, it is difficult to elucidate whether these mechanisms are independent, partially, or totally interdependent. In other terms, the transcriptional regulation of appetite-markers might take place before changes in microbiota diversity, and therefore could be produced by other means. In this regard, our results showed that the probiotic *L. rhamnosus* did not significantly influence microbiota diversity after 24 h of treatment. Nonetheless, changes in the mRNA levels of appetite-related markers and melatonin receptors were present, as previously mentioned. In particular, differently from the DARK photoperiod, no significant changes were identified at the higher levels of taxonomy (i.e., phylum, class) with the PROB treatment, although some of them were

Table 2
Pairwise comparison of differentially abundant species between groups.

DARK-CT			DARK-PROB			PROB-CT		
Species	FDR	log2FC	Species	FDR	log2FC	Species	FDR	log2FC
V. paradoxus	2.55×10^{-19}	- 7.8	*V. paradoxus*	1.14×10^{-23}	- 6.4	*P. saccarophila*	2.01×10^{-15}	3.1
H. daqingensis	2.70×10^{-12}	- 13.9	*P. alcaligenes*	3.03×10^{-6}	- 3.7	*P. alcaligenes*	6.00×10^{-14}	3.1
N. subterraneum	1.32×10^{-5}	20.5	*D. invisus*	8.27×10^{-6}	20.9	*H. daqingensis*	5.80×10^{-5}	- 2.9
D. invisus	2.02×10^{-5}	21.3	*G. obscuriglobus*	4.63×10^{-5}	- 10.7	*P. peli*	7.57×10^{-5}	9.1
S. oneidensis	2.02×10^{-5}	−9	*N. subterraneum*	5.43×10^{-5}	20.1	*P. copri*	1.47×10^{-3}	8.2
P. fluviatilis	2.02×10^{-5}	20.1	*H. daqingensis*	1.12×10^{-4}	- 10.5	*G. obscuriglobus*	1.65×10^{-3}	8.1
S. aquatica	2.02×10^{-5}	- 10.9	*P. simplex*	1.17×10^{-4}	19.6	*B. producta*	3.07×10^{-3}	- 7.8
P. simplex	2.25×10^{-5}	19.9	*C. amoebophila*	1.17×10^{-4}	19.4	*A. johnsonii*	8.49×10^{-3}	2.9
P. amoebophila	2.25×10^{-5}	19.9	*S. oneidensis*	1.69×10^{-4}	- 8.3			
L. rhamnosus	1.09×10^{-4}	8.1	*W. falsenii*	8.13×10^{-4}	−10			
M. organophilum	9.59×10^{-4}	5.8	*S. aquatica*	1.35×10^{-3}	- 9.9			
S. putrefaciens	1.57×10^{-3}	- 9.4	*S. amazonensis*	1.36×10^{-3}	- 10.1			
H. effusa	3.23×10^{-3}	−7	*A. johnsonii*	2.08×10^{-3}	−4			
P. mexicana	3.33×10^{-3}	6.1	*S. granuli*	2.41×10^{-3}	7.9			
R. gelatinosus	3.66×10^{-3}	- 4.1	*M. organophilum*	2.67×10^{-3}	4			
S. decolorationis	8.06×10^{-3}	- 9.6	*S. putrefaciens*	4.43×10^{-3}	- 8.1			
			E. oligotrophica	7.08×10^{-3}	- 9.1			

Species whose abundance was greater than 1% across all the experimental conditions. FDR, false discovery rate. Determined by a pairwise test adjusted for multiple comparisons (Benjamini-Hochberg) (n = 2–3).

found at the genera and species level only after a pairwise comparison. However, the lack of knowledge on how these taxa might influence physiological processes in fish makes it difficult to attribute particular effects to specific changes in genera or species diversity.

It is important to note that one possible explanation for the limited impact of the probiotic on microbiota diversity in our study could be that 24 h might not be enough to cover a window of time for *L. rhamnosus* to alter the gut microbial community. However, the DARK condition showed a more diversified microbiota already at the phylum level were the abundance of Proteobacteria, the most abundant microbiota phylum found in zebrafish larvae and juveniles (Stephens et al., 2016), was significantly reduced compared to both CT and PROB, suggesting that only the DARK photoperiod was able to drastically modulate larvae microbiome after a short treatment. These findings are in accordance with a previous study from our group where no effects of *L. rhamnosus* on microbiota composition were found, whereas a significant impact of photoperiod was reported (Basili et al., 2019). In the present study, even though both PROB and DARK conditions showed the presence of the *Lactobacillus* genera, the species *L. rhamnosus* could not be identified in PROB, suggesting altogether that the reported effects of the probiotic on appetite regulatory markers may not be mediated by the probiotic colonization nor significant changes in the gut microbiome, but simply from its passage in the intestine as previously observed in other species (Pérez-Sánchez et al., 2011). In this regard, contrary to what it has been previously reported by other authors (Falcinelli et al., 2017, Falcinelli et al., 2016), our results suggest that the effects of the probiotic on appetite regulation might not be primarily related to changes in microbiota composition after a short-term trial, or at least not through *L. rhamnosus* colonization in the gut, and that other direct mechanisms might be in place. We hypothesize that the bacterial strain used in this study could be able of releasing compounds capable of modulating appetite signals or, somehow directly regulate such functions by other means than changes in the gut microbiota. In this regard, recent findings have indicated that surface components and metabolites produced by probiotics such as secreted proteins and short-chain fatty acids, can constitute molecular patterns and specifically bind to certain receptors, regulating key signaling pathways (Liu et al., 2020; Plaza-Diaz et al., 2019). It is important to mention that while the use of a short-term approach allowed identifying a non-concomitant effect of the probiotic on the regulation of appetite markers and microbiota changes, as well as a potential involvement of melatonin that have not been described in similar studies, also limited our ability to answer other scientific questions, including the fact that a longer exposure to the probiotic could have a stronger impact on the microbiota diversity, and/or different effects on the gene markers analyzed. Particularly, although a tendency toward an upregulation of growth-related markers was observed, a 24 h treatment appeared to be not sufficient to trigger a significant change on most of the genes evaluated. Moreover, a longer-term experiment could also confirm that the observed effects on melatonin receptors' expression are not only transient, which could have important implications considering the role of this hormone in appetite control. Therefore, further studies need to be performed in order to fully elucidate both, the mechanisms of action by which the probiotics exert their effects on appetite regulation as well as the potential involvement of melatonin in this process.

5. Conclusions and future perspectives

In summary, the present study demonstrated for the first time the short-term effects of the probiotic *L. rhamnosus* on the modulation of appetite markers and melatonin receptors' expression in zebrafish larvae, highlighting a high similarity to the dark-driven effects produced by a photoperiod shift to a constant darkness. The microbiome analysis revealed that the probiotic regulation of the appetite-related genes and melatonin receptors' expression appeared to be independent of drastic changes in microbiota composition. In addition, although both PROB and DARK conditions showed the presence of the *Lactobacillus* genera, only in the DARK condition the species *L. rhamnosus* could be identified, suggesting altogether that the aforementioned effects of the probiotic may not be mediated by its colonization in the gut microbiome in the first place. Overall, our findings represent an important first step in the characterization of the mechanisms of action by which the probiotic *L. rhamnosus* exerts its effects on appetite and melatonin regulation. However, we anticipate that in order to generate mechanistic data that could provide more evidence on the role of melatonin in mediating the probiotic effects, new experiments using relevant zebrafish lines or genetically modified individuals with, for instance, a lack of melatonin production or receptors expression should be performed.

Data availability

The Raw sequencing data can be accessed through the NCBI Sequence Read Archive (SRA) database under the Bioproject number PRJNA528701.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpb.2021.110634.

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