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Probiotic Strain *Bifidobacterium animalis* subsp. *lactis* CECT 8145 Reduces Fat Content and Modulates Lipid Metabolism and Antioxidant Response in *Caenorhabditis elegans*

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Supporting Information

ABSTRACT: Recently, microbial changes in the human gut have been proposed as a possible cause of obesity. Therefore, modulation of microbiota through probiotic supplements is of great interest to support obesity therapeutics. The present study examines the functional effect and metabolic targets of a bacterial strain, *Bifidobacterium animalis* subsp. *lactis* CECT 8145, selected from a screening in *Caenorhabditis elegans*. This strain significantly reduced total lipids (40.5% ± 2.4) and triglycerides (27.6% ± 0.5), exerting antioxidant effects in the nematode (30% ± 2.8 increase in survival vs control); activities were also preserved in a final food matrix (milk). Furthermore, transcriptomic and metabolomic analyses in nematodes fed with strain CECT 8145 revealed modulation of the energy and lipid metabolism, as well as the tryptophan metabolism (satiety), as the main metabolic targets of the probiotic. In conclusion, our study describes for the first time a new *B. animalis* subsp. *lactis* strain, CECT 8145, as a promising probiotic for obesity disorders. Furthermore, the data support future studies in obesity murine models.

KEYWORDS: *obesity, metabolic syndrome, Bifidobacterium strain, Caenorhabditis elegans, probiotic*

BACKGROUND

Recent advances in sequencing technology have shown the complexity of microbiota within metaorganisms and how microbiome modulation impacts health. Based on such information, therapeutic strategies can be designed to prevent different disorders.[1] Among them, obesity is a significant public health concern affecting more than half a billion people worldwide, and not limited to developed countries but in developing nations as well. Moreover, obesity is detrimental to the quality of life as a whole and implies high health costs as a consequence of its associated morbidities. It results from a long-term imbalance between energy intake and expenditure; however, the mechanisms underlying obesity seem to go beyond the long-held belief in caloric intake and lifestyle factors. It is becoming evident that host genetics, environment, diet and lifestyle, and systemic and adipose tissue inflammation play important roles in the development of this pathology. In recent years, microbial changes in the human gut were proposed as a possible cause of obesity.[2−4] Studies in mice have found a higher abundance of *Firmicutes* in obese mice and those fed on western diets, concomitant with a decrease in the abundance of *Bacteroidetes*.[5] Within the phylum *Firmicutes*, the class *Mollicutes* was the most common in obese mice.[5] Studies in humans found varying *Firmicutes*/*Bacteroidetes* ratios in obese individuals. Some supported the finding of a high *Firmicutes*/*Bacteroidetes* ratio,[2,6,7] and some did not find any correlation between body mass index and the *Firmicutes*/*Bacteroidetes* ratio,[8] while others found an opposite ratio.[9]

Probiotics are live microbial dietary supplements that beneficially affect consumers through their effects on the intestinal tract.[10] Inoculation of the gut microbiota of obese mice into axenic mice induces significant mass gain when compared with that of the gut microbiota of lean animals, paving the way to use selected bacteria (i.e., probiotics) for anti-obesity treatment.[11] Several probiotic strains of the genera *Bifidobacterium* and *Lactobacillus* have anti-obesity effects on different mouse models of induced obesity.[12−14] In all these cases, the mechanisms of action are only partially described but appear to be related with fat metabolism, insulin sensitivity, inflammation, and intestinal mucosal adherence. Also very recently, a clinical trial has demonstrated sustainable weight loss in obese women consuming a *Lactobacillus rhamnosus* probiotic strain.[15] In this case, changes were detected in circulating leptin concentrations and the relative abundance of bacteria of the *Lachnospiracea* family. Therefore, further studies of the molecular mechanism of action of probiotics are essential to shed light on the impact of microbiome modulation on the host.

The worm *Caenorhabditis elegans* is a model organism used to study human metabolic disorders, and it has become a powerful tool to study obesity. Its bacterial diet provides a

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relatively simple and genetic tractable model to study the effects of nutrients.[16] This nematode stores lipid in hypodermic and intestinal cells, easy to detect by staining. Moreover, some genes involved in the synthesis, degradation, and transport of fats are conserved in mammals and have been identified by RNAi. Accordingly, studies using *C. elegans* have explored its use to evaluate potential obesity therapeutics, explore the mechanisms behind single-gene mutations related to obesity, and define the mechanistic details of fat metabolism.[17−22] Furthermore, the differential compositions of gut microbiota affect the worm's health, which requires microbiota for normal growth and metabolism.[23] The intestinal microbiota in *C. elegans* exert protective, structural, and metabolic effects such as protection against pathogens, immune system enhancement, vitamin synthesis, and promotion of development. Thus, probiotics can enhance immune defenses and increase lifespan in *C. elegans*.

In this study, we used *C. elegans* to screen a collection of bacteria to search for a strain with fat-reducing properties. The strains were isolated from the feces of healthy breast-fed babies. There are previous reports that human breast milk (HM) helps to avoid rapid infant weight gain and obesity in later life. Thus, research shows, on one hand, a protective effect of breastfeeding against obesity risk[24] and, on the other hand, a positive correlation between rapid weight gain and obesity risk.[25] We found a *Bifidobacterium animalis* subsp. *lactis* probiotic strain (CECT 8145) to have a strong fat-reduction capacity in the nematode, either alone or in fermented milk. Furthermore, this strain modulates the lipid metabolism, the antioxidant response, and the feeding behavior in *C. elegans*. These findings highlight the potential of the *Bifidobacterium* strain CECT 8145 as a suitable probiotic for dietary supplements or food formulations to help body fat reduction.

MATERIALS AND METHODS

Chemicals. The bacterial strains analyzed in this study were grown in de Man, Rogosa, and Sharpe medium (MRS; Oxoid, Basingstoke, United Kingdom), supplemented with cysteine (0.05% wt/vol; Sigma, St. Louis, MO, USA; MRS-C) for bifidobacteria.

Nile Red dye (9-diethylamino-5*H*-benzo[α]phenoxazin-5-one) obtained from Sigma was used to monitor lipid storage in live worms. The anti-obesity drug Orlistat was purchased from Sigma-Aldrich (Madrid, Spain) and used as positive control in the Nile Red staining assays.

Ascorbic acid (0.1 μg/mL, Sigma-Aldrich) was used as an antioxidant positive control.

Nematode and Bacterial Strains. *Caenorhabditis elegans* strain N2, Bristol (wild-type), and the 13 mutant strains VC1785, *acox-1* (ok2257); RB2015, *Acs-5* (ok2668); RB859, *daf-22* (ok693), BX153, *Fat-7*, (wa36); RB1716, *nhr-49* (ok2165); GR1307, *daf-16* (mgDf50); BX106, *fat-6* (tm331); VC175, *sod-4* (gk101); RB1764, *trxr-2* (ok2267); RB2434, *asg-2*, (ok3344); HY520, *pod-2* (ye60); GR1321, *tph-1* (mg280); and CB1370, *daf-2* (e1370) were obtained from the *Caenorhabditis* Genetics Centre (CGC) at the University of Minnesota (USA). Strains were maintained at 20 °C on nematode growth medium (NGM), and *Escherichia coli* OP50 strain was used as nematode diet.

We analyzed a total of 23 and 15 strains belonging to *Lactobacillus* or *Bifidobacterium* genera, respectively, two of which correspond to commercial strains LGG (*L. rhamnosus*) and Bb12 (*B. animalis* subsp. *lactis*). All strains deposited in the Biopolis SL microbial culture collection were initially isolated from faeces of healthy babies under breast-milk feeding. The bacteria were grown at 37 °C for 18 h on MRS-C medium for bifidobacteria, in an anaerobic atmosphere generated by means of an AnaeroGen system (Oxoid).

Identification and Taxonomic Characterization of Isolates by Sequencing. Strains were taxonomically identified by 16S ribosomal DNA (rDNA) sequencing as reported previously.[26] The resulting sequences were automatically aligned, inspected visually, and compared with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The strains were identified on the basis of highest scores.

Body Fat Reduction. The 38 bacterial strains were evaluated for their fat-reduction effect on *C. elegans* (N2). Concentrated cultures (50 μL, OD = 30) were added to the NGM surface, previously seeded with *E. coli* OP50 to ensure standard nutrition conditions.

The nematode fat content was measured by Nile Red staining as previously described.[21] Experiments involved synchronizing worms in NGM with *E. coli* OP50 or NGM with the corresponding *Lactobacillus* or *Bifidobacterium* strain. Positive controls were NGM plates with 6 μg/mL of Orlistat.

Nile Red was used to monitor lipid storage in live worms. The dye was added on the top of the NGM agar plates, pre-seeded with the corresponding bacteria, to a final concentration of 0.05 μg/mL. Worms were incubated at 20 °C for 3 days until young adult stage. After this incubation period, nematode samples were placed in M9 buffer, and fluorescence was measured in an FP-6200 system (JASCO Analytical Instruments, Easton, MD, USA) using λ_{ex} = 480 nm and λ_{em} = 571 nm. A total of 120 worms per condition were analyzed in two different experiments.

Additionally, this assay was performed with inactivated cells of *B. animalis* subsp. *lactis* CECT 8145 strain by heat treatment (70 °C for 18 h). Likewise, the above-mentioned *C. elegans* mutant strains (described in *C. elegans* strains section) were analyzed to determine the molecular target of the *B. animalis* subsp. *lactis* CECT 8145 strain.

Triglyceride (TG) Quantification. The effect of the strain *B. animalis* subsp. *lactis* CECT 8145 on TG reduction was tested in *C. elegans* (N2). Nematode triglyceride content was measured using a Triglyceride Quantification Kit (Biovision, Mountain View, CA, USA). Age-synchronized nematodes were fed with *E. coli* OP50 or *B. animalis* subsp. *lactis* CECT 8145 until young adult stage. Worms were then collected and washed with PBS buffer. After worm settling, supernatant was removed, and 400 μL of the triglyceride assay buffer was added to worm pellet. Worms were sonicated with a digital sonifier (Branson Ultrasonics Corp., Danbury, CT, USA) using 4 pulses for 30 s at 10% power. Total protein content was estimated by bicinchoninic acid assay. Samples were slowly heated twice at 90 °C for 5 min in a thermomixer (ThermoFisher) to solubilize all TG in the solution. After brief centrifugation, samples were used for the triglyceride assay (50 μL/well) following the manufacturer's instructions. Five different biological replicates were included for each condition in three independent experiments.

Antioxidant Activity. The antioxidant activity of *B. animalis* subsp. *lactis* CECT 8145 strain was evaluated through the previously described bioassay in *C. elegans*.[27] *C. elegans* wild type N2, and mutant strains GR1307, daf-16 (mgDf50), and CB1370, *daf-2* (e1370), were used.

Experiments involved culturing the nematodes for 7 days in NGM medium with *E. coli* OP50 or the *B. animalis* subsp. *lactis* CECT 8145 strain. During this time, nematodes were transferred to new plates containing fresh cultures every 2 days. Next, acute oxidative stress was applied with H_2O_2 (2 or 1.75 mM) for 5 h, and worm survival was evaluated for each feeding condition after this time. Ascorbic acid (0.1 μg/mL) was used as an antioxidant positive control. Experiments were done in duplicate, analyzing 200 worms per condition.

Fermentation Assays. The ability of *B. animalis* subsp. *lactis* CECT 8145 to ferment milk was analyzed. We used liquid UHT cow milk which was inoculated with different doses of this bacterial strain (10^6, 10^7, and 10^8 CFU/mL) and incubated for 24 h at 37 °C. Bacterial inoculums were prepared from an overnight culture of the strain in MRS with Cys in anaerobic conditions.

Functional yogurts were made (with cow milk and with soy milk). *B. animalis* subsp. *lactis* CECT 8145 strain (10^8 CFU/mL) and commercial yogurt starters (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) were added to commercial skim milk and powdered milk (0.6%), and to soy milk. Control yogurt was made

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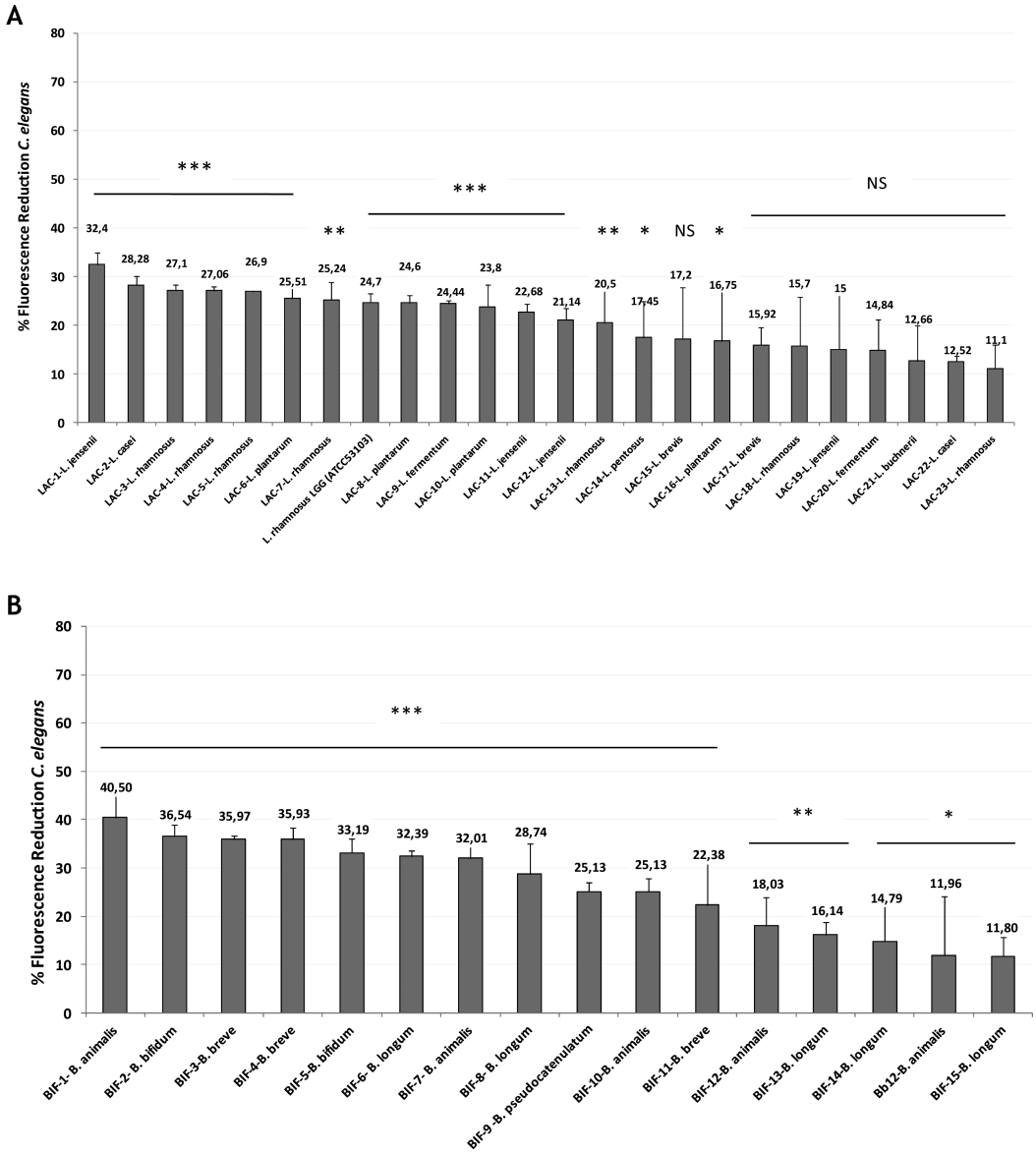

Figure 1. Screening for body-fat-reducing bacterial strains in *C. elegans*. Percentage of fluorescence reduction in bacterial-fed nematodes versus control (NGM) feeding is represented. Bacterial species were identified by 16S rDNA sequencing. Nile Red was quantified at young adult stage. (A) Screening of the 23 *Lactobacillus* strains studied. (B) Screening of the 15 *Bifidobacterium* strains studied. ***, significant at $P \leq 0.001$; **, significant at $P \leq 0.01$; *, significant at $P \leq 0.05$; NS, not significant.

with commercial yogurt starters alone. Growth of the inoculated *Bifidobacterium* strain was checked at the end of fermentation by plate counting in MRS-C medium. After that, the fermented products were evaluated in the *C. elegans* model to assess their effect on body fat reduction by adding 200 μL of each product on top of the plates.

Gene Expression Analysis in *C. elegans*. Gene expression in worms fed with the strain *B. animalis* subsp. *lactis* CECT 8145 was analyzed in the *C. elegans* wild-type strain (N2). The nematodes were cultured until young adult stage on NGM with *E. coli* OP50 or *B. animalis* subsp. *lactis* CECT 8145 strain, as control and treated condition, respectively. Worms were recovered with M9 buffer, washed three times, and collected in eppendorf tubes for disruption by sonication (3 pulses at 10 W, 20 s/pulse). Total RNA was isolated with RNeasy Kit (Qiagen, Hilden, Germany) and processed for hybridization using the GeneChip *C. elegans* Genome Array of Affymetrix (UCIM, University of Valencia), containing oligonucleotide probe sets to assess over 22 500 transcripts from the *C. elegans* genome. Four biological replicates per condition were examined by bioinformatics.

Metabolomic Analysis in *C. elegans*. The changes in the metabolic profile of *C. elegans* after ingestion of *Bifidobacterium* strain CECT 8145 were studied and compared with the profile of nematodes fed NG medium and *E. coli* OP50. Three-day-old nematodes were subjected to metabolite extractions and analysis by LC-MS/MS (ESI +) (-ESI) and GC-MS, and subsequent bioinformatic data processing.

Experiments were carried out using the *C. elegans* wild-type strain N2. Worms were age-synchronized by isolating eggs from gravid adults and hatching them overnight in NGM plates with the different feeding conditions: NGM with *E. coli* OP50 (standard diet) or NGM with CECT 8145 strain. A total of 30 plates/condition (200−300 worms/plate) were prepared to obtain around 6000−9000 worms/sample (around 300 μL of worm pellet). The pellets obtained from six independent experiments were fast-frozen and stored at −80 °C until analysis.

Samples were processed and analyzed as described in refs 28 and 29.

Statistical Analysis. The levels of significance of *C. elegans* body fat decline and TG reduction, as well as nematode viability after oxidative stress in control and treatment conditions, were evaluated by

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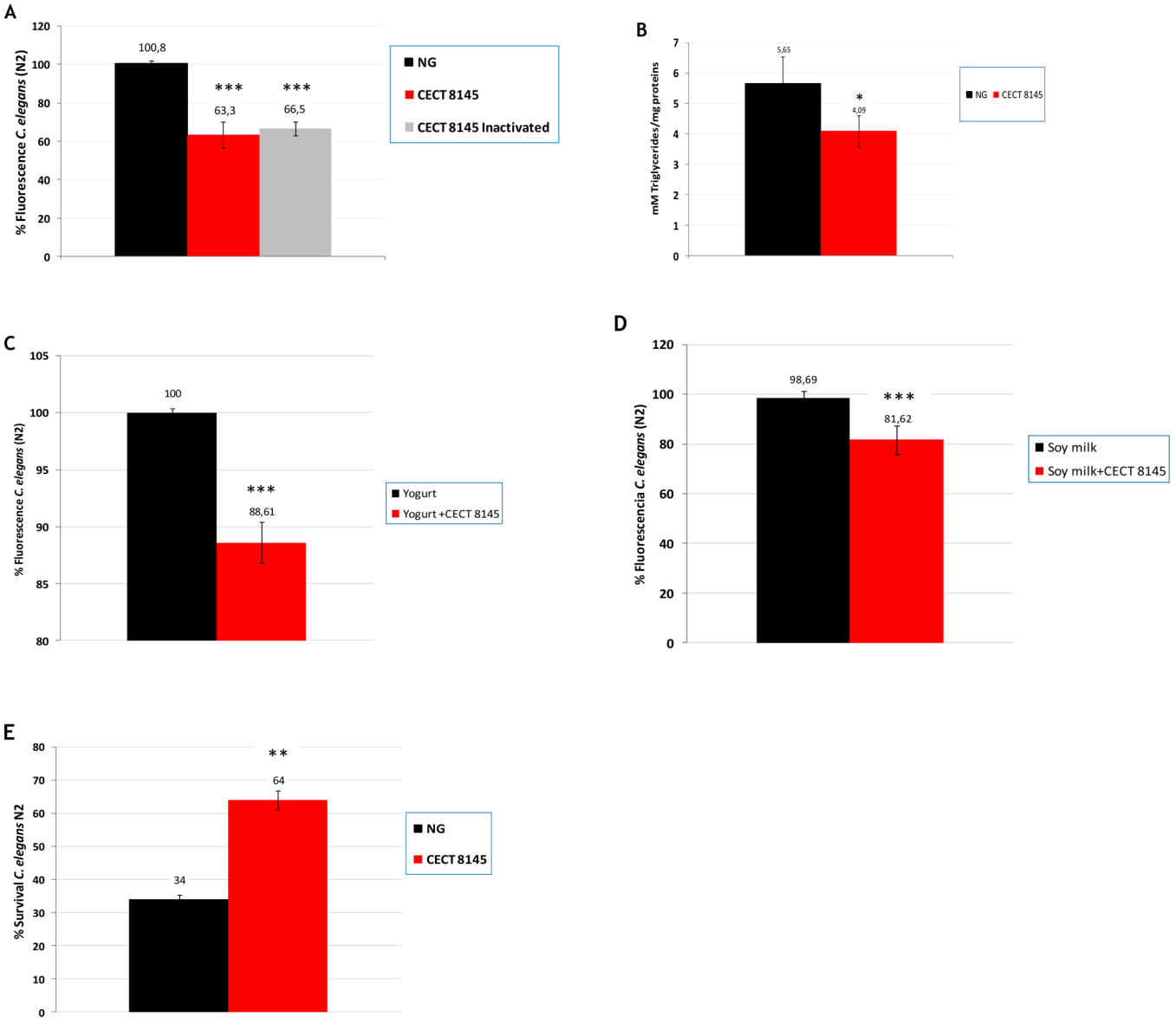

Figure 2. Protective effect of *Bifidobacterium animalis* subsp. *lactis* CECT 8145 on obesity and oxidative stress parameters in *C. elegans* wild type N2. (A) Body fat reduction (measured as percentage of fluorescence) in *C. elegans* obtained in fresh and inactivated CECT 8145 cells. (B) Quantification of triglyceride content (mM TG/mg protein) in *C. elegans* fed with CECT 8145 cells and NGM. (C) Body fat reduction in *C. elegans* fed with yogurt fermented with commercial starters and CECT 8145 strain. (D) Body fat reduction in *C. elegans* fed with soy milk fermented with commercial starters and CECT 8145 strain. (E) Antioxidant activity (percentage of survival) of strain CECT 8145 estimated after oxidative stress with 2 mM H_2O_2 in *C. elegans* (N2). Data are the average of at least two independent experiments. ***, significant at $P \leq 0.001$; **, significant at $P \leq 0.01$; *, significant at $P \leq 0.05$.

one-way analysis of variance (ANOVA) using Statgraphics plus (version 5.1) software (Manugistics, Rockville, MD).

Raw data obtained from Affymetrix arrays were background corrected using RMA methodology.[30] Signal intensity was standardized across arrays via the quantile normalization algorithm. Gene expression was analyzed to determine differences in mRNA between biological conditions. For each comparison of interest, the difference between treated samples and controls was statistically tested using limma moderated t-statistic. To control the false discovery rate, *p*-values were corrected for multiple testing as in ref 31. Finally, gene set was analyzed for each comparison using logistic regression models.[32]

RESULTS AND DISCUSSION

The Strain *Bifidobacterium animalis* subsp. *lactis* CECT 8145 Is the Most Effective for *C. elegans* Body Fat Reduction. A total of 38 probiotic strains, 23 belonging to the *Lactobacillus* genus and 15 to *Bifidobacterium* genus, were screened in *C. elegans* for their body fat reduction activity. As mentioned above, two of them are commercial strains, LGG (*L. rhamnosus*) and Bb12 (*B. animalis* subsp. *lactis*).

Figure 1A shows the percentage of fluorescence reduction (fat reduction) obtained in *C. elegans* fed with the different *Lactobacillus* strains (LAC-1 → LAC-23) versus control-fed nematodes (NGM). The observed percentages on fluorescence reduction ranged between 11.1 and 32.4%. The most effective strain was LAC-1, reducing fluorescence by 32.4%. More interestingly, the activity on body fat reduction was higher for *Bifidobacterium* strains (BIF-1 → BIF-15) (Figure 1B), with a fluorescence reduction between 11.8 and 40.5%. BIF-1 was the most effective *Bifidobacterium* strain on fluorescence reduction (40.5% of body fat reduction). Conversely, the commercial

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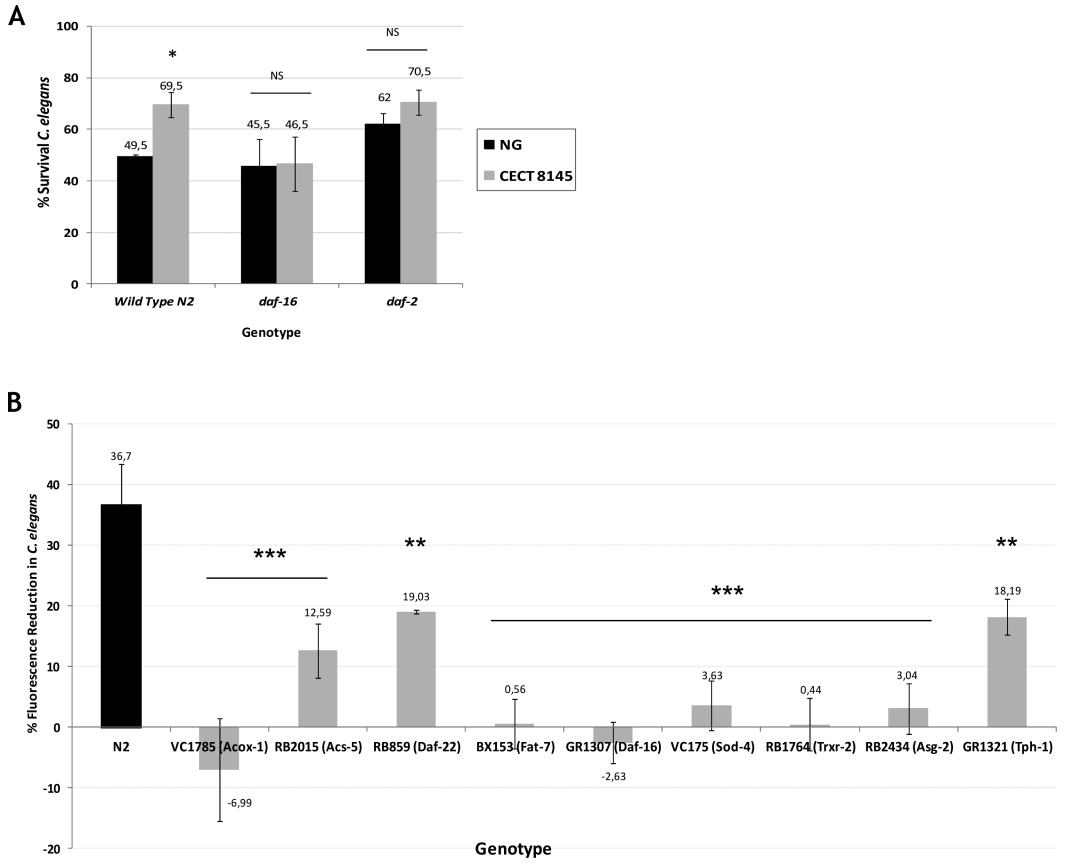

Figure 3. Study in *C. elegans* mutant strains to validate molecular targets of *Bifidobacterium* CECT 8145 strain. (A) Antioxidant activity in N2, and DAF-16 and DAF-2 mutants fed with the probiotic *Bifidobacterium* CECT 8145. Worms were subjected to H_2O_2 (1.75 mM). Phenotype was lost in both mutant strains. (B) Effect of *Bifidobacterium* CECT 8145 strain on body fat reduction in *C. elegans* mutant strains. Percentage of fluorescence reduction is represented in wild-type strain (N2) and the loss-of-function strains for ACOX-1, ACS-5, DAF-22, FAT-7, DAF-16, SOD-4, TRXR-2, ASG-2, and TPH-1 genes. The values were calculated taking into account as reference the fluorescence obtained in *C. elegans* (N2) cultured in standard NG plates (*E. coli* OP50). ***, significant at $P \leq 0.001$; **, significant at $P \leq 0.01$; *, significant at $P \leq 0.05$; NS, not significant differences.

strains *Lactobacillus* GG and *Bifidobacterium* Bb12 were less effective (only 24.7% and 11.9% fluorescence reduction, respectively).

Therefore, further studies were performed with the *Bifidobacterium* strain BIF-1 due to its potential fat-reduction activity.

Identification of BIF-1 at species level was performed using 16S rDNA sequencing. Search of sequence homology using BLAST provided the highest homology (99%) with *B. animalis* subsp. *lactis* species. Identification was confirmed by whole genome sequencing.[33] The 16S rRNA sequence corresponding to *B. animalis* subsp. *lactis* CECT 8145 has been deposited in the EMBL nucleotide database under accession number KP202873. The strain was deposited at the Spanish Type Culture Collection (CECT) under accession number CECT 8145.

Afterward, the inactivated cells of the strains CECT 8145 were evaluated for its anti-obesity effect on *C. elegans*. Results indicated that inactivated cells showed similar effectiveness on body fat reduction as fresh cultures compared with control ($P \leq 0.001$) (Figure 2A). These would support the ideas that probiotic efficacy still remains in not viable cells and that cell wall components could contribute to the functional activity.

TG levels were then quantified in nematodes fed CECT 8145 cells. Results indicate a significant reduction in total TG in animals fed with the probiotic strain CECT 8145 ($P \leq 0.05$) compared with the control-fed nematodes (Figure 2B). TG are the main constituents in lipid droplets stored in *C. elegans*, and lipid accumulation has been associated with increase in TG in this nematode.[34] Therefore, the TG reduction in nematodes treated with the *Bifidobacterium* strain CECT 8145 supports total fat reduction, consistent with recent studies indicating the potential of probiotics as anti-obesity agents. Specifically, triglyceride reduction is evidence of body fat reduction, and is a mechanism of action previously reported for *Bifidobacterium* strains.[35]

The Probiotic Strain CECT 8145 in Fermented Milk Affects *C. elegans* Body Fat Reduction. Previous research has reported the beneficial effects of yogurt and soy products fermented with probiotics in preventing obesity.[36,37] Cow and soy milk samples were fermented with the CECT 8145 strain by inoculating the culture at different doses, confirming its ability to ferment cow milk. All the doses used (10^6, 10^7, and 10^8 CFU/mL) were sufficient to produce a fermented product, the optimal being 10^8 CFU/mL. Then, a functional yogurt was made by adding strain CECT 8145 and commercial yogurt starters (*L. delbrueckii* subsp. *bulgaricus* and *St. thermophilus*). A control yogurt without CECT 8145 was also included in the study. The effect of these yogurts on *C. elegans* body fat reduction was evaluated (see Figure 2C). Results showed the highest body fat reduction corresponded to the yogurt

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fermented with strain CECT 8145 (11.4% more than the control yogurt; $P \leq 0.001$).

A similar experiment was performed using soy milk as a substrate for CECT 8145 fermentation. Figure 2D shows that feeding nematodes with fermented soy milk with CECT 8145 strain led to a significant fat reduction (17.07%; $P \leq 0.001$) compared with control fermented soy. These results clearly indicate the effectiveness of the strain in the final food matrix.

The *Bifidobacterium* Strain CECT 8145 Provides Antioxidant and Anti-inflammatory Activity in *C. elegans*. There is evidence that some energy homeostasis mechanisms are also involved in stress response mechanisms in *C. elegans*.[38] Therefore, we studied whether the selected CECT 8145 strain has "in vivo" antioxidant effect by measuring resistance to acute oxidative stress in worms. As shown in Figure 2E, nematodes fed with strain CECT 8145 were more resistant to oxidative stress ($P \leq 0.01$), as worm survival was higher (64%) than in control feeding conditions (34%). These results indicate a marked antioxidant effect of the *Bifidobacterium* strain CECT 8145 and are in agreement with previous studies reporting the close relationship between lipid metabolism and oxidative stress response in the nematode.[20]

Furthermore, we explored whether the strain *B. animalis* CECT 8145 exerts anti-inflammatory effect on *C. elegans*. The nematode has been used to study the effect of a non-steroidal anti-inflammatory drug, Celecoxib. This drug extends lifespan in *C. elegans*, and this requires the activity of DAF-16, the FOXO transcription factor known to regulate longevity in response to insulin/IGF-I-like signaling pathway (IIS).[39] Interestingly, the DAF-2(insulin receptor)/DAF-16 pathway was also modulated by an anti-inflammatory *Lactobacillus rhamnosus* strain in the nematode,[40] suggesting an important role of IIS pathway as target for testing anti-inflammatory compounds. Our results clearly showed increased worm survival in N2 nematodes fed with the *B. animalis* CECT 8145 strain after acute oxidative stress (Figures 2E and 3A); however, this phenotype was absent in DAF-16 and DAF-2 mutants fed with the probiotic (Figure 3A). This indicate that the antioxidant activity of *B. animalis* CECT 8145 is dependent on the IIS pathway, at least, suggesting the potential anti-inflammatory effect of this probiotic.

Our results correlate well with a study showing the effects of a probiotic mixture in decreasing fat mass, oxidative stress, and inflammatory liver damage in rats.[41]

Transcriptional Changes in *C. elegans* Fed with the *Bifidobacterium* Strain CECT 8145. To identify gene expression changes associated with CECT 8145 strain intake, we used *C. elegans* DNA microarrays. Young adults fed with the *Bifidobacterium* strain were compared with nematodes in NGM (with *E. coli* OP50 alone). Microarray data are available through the NCBI Gene Expression Omnibus (GEO) data repository under accession number GSE63531 (http://www.ncbi.nlm.nih.gov/geo/). The diet with strain CECT 8145 produced 296 highly expressed genes and 26 repressed genes in comparison with the control (from a total of 22 625 genes). Table S1 shows a selected list of 40 genes with the highest score of up-regulation in nematodes fed CECT 8145. These genes were selected according to function from a total of 296 genes. The complete list is available at the GEO database (accession no. GSE63531). The highly expressed genes were related, among others, with aromatic amino acids, fatty acids, glutathione, carbohydrate, protein metabolism, proteolysis, reproduction, moulting, locomotion, oxidation−reduction processes, and neuropeptide signaling pathways. The repressed genes were mainly related with the positive regulation of growth rate and the xenobiotic metabolism.

Concerning metabolic pathways, 23 different KEGG pathways were identified to be significantly up-regulated ($P \leq 0.05$) in nematodes fed CECT 8145 (Table 1). One group of pathways up-regulated in *C. elegans* under *Bifidobacterium* CECT 8145 feeding was related with carbohydrate metabolism. Pyruvate is a metabolite produced from glucose by glycolysis, and is the metabolic intersection of different pathways, such as propanoate metabolism, butanoate metabolism, leucine and lysine biosynthesis, and citrate cycle. Moreover, pyruvate decarboxylation produces acetyl-CoA, which is the key substance for ATP synthesis through citrate cycle. This up-regulation of carbohydrate metabolism was in accordance to the up-regulation of energy metabolism, through oxidative phosphorylation and nitrogen metabolism. Oxidative phosphorylation is a pathway in the mitochondrial electron transport chain and involves nutrient oxidation to produce ATP. This is also supported by the observed up-regulation of biological processes related with ATP synthesis and central metabolism (like mitochondrial ATP synthesis coupled electron transport, ion transmembrane transport, or proton transport, among others) (Table 2). All these results suggest that the *Bifidobacterium* strain CECT 8145 up regulates metabolic pathways for energy production, explaining the body fat reduction in treated nematodes. This is in agreement with a previous report showing the impact of the administration of a

Table 1. List of 23 Significantly Up-Regulated KEGG Metabolic Pathways in *C. elegans* Fed with CECT 8145

KEGG ID	up-regulated metabolic pathway	*P*-value
00190	oxidative phosphorylation	0
00480	glutathione metabolism	0
00982	drug metabolism, cytochrome P450	0
00980	metabolism of xenobiotics by cytochrome P450	0
00983	drug metabolism, other enzymes	0
00670	one-carbon pool by folate	0
04142	lysosome	0
00260	glycine, serine, and threonine metabolism	0.0004
00330	arginine and proline metabolism	0.0004
00860	porphyrin and chlorophyll metabolism	0.0004
00270	cysteine and methionine metabolism	0.0007
01040	biosynthesis of unsaturated fatty acids	0.0009
00040	pentose and glucuronate interconversions	0.001
04146	peroxisome	0.001
00590	arachidonic acid metabolism	0.001
00053	ascorbate and aldarate metabolism	0.003
00514	other types of O-glycan biosynthesis	0.005
00910	nitrogen metabolism	0.008
00250	alanine, aspartate,and glutamate metabolism	0.01
00380	tryptophan metabolism	0.01
00620	pyruvate metabolism	0.03
00650	butanoate metabolism	0.04
00410	β-alanine metabolism	0.05
00360	phenylalanine metabolism	0.05
00280	valine, leucine, and isoleucine degradation	0.05
00030	pentose phosphate pathway	0.05
00230	purine metabolism	0.05
00071	fatty acid metabolism	0.07
00020	citrate cycle (TCA cycle)	0.07

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Table 2. List of 26 Significantly Up-Regulated Biological Processes in *C. elegans* Fed with CECT 8145 Strain

gene ontology	up-regulated biological process	*P*-value
006937	regulation of muscle contraction	0
030259	lipid glycosylation	0
042775	mitochondrial ATP synthesis coupled with electron transport	0.001
009156	ribonucleoside monophosphate biosynthetic process	0.001
009072	aromatic amino acid family metabolic process	0.003
034220	ion transmembrane transport	0.003
030241	skeletal muscle myosin thick filament assembly	0.004
009112	nucleobase metabolic process	0.004
015992	proton transport	0.006
006508	proteolysis	0.006
034607	turning behavior involved in mating	0.008
007218	neuropeptide signaling pathway	0.008
040018	positive regulation of multicellular organism growth	0.008
046942	carboxylic acid transport	0.009
072529	pyrimidine-containing compound catabolic process	0.01
042398	cellular-modified amino acid biosynthetic process	0.02
015833	peptide transport	0.02
006754	ATP biosynthetic process	0.02
009063	cellular amino acid catabolic process	0.02
048521	negative regulation of behavior	0.02
055074	calcium ion homeostasis	0.03
006637	acyl-CoA metabolic process	0.03
042338	cuticle development involved in collagen and cuticulin-based cuticle molting cycle	0.04
006814	sodium ion transport	0.04
036293	response to decreased oxygen levels	0.04
009069	serine family amino acid metabolic process	0.05

Lactobacillus probiotic strain in the modulation of carbohydrate metabolism in mice.[42]

A second group of up-regulated pathways were those involved in lipid metabolism (biosynthesis of unsaturated fatty acids and fatty acid metabolism). Taking into account that peroxisome was also up-regulated, these results could indicate an up-regulation of fatty acid β-oxidation. This is in agreement with the significant over-expression of some β-oxidation genes as acox-1 (see Table S1). Lipid glycosylation was also over-expressed in *C. elegans* by strain CECT 8145, supported by the increase in O-glycan biosynthesis (Table 1) and lipid glycosylation processes (Table 2). Both processes are related with structural and functional roles in cellular membrane. Lipid metabolism is a target of several lactic acid bacteria and bifidobacteria. Accordingly, *L. curvatus* HY7601, *L. plantarum* KY1032, and *B. breve* B-3 up-regulate genes related with fat metabolism and fatty acid oxidation in mice.[18,19] Therefore, our results support that a regulatory mechanism of probiotics is through the modulation of energy metabolism.

Furthermore, different amino acid metabolism pathways were up-regulated by the strain CECT 8145, probably as a consequence of the increased proteolysis processes (Table 2). This was also observed in biological processes analysis, where the aromatic amino acid family was found to be up-regulated. Among them, tryptophan is an amino acid leading to serotonin synthesis, which has been related with important functions in nervous system and other tissues in *C. elegans*, and is also involved in behaviors such as egg laying, pharyngeal pumping, male mating, and regulating locomotion,[43] as well as the feeding behavior.[44] Mutants bearing a TPH-1 deletion do not synthesize serotonin, and tph-1 mutants display abnormalities in behavior and metabolism, such as storing large amounts of fat and decreased feeding rate.[45] The treatment of *C. elegans* with the strain CECT 8145 also up-regulates the neuropeptide signaling pathway, in agreement with the over-expression of genes like flp-8, a short peptide neurotransmitter involved in nematode pumping-rate regulation. These results would indicate the impact of the *Bifidobacterium* strain CECT 8145 on *C. elegans* feeding behavior through serotonergic system, neuropeptide signaling, and their relation with obesity. This is in agreement with other studies suggesting a direct effect of lactic acid bacteria and bifidobacteria on feeding behavior and satiety.[38,46,47]

Moreover, glycine, serine, and threonine metabolism was also induced after feeding on CECT 8145, which was also determined in the analysis of biological processes (Table 2). Serine has many important biological roles, including the biosynthesis of phospholipids, pyruvate, and cysteine, while glycine contributes to the one-carbon pool, to formation of glutathione, purine nucleotides, and porphyrins (organic compounds the best-known being hemo, a cofactor of the protein hemoglobin). These results were consistent with the up-regulation of purine and pyrimidine nucleotides, porphyrins, gluthatione, cysteine, and pyruvate observed after *Bifidobacterium* CECT 8145 intake. Specifically, the observed increase in nucleotide synthesis would be consistent with a simultaneous up-regulation of the pentose phosphate pathway.

Xenobiotic metabolism was also over-induced in nematodes treated with CECT 8145, together with an up-regulation of lysosome. These are both related with catalysis of exogenous compounds and digestion of substances, which contribute to molecule recycling. Further, lysosomes are responsible for cellular homeostasis due to their involvement in secretion, plasma membrane repair, cell signaling, and energy metabolism.

Additionally, strain CECT 8145 induced muscle contraction processes, turning behavior involved in mating, and cuticle development, as shown in Table 2. These findings are consistent with the over-expression of different genes involved in reproduction, locomotion, and body morphogenesis (COL genes) (Table S1).

Taking into account the main processes up-regulated under *B. animalis* CECT 8145 treatment, a genetic approach with *C. elegans* knockout strains was undertaken to validate the role of different genes in the functional activity of the strain. The genes selected are involved in peroxisome fatty acid β-oxidation, fatty acid desaturation, REDOX homeostasis mechanisms, and also oxidative phosphorylation (Figure 3B and Table S2). Results showed ACOX-1 as a target gene of fatty acid β-oxidation, as no body fat reduction was observed in the *C. elegans acox-1* mutant strain (Figure 3B). A partial phenotype loss was observed with ACS-5 and DAF-22, showing these genes to be targets of CECT 8145. Moreover, FAT-7 (a gene with significant over-expression in *C. elegans* fed on CECT 8145) and DAF-16 were also molecular targets, demonstrating that fatty acid desaturation is one of the metabolic functions affected by this probiotic strain. Furthermore, genes like SOD-4 and TRXR-2, involved in the maintenance of REDOX homeostasis, also play an important role in body fat content, as *C. elegans* strains lacking these genes display the same fat content as the wild-type strain fed on the probiotic. Finally, the total loss of function of ASG-2 gene agrees with the up-regulation of oxidative phosphorylation after treatment with CECT 8145; while a partial body fat reduction was observed in the tph-1

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Table 3. Metabolite Changes Determined in *C. elegans* Fed with CECT 8145 Strain

metabolic pathway	metabolite name	fold change (CECT 8145/control)[a]	*P*-value
peptide (γ-glutamyl)	γ-glutamyl-leucine	2.05	≤0.05
	γ-glutamyl-methionine	3.6	≤0.05
glutathione metabolism	glutathione, reduced (GSH)	0.8	NS
	glutathione, oxidized (GSSG)	0.7	0.05 < *p* < 0.10
	ophthalmate	0.6	≤0.05
	cysteine-glutathione disulfide	0.65	NS
carbohydrate (fructose, mannose, galactose, starch, and sucrose metabolism)	maltotetraose	2.3	≤0.05
	maltopentaose	2.4	0.05 < *p* < 0.10
carbohydrate (glycolysis, gluconeogenesis, pyruvate metabolism)	glucose	0.7	0.05 < *p* < 0.10
carbohydrate (nucleotide sugars, pentose metabolism)	6-phospho-gluconate	1.6	≤0.05
	ribose	1.5	≤0.05
	ribulose-5-phosphate	0.8	0.05 < *p* < 0.10
xenobiotic metabolism (sugar)	trehalose 6-phosphate	0.5	≤0.05
nucleotide metabolism (pyrimidine)	*N*-carbamoyl-aspartate	2.2	≤0.05
	orotate	1.1	NS
nucleotide metabolism (purine)	allantoin	0.6	≤0.05
	adenosine	1.5	NS
	guanosine	1.07	NS
	adenine	1.5	≤0.05
	hypoxanthine	1.3	≤0.05
	adenosine 5′-monophosphate (AMP)	1.1	NS
	guanosine 5′- monophosphate (5′-GMP)	1.1	NS
amino acid metabolism (glutamate)	glutamate	1.08	NS
	glutamine	1.2	NS
lipid metabolism (glycerolipid)	choline	1.2	NS
lipid metabolism (sterol)	7-dehydrocholesterol	1.8	≤0.05
amino acid metabolism (tryptophan)	tryptophan	0.8	0.05 < *p* < 0.10
cofactors and vitamins	phosphopantetheine	1.04	NS
	coenzyme A	1.4	0.05 < *p* < 0.10
	3′-dephosphocoenzyme A	1.4	NS
	flavin adenine dinucleotide (FAD)	1.1	NS
	flavin mononucleotide (FMN)	1	NS

[a]Fold change <1.00 indicates lower amount of the metabolite in *C. elegans* treated with *Bifidobacterium* strain CECT 8145.

strain, indicating that tryptophan metabolism is also a metabolic target of CECT 8145.

Metabolomic Analysis in *C. elegans* Fed with *Bifidobacterium* Strain CECT 8145. The changes in the metabolic profile of *C. elegans* after ingestion of *Bifidobacterium* strain CECT 8145 were studied and compared with the profile of nematodes fed NG medium and *E. coli* OP50. Table 3 summarizes the main metabolite changes in *C. elegans* under CECT 8145 treatment.

The levels of γ-glutamyl-leucine and γ-glutamyl-methionine were higher in nematodes fed with the CECT 8145 strain, which would be consistent with a possible increase in γ-glutamyl-transferase (GGT) activity and, thus, recycling of glutathione (GSH). Furthermore, ophthalmate, a metabolite used for GSH synthesis, decreased significantly in the group fed with the *Bifidobacterium* strain, which is consistent with a decrease in GSH biosynthesis. This is probably due to lower glutathione demand due to lower levels of oxidative stress. This fact is supported by the observation of lower levels of oxidative stress biomarkers, namely GSSG (oxidized GSH) and cysteine-glutathione disulfide, in the group fed with CECT 8145.

The group fed with *B. animalis* subsp. *lactis* CECT 8145 displayed changes in many of the metabolites involved in carbohydrate metabolism. Maltotetraose and maltopentaose exhibited higher levels (which are associated with the changes observed in glycogen metabolism), whereas both trehalose-6-

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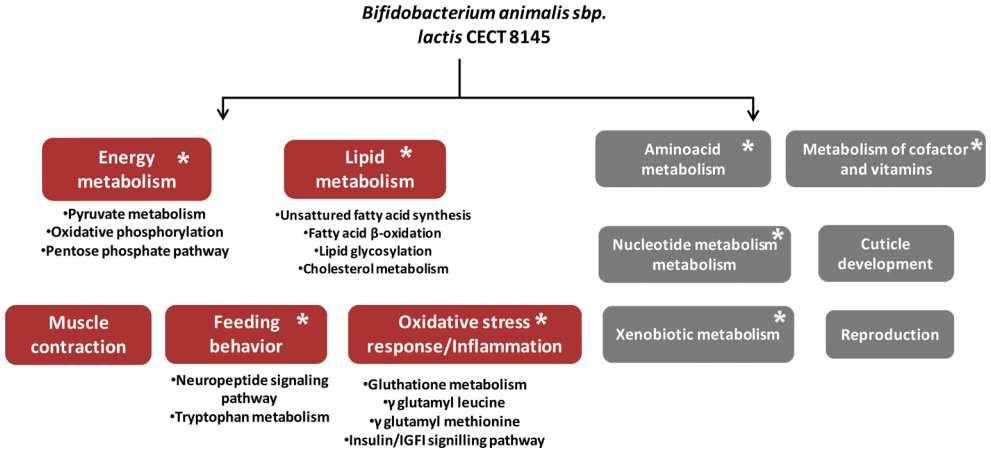

Figure 4. Regulatory mechanism of the strain *B. animalis* subsp. *lactis* CECT 8145 in *C. elegans* assessed by transcriptomic and metabolomic approaches. In red: obesity-related mechanisms. In gray: other mechanisms.* indicates target mechanism of strain CECT 8145 obtained from both transcriptomic and metabolomic approaches.

phosphate and glucose levels were lower in the group fed with CECT 8145. Other affected pathways were glycogen metabolism and pentose phosphate pathway. Thus, 6-phospho-gluconate showed a significant increase in the probiotic-treated group. This fact together with high levels of ribose and low levels of ribulose-5-phosphate are consistent with a possible up-regulation of the pentose phosphate pathway on exposure to CECT 8145.

Changes in nucleotide metabolism are a consequence of the changes observed in the activity of the pentose phosphate pathway. Nematodes fed with *B. animalis* subsp. *lactis* CECT 8145 showed higher levels of *N*-carbamoyl-aspartate and orotate, two intermediaries in pyrimidine synthesis. Similar changes were seen in purine metabolism. Thus, treated nematodes showed lower levels of allantoin (a product of purine degradation). In addition, the group treated with the probiotic strain had higher levels of purine nucleosides (adenosine and guanosine), bases (adenine and hypoxanthine), and nucleotides [adenosine 5′-monophosphate (AMP) and guanosine 5′-monophosphate (GMP)]. These results, together with the observed increase in precursor amino acids (glutamate and glutamine) and the possible up-regulation of the pentose phosphate pathway, support a possible increase in purine biosynthesis, accompanied by a decrease in purine degradation.

Also, increased levels of choline and acetylcholine, which are involved in glycosylation processes and membrane metabolism, were detected in nematodes fed with CECT 8145. Moreover, levels of 7-dihydrocholesterol, an intermediary in cholesterol biosynthesis, were increased in nematodes fed CECT 8145, which is consistent with its modulation of cholesterol biosynthesis. Changes in cholesterol content in the membrane may affect the receptor environment, ion channels, and other membrane proteins, and thereby alter their function. Furthermore, cholesterol metabolism affects lipid and hormone-related processes.

Additionally, a significant reduction of tryptophan was determined in nematodes treated with CECT 8145, possibly indicating an increase in serotonin synthesis. This is in accordance with the transcriptomic results showing an up-regulation of tryptophan metabolism. Finally, in *C. elegans* treated with *B. animalis* subsp. *lactis* CECT 8145 there was an increase in phosphopantetheine, 3′-dephospho-coenzyme-A, and coenzyme A (CoA). Moreover, CECT 8145 intake led to increased flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), consistent with the up-regulation of FAD biosynthesis. CoA and FAD are involved in the metabolism of carbohydrates, lipids, and amino acids.

Therefore, these results show that feeding *B. animalis* subsp. *lactis* CECT 8145 to *C. elegans* produces a series of metabolic changes related to the antioxidant, carbohydrate, and nucleotide metabolism. Glutathione metabolism appears to be a target of the probiotic, resulting in reduced oxidative stress levels. Furthermore, CECT 8145 intake led to an up-regulation of the pentose phosphate and glycosylation pathways. Additional changes were observed in the metabolism of glycogen, nucleotides, lipids, and cofactors. It is noteworthy that all these results are consistent with those observed in the transcriptomic study.

In summary, our study describes for the first time a new *B. animalis* subsp. *lactis* strain, CECT 8145, that is able to reduce body fat and triglycerides in *C. elegans*. The probiotic also exerts antioxidant and anti-inflammatory activity in the nematode by promoting an increase in oxidative stress defense. This could be interpreted as a consequence of adaptive responses, commonly defined as mitohormesis.[47] In this respect, further studies of ROS production in nematodes fed with the probiotic would help to support this hypothesis. Furthermore, a good correlation in data from both transcriptomics and metabolomics in nematodes fed with *B. animalis* CECT 8145 was found, suggesting a regulatory mechanism of the probiotic based on different molecular targets (Figure 4). Thus, the energy and lipid metabolism, as well as feeding behavior and oxidatives stress response, are the major molecular mechanisms modulated by this strain. In addition, the fat-reduction activity of the *Bifidobacterium* strain is maintained when added to a food matrix, such as fermented yogurts. Our data support the potential use of the probiotic strains as supplement or ingredient to manage fat reduction. Currently studies are underway in a murine model of obesity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b05934.

Table S1, listing the first 40 overinduced genes ($P \leq 0.05$) in *C. elegans* fed with CECT 8145 strain (PDF)

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Table S2, giving body fat reduction and antioxidant activity in *C. elegans* knockout strains fed with CECT 8145 strain(PDF)

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Notes

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