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Dietary fiber supplementation increases *Drosophila melanogaster* lifespan and gut microbiota diversity†

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Dietary fiber has been shown to have multiple health benefits, including a positive effect on longevity and the gut microbiota. In the present study, *Drosophila melanogaster* has been chosen as an *in vivo* model organism to study the health effects of dietary fiber supplementation (DFS). DFS extended the mean half-life of male and female flies, but the absolute lifespan only increased in females. To reveal the underlying mechanisms, we examined the effect of DFS on gut microbiota diversity and abundance, local gut immunity, and the brain proteome. A significant difference in the gut microbial community was observed between groups with and without fiber supplementation, which reduced the gut pathogenic bacterial load. We also observed an upregulated expression of *dual oxidase* and a modulated expression of *Attacin* and *Diptericin* genes in the gut of older flies, possibly delaying the gut dysbiosis connected to the age-related gut immune dysfunction. Brain proteome analysis showed that DFS led to the modulation of metabolic processes connected to mitochondrial biogenesis, the RhoV-GTPase cycle, organelle biogenesis and maintenance, membrane trafficking and vesicle-mediated transport, possibly orchestrated through a gut–brain axis interaction. Taken together, our study shows that DFS can prolong the half-life and lifespan of flies, possibly by promoting a healthier gut environment and delaying the physiological dysbiosis that characterizes the ageing process. However, the RhoV-GTPase cycle at the brain level may deserve more attention in future studies.

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†Electronic supplementary information (ESI) available: Table 1: List of primers for PCR. Table 2: List of identified proteins together with their MW, pI, peptides and coverage values of MS/MS, ratios and *p* values. Table 3: List of *p*-values obtained for each main effect and their interaction in the two-way ANOVA analysis of the results of the RT-PCR assay. Fig. 1–3: Relative abundances (log) of beneficial or pathogenic microbes (family, genus and phylum, respectively). Fig. 4: Sample images relative to the 2DE gels (CTRL *vs.* M0.25%). See DOI: https://doi.org/10.1039/d4fo00879k

1. Introduction

It has been reported that the commensal microbial community may impact a diverse range of host physiologies including regulation of immunity, metabolism, and brain functionality.[1,2] The gut microbiota, the collection of bacteria, archaea, eukarya and viruses colonising the GI tract, could be considered a 'dynamic endocrine organ'[3] in which trillions of microbes interact with the innate and adaptive local and systemic immune systems.[4,5] Recently, the gut microbiota has also been reported to communicate with the central nervous system *via* biochemical, endocrine, and neurological pathways that work bi-directionally through the so-called gut–brain axis (GBA).[3,6]

Drosophila melanogaster has emerged as a powerful model to study the function of gut microbes,[7] and its relatively simple microbial community and ease of physiological and genetic manipulation make it an ideal candidate to study complex GBA interactions.[8] For instance, the adult *Drosophila* midgut is typically in stable contact with a symbiotic commensal community composed of 5–20 different microbial species that

mainly comprise yeasts and commensal bacterial species such as acetic acid bacteria (*Acetobacter*, AABs) and lactic acid bacteria (*Lactobacillus*, LABs), together with less abundant but highly prevalent *Enterobacteriaceae* and *Leuconostoc* as major host strain-specific bacterial genera.[9] Interindividual differences in the microbiota are however observed,[8,10] and are purported to relate to numerous factors, including host genetics, environmental exposure, sex, age, and dietary regime.[8,11] Indeed, *Drosophila* dietary interventions comprising probiotic and symbiotic formulations have been reported to ameliorate deficits in managing inflammation, metabolic stress and oxidative stress associated with ageing, promoting longevity through the mechanisms of the GBA.

Diets rich in dietary fiber have been shown to exert multiple health benefits, including a positive effect on longevity and boosting the gut microbiota.[12] Gut microbes, by digesting fiber, produce short chain fatty acids (SCFAs) such as butyrate, propionate and acetate that affect the brain in a number of ways,[13] although there may also be some connections through the immune system.[14] For example, gut immune defence mechanisms and gut microbes play an important role in the immune system and inflammation by controlling what is passed into the body and what is excreted.[15] The current understanding of the impact of the gut microbiota on host physiology is strictly conditioned by the technical difficulties associated with an in-depth integrated genetic analysis of both the microbes and the host.[2]

Whole grains and legumes are particularly rich in dietary fiber, with a significant amount contained in their brans or hulls. These terms usually refer to the outer layers of the grain or caryopsis of seeds such as cereals or legumes. Importantly, opting for cereal and legume bran utilisation instead of isolated dietary fiber provides economic and environmental advantages: the use of agricultural by-products avoids the extra costs of fiber extraction, and waste materials are recycled from an environmentally sustainable perspective. Bran and hull are excellent sources of insoluble dietary fibers such as cellulose, hemicellulose, and lignin, together with soluble raffinose, fructans, xylanes and β-glucans.[16–18]

A commercial syrup with a consistency like honey (MELTEC® by HIFOOD S.p.A. Parma, Italy) rich in fiber obtained from legumes and cereals is available on the market and is a clean label ingredient proposed for partial/total sugar substitution in bakery applications.[19]

On the basis of the above, our study aimed to assess the effect of dietary fiber supplementation (at medium and very high intake levels) on longevity, gut microbiota diversity and abundance, local gut immunity, and the brain proteome of *D. melanogaster*.

2. Materials and methods

2.1. *Drosophila melanogaster* husbandry

After eclosion, *D. melanogaster* (CantonS strain) were separated under FlyNap (Carolina Biological Supply, Burlington, USA)

anaesthesia according to the sex (males: M and females: F) as previously reported.[20] Flies were reared in plastic tubes containing standard media (Formula 4-24®, Carolina Biological Supply, Burlington, USA) and yeast (*Saccharomyces cerevisiae*), considered the control diet (CTRL), and were allowed to grow under a 12 h : 12 h light–dark cycle at 25 °C and 60% (±5%) relative humidity.

2.2. Dietary fiber supplementation

Dietary fiber supplementation was implemented by the inclusion of a commercial fiber syrup (MELTEC®, HIFOOD, Parma, Italy) obtained from corn (*Zea mays*) dextrin and seed coats of chickpeas (testa of *Cicer arietinum* seed).[19] MELTEC® composition was about 66% (g fiber per 100 g sample) dietary fiber content [of which, 27.4 g per 100 g was high molecular weight dietary fiber and 35.86 g per 100 g was low molecular weight dietary fiber], 9% (g per 100 g sample) carbohydrates (with sugars <0.5% g per 100 g sample), fats < 0.2% (g per 100 g sample), 0.2% (g per 100 g sample) proteins, and a moisture content of ≈25% (g water per 100 g sample).[19] In the present study, the MELTEC® syrup was solubilized in water at two different concentrations (0.25% or 1% w/v: M0.25% or M1%, respectively) and added or not (CTRL diet received only water) to soak the standard medium (Formula 4–24®; Carolina Biological, Burlington, NC, USA). The ingredients of the standard medium (as reported by the manufacturer) are as follows: oat flour, soy flour, wheat flour, other starches, dibasic calcium phosphate, calcium carbonate, citric acid, niacinamide, riboflavin, sodium chloride, sodium iron pyrophosphate, sucrose, thiamine, mononitrate, emulsifier preservatives, mould inhibitor, and food colouring.

The concentrations M0.25% and M1%, relative to the fiber content present in Formula 4-24® (which we cannot publish as it is proprietary to the supplier), represent an increase of approximately 40% and 160% in fiber content compared to control flies, representing medium fiber and very high fiber intakes,[21] and if the fly weight is scaled to that of 70 kg men, these concentrations would correspond to 12.5 or 50.0 g of dietary fiber per day in a human diet.

2.3. *Drosophila melanogaster* longevity assay

A total of nearly 1200 male and female flies were used for the evaluation of the effect of DFS on life span. Flies were randomly divided into 3 groups (*N* = 200 per sex per group): CTRL, M0.25% and M1%, and flies were transferred into vials containing fresh food every 3–4 days. The number of dead flies was counted each time, and it was repeated until all flies died.

2.4. Body weight determination

To assess the effect of DFS on body weight gain, flies were weighed on days 3, 15, and 30. Briefly, 20 flies (4 replicates) in each group of flies reared for the longevity assay were transferred into empty tubes to be weighed on an electronic scale and then they were put back in their experimental tubes. The mean body weights of the flies in each group/age were calculated.

2.5. Microbial 16S rRNA extraction and sequencing

For the evaluation of DFS on the gut microbiota, flies were fed with different concentrations of experimental diets alongside a control diet for 15 days from birth. Microbial DNA was then isolated from five gut pools (10 whole gut pools per group per sex), using a JetFlex Genomic DNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, prior to dissecting, flies were anesthetized with FlyNap and their body surfaces were sterilized with 70% ethanol for 1 min and rinsed twice with sterile PBS. The foreguts and midguts were dissected with special care to reduce contamination and were kept on ice until sampling was completed. The pooled guts were successively homogenized with sterile 0.5 mm wide glass beads (Bertin) for 30 s at 6800 rpm in an automatic tissue homogenizer (Precellys 24, Bertin).[22] The quantity of DNA was assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific). The V3–V4 regions of bacterial 16S rDNA were amplified using bacterial/archaeal degenerate primers 515F/806R, and 16S rRNA amplicon sequencing was performed with an Illumina NovaSeq 6000 PE250. Sequence analysis was performed using Uparse software (Uparse v7.0.1001), using all the effective tags. Sequences with ≥97% similarity were assigned to the same OTUs. A representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was used against the SSUrRNA database of SILVA Database 1.38. Information on the abundance of OTUs was normalised using a standard sequence number corresponding to the sample with the least sequences. Alpha-diversity was assessed using both Chao1 and Shannon H diversity indices whilst beta diversity was assessed using the Bray–Curtis distance. Statistical significance was determined by Kruskal–Wallis or Permutational Multivariate Analysis of Variance (PERMANOVA). Comparisons at the phylum, family and genus levels were made using DESeq2 combined with a false discovery rate (FDR) approach used to correct for multiple testing ($q < 0.05$).

2.6. Gut RNA extraction and analysis of mRNA levels by reverse transcriptase polymerase chain reaction

For the evaluation of the DFS effect on gut anti-microbicidal gene expression, further 1200 male and female flies were lifelong supplemented (M0.25% or M1%, respectively) with the fiber syrup alongside the control. The gut pools were collected on days 15, 30 and 45 in female flies, whereas on days 15 and 30 in male flies (four pools of 10 guts at each time point). RNA was extracted from the whole gut pools of flies using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany), and to measure the yield and purity of RNA, a NanoVue spectrophotometer (GE Healthcare, Milano, Italy) was used. Only samples with ratios A260/A280 > 1.8 were used. For each sample, 1 μg of total RNA was reverse transcribed (RT) to obtain cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. The subsequent polymerase chain reaction (PCR) was performed in a total volume of 10 μL containing 2 μL of RNAse free dH$_2$O, 2.5 μL (12.5 ng) of cDNA, 5 μL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories), and 0.5 μL (500 nM) of each primer. The primers used were purchased from Sigma-Aldrich and are reported in ESI Table 1.† *Rpl32* was used as the reference gene.

2.7. Brain proteomic analysis

For proteomic analysis, *Drosophila* head samples (three replicates of 15 heads each) collected on day 45 in female flies were resuspended in rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 60 mM dithiothreitol and 0.002% bromophenol blue) using a microtube pestle and sonicated for 1 min 3 times in an ultrasonic bath. After sonication (1 min, 2 times), homogenates were allowed to rehydrate for 1 h at room temperature (RT) with occasional stirring. Thereafter, the solution was centrifuged at 16 000g for 20 min at RT. The protein concentration of the resulting supernatant was determined using the Pierce 660 protein assay (Thermo Fisher Scientific, Waltham, MA, USA). BSA was used as a standard. 2DE was carried out as previously described.[23] Briefly, 75 μg of proteins were filled up to 350 μL in rehydration solution. Immobiline IPG BlueStrips (SERVA Electrophoresis, GmbH, Heidelberg, Germany) of 18 cm with a linear gradient (pH 3–10) were rehydrated overnight in the sample and then transferred to the Ettan IPGphor 2 (GE Healthcare Europe, Uppsala, Sweden) for isoelectrofocusing (IEF). The second dimension (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; SDS-PAGE) was carried out by transferring the proteins to 12% polyacrylamide, running at 45 mA per gel and 14 °C for about 7 h, using the Protean® Plus Dodeca Cell (BioRad). The gels were stained with ruthenium II tris(bathophenanthroline disulfonate) and tetrasodium salt (Cyanagen Srl, Bologna, Italy) (RuBP). ImageQuant LAS4010 (GE Healthcare) was used for the acquisition of images. The analysis of images was performed using SameSpot software (v4.1, TotalLab; Newcastle Upon Tyne, UK).

Gel spots were excised and *in vitro* digested as reported by Giusti *et al.* in 2018.[24] Samples were analysed by LC-MS/MS using an UltiMate3000 RSLCnano chromatographic system coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), operating in positive ionization mode, equipped with a nanoESI source (EASY-Spray NG). Peptides were loaded on a PepMap100 C18 pre-column cartridge (5 μm particle size, 100 Å pore size, 300 μm i.d. × 5 mm length, Thermo Fisher Scientific, Waltham, MA, USA) and subsequently separated on an EASY-Spray PepMap RSLC C18 column (2 μm particle size, 100 Å pore size, 75 μm i.d. × 15 cm length, Thermo Fisher Scientific, Waltham, MA, USA) at a flow rate of 300 nL min^{-1} and a temperature of 40 °C using 0.1% FA in water (eluent A) and 99.9% ACN and 0.1% FA (eluent B). Chromatographic separation was achieved by a two-step linear gradient from 5% to 30% eluent B in 40 min, and from 30% to 55% in 5 min followed by an increase to 90% in one minute, for a total runtime of 56 min.

Precursor (MS1) survey scans were recorded in the Orbitrap at resolving powers of 240 K (at m/z 200). Data-dependent MS/

MS (MS2) analysis was performed in top speed mode with a 3 s cycle time, during which the most abundant multiple-charged (2+–5+) precursor ions detected within the range of 375–1500 m/z were selected for HCD activation in order of abundance and detected in the ion trap at a rapid scan rate after fragmentation using 30% normalized collision energy. Quadrupole isolation with a 1.6 m/z isolation window was used, and dynamic exclusion was enabled for 60 s after a single scan. Automatic gain control targets and maximum injection times were standard and auto for MS1 and 150% and 70 for MS2. For MS2, the signal intensity threshold was 5.0×10^3, and the option "Injection Ions for All Available Parallelizable Time" was set.

Raw data were directly loaded in PEAKS Studio Xpro software (Bioinformatics Solutions Inc., Waterloo, Canada) using the 'correct precursor only' option. The mass lists were searched against the Uniprot/SwissProt database (downloaded January 2022) restricted to fruit fly taxonomy to which a list of common contaminants was appended (42 757 searched entries). Non-specific cleavage was allowed at one end of the peptides, with a maximum of 2 missed cleavages and 2 variable PTMs per peptide. 10 ppm and 0.5 Da were set as the highest error mass tolerance values for precursors and fragments, respectively. $-10\lg P$ threshold for PSMs was manually set to 35. The FDR was <0.1%.

2.8. Statistical analysis

Survival curves were prepared using a Kaplan–Meier survival estimator and analysed using the OASIS2 software.[25] The values relative to RT-PCR (female guts) and body weights (male and female weights, separately considered) are represented as means ± SEM and two-way ANOVA analysis was used to compare differences among groups followed by Tukey's test (Prism 8.4.2, GraphPad software, San Diego, CA). The values of $p < 0.05$ were considered statistically significant.

For proteomic experiments, the spot volume ratios between the different conditions (CTRL and DFS) were calculated using the average spot normalized volume of three biological replicates. Comparison analysis was performed using one-way ANOVA. Spots that exhibited both the p-value and q value of <0.05 were taken into consideration for further protein identification. Bioinformatics analysis of the identified proteins was carried out using Metascape software.

3. Results

3.1. Dietary fiber supplementation impacts the lifespan of *Drosophila melanogaster*

To examine the effect of lifelong dietary fiber supplementation (DFS) on the lifespan of flies, male and female *Drosophila* were reared on a standard diet supplemented with different DFS concentrations (M0.25% and M1%) (Fig. 1). DFS influenced the half-life of both male (CTRL *vs.* M0.25%: $p < 0.002$; CTRL *vs.* M1%: $p < 0.01$) and female flies (CTRL *vs.* M0.25% or M1%: $p < 0.0001$), although the absolute lifespan was increased in
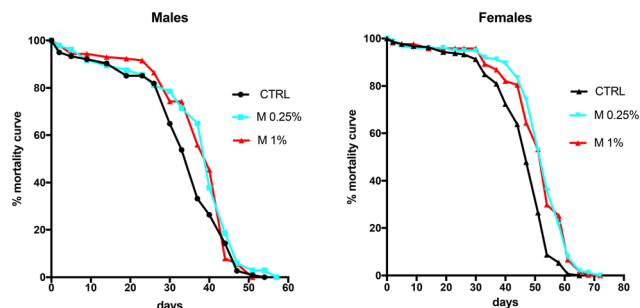
Fig. 1 Longevity curves of *Drosophila* (female and male) subjected to DFS (M0.25% or M1%). Data are presented as the percentage of survival of flies as a function of time (in days). The Kaplan–Meier test was used to detect the significant differences among the three groups.

female flies (about 11% longer in flies supplemented with both M0.25% and M1%).

## 3.2. Impact of dietary fiber supplementation on body weight

Caloric restriction has been previously reported to increase the lifespan of *Drosophila melanogaster*.[26,27] The weight of flies was, therefore, monitored over time to rule out that the observed effect on longevity was not due to a decrease in food intake and energy induced by high fibre supplementation. Whilst the body weight significantly increased during ageing (age effect: $p < 0.0028$ and 0.0001, in female and male flies, respectively), it did not significantly fluctuate across the experimental groups (effect of supplementation: p = n.s.; Fig. 2). The only exception is represented by male flies supplemented with M1% that, on day 30, showed a significant increase in their body weight *vs.* CTRL flies of the same age (interaction of age × supplementation: $p < 0.0151$; Fig. 2). Therefore, the improvement of the mean lifespan observed both in male and female flies was not related to the caloric restriction (CR) effect,[28] fiber, but rather to other mechanisms as hypothesised below.

## 3.3. Dietary fiber supplementation affects the gut microbiota

Using 16S rRNA gene sequencing, differences in microbial diversity among control (n = 4), DFS 0.25% (n = 4) and DFS 1% (n = 4) fed flies were first analysed. The relative abundances (log) of beneficial or pathogenic microbes are reported in ESI Fig. 1–3.† Although not reaching significance, flies receiving DFS had a dose-dependent higher diversity (p-value: 0.23638) and a lower microbiota richness (p-value: 0.55093) as assessed by the Shannon H diversity index and the Chao1 index, respectively (Fig. 3A). A significant difference in the gut microbial community was observed between groups based on the Bray–Curtis distance; $P < 0.05$ by PERMANOVA (Fig. 3B and C). Subsequently, differential abundance in bacterial groups that could be driving this separation was tested by means of the DESeq2 algorithm. At the genus level, we detected 6 bacteria which were strongly modulated (DSEq2 $q < 0.05$). Among those, a 2.9-fold and 4.2-fold increase in *Asaia* (q = 0.041) and *Alloprevotella* (q = 0.049) in DFS groups were observed, whilst the abundances of *Bacillus*, *Pseudomonas*, *Listeria* and
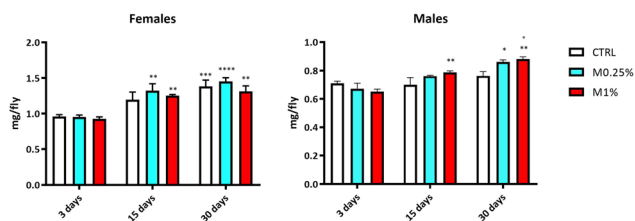
Fig. 2 Body weight measurement of flies with or without (CTRL) DFS (M0.25% or M1%) on days 3, 15 and 30 (in male and female flies, respectively). Twenty flies (4 replicates) in each group were weighed. Each bar represents the mean $\pm$ SEM. Data were analysed by two-way ANOVA with repeated measures, with groups and time as the main factors (Prism 8.4.2, GraphPad software, San Diego, CA). *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, and ****$p < 0.0001$ vs. 3 days (age effect: $p < 0.0028$ and 0.0001, in female and male flies, respectively) or °$p < 0.05$ vs. the control of the same age (interaction of age × supplementation: $p < 0.0151$, only in male flies).
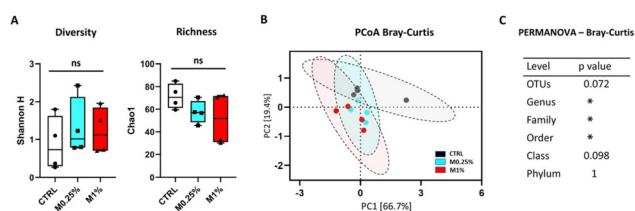
Fig. 3 Impact of M0.25% or M1% on the gut microbiota structure. (A) Diversity (Shannon H index) and richness (Chao1 index) of the microbiota in flies fed with a control diet or fibre syrup at 0.25% (M0.25%) or 1% (M1%). (B) Principal components of analysis (PCoA) based on their treatment at the OTU level, using the Bray–Curtis distance. (C) Significant differences in the gut microbial community structure as determined by the Bray–Curtis distance-based permutational multivariate analysis of variance (PERMANOVA). * = $P < 0.05$. Bars show the mean $\pm$ SEM. Symbols represent individual flies ($n = 4$ per group). NS = not significant. PC1/2 = principal component 1/2.

Enterococcus were all strongly decreased (−4 to −6-fold decrease) in this group when compared to the control (Table 1).

3.4. Dietary fiber supplementation influences the gut immune defence mechanisms in female *Drosophila melanogaster*

To better explore, at the molecular level, the mechanisms underlying the life-span-extending effect of DFS, the

Table 1 Genera significantly different (DESeq2, $q < 0.05$) between the control and CDSF groups

| Genus	log2FC	P values	FDR
g__*Bacillus*	−6.5323	1.2×10^{-6}	6.21×10^{-5}
g__*Pseudomonas*	−4.4395	0.000349	0.009086
g__*Listeria*	−4.7008	0.001469	0.025465
g__*Enterococcus*	−3.4396	0.003066	0.039861
g__*Asaia*	2.9055	0.003991	0.041509
g__*Alloprevotella*	4.2581	0.005674	0.049171

expressions of *Duox* and antimicrobial peptides (AMPs) *Attacin A* and *Diptericin A* (*AttA* and *DptA*, respectively) genes related to gut immune defence mechanisms were investigated. Given that DFS was more effective in increasing the longevity of female flies, mRNA level analysis was limited to samples taken from female *Drosophila* supplemented or not (CTRL) with M0.25% or M1%. Data were normalised vs. the gene expressions of CTRL flies on day 15.

The modulation of the gut immune responses concomitant with ageing was observed in CTRL. The two AMPs considered were found to increase (in particular *AttA*, age effect: $p < 0.0001$) while the *Duox* ($p < 0.0001$) gene underwent significant downregulation (Fig. 4a).[3] In contrast, supplemented flies did not show a *Duox* decreasing expression trend with age, whereas the different AMP coding genes, regardless of age, were particularly upregulated (the effect of supplementation is represented in Fig. 4b). The *p*-values are summarized in ESI Table 3† for each main effect and their interactions.

3.5. Dietary fiber supplementation modulates brain protein expression in aged female *Drosophila melanogaster*

The brains of 45-day-old female flies supplemented with M0.25% were analysed by proteomic analysis. Since DFS had comparable effects on increasing longevity at both supplementation levels, only the M0.25% concentration was considered. The list of the identified proteins together with their MW, pI, peptides and coverage values of MS/MS, ratios and *p* values are reported in the ESI (ESI Table 2†).

Twenty-five spots were differentially expressed ($p < 0.05$) between CTRL and M0.25% groups, of which 18 spots showed
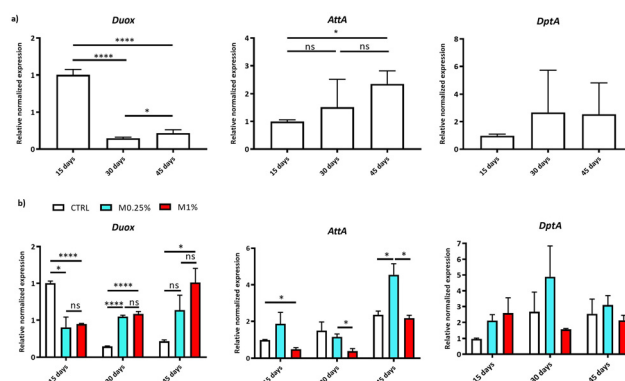
Fig. 4 Impact of age ((a) CTRL group) or DFS (M0.25% and M1%) (b) on gut immune gene expressions. Pools of the gut from female flies fed with a control diet (CTRL) or MELTEC® 0.25% (M0.25%) or 1% (M1%) were collected on days 15, 30 and 45. Quantification of mRNA was performed, and the mRNA levels of *Attacin A* (*AttA*), *Diptericin A* (*DtpA*) and *dual oxidase* (*Duox*) were normalized to the *Rpl32* reference gene. Triplicate reactions were performed for each experiment. Gene expressions were further normalized vs. CTRL gene expression on day 15. Each bar represents the mean $\pm$ SEM of at least three independent experiments. Data were analysed by two-way ANOVA followed by Tukey's test (Prism 8.4.2, GraphPad software, San Diego, CA). *$p < 0.05$ and ****$p < 0.0001$.
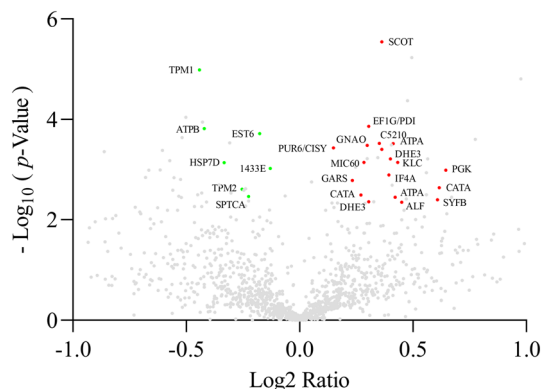
Fig. 5 Scatter plot of fold change (*x*-axis) against the log *p* value (*y*-axis) of all quantified proteins. Upregulated and downregulated proteins are coloured red and green, respectively. Only proteins that showed both the *p*-value and *q*-value of <0.05 were identified.

an increase in expression, whereas 7 were reduced after M0.25% supplementation (Fig. 5).

The brain proteins significantly modulated by supplementation were analysed using Metascape Resource (Reactome gene set analysis)[29] to unveil the metabolic pathways in which they exert their function. Mitochondrial biogenesis ($\log10(P)$ −5.11), the RhoV GTPase cycle ($\log10(P)$ −4.61), organelle biogenesis and maintenance ($\log10(P)$ −4.09), membrane trafficking ($\log10(P)$ −3.04), vesicle-mediated transport ($\log10(P)$ −3.03), signalling by Rho GTPases ($\log10(P)$ −2.90) and the citric acid cycle and respiratory electron transport ($\log10(P)$ −2.46) were the metabolic processes in which these proteins (whose expression was modulated by fiber supplementation) were involved (Fig. 6).

4. Discussion

In this study, the effect of long-term supplementation of a commercial syrup (MELTEC®) rich in fiber on the life span

Fig. 6 Reactome gene set analysis using the METASCAPE resource executed by choosing the brain proteins differently expressed in flies receiving DFS. The main metabolic pathways in which these proteins exert their functions are shown.

and gut modulation of *D. melanogaster* was investigated due to the widely acknowledged positive impacts of dietary fibers on health.

Fibre supplementation (at both M0.25% and M1% concentrations) significantly extended the half-life of female and male flies, although the absolute life span was only extended in female flies.

Such observed effects may not be attributed to a potential CR mechanism, as no change in the body weight of flies or food intake was associated with high fiber supplementation.[30]

DFS (at both M0.25% and M1% concentrations) also affected the gut microbiota diversity after 15 days of supplementation, with a significant difference in the gut microbial community and an elevated bacterial load of *g. Asaia* and *g. Alloprevotella* in comparison with the control group, suggesting a prebiotic action of DFS. The presence of these two bacteria is novel in *D. melanogaster* even if the acetic acid bacterium *Asaia* has been previously found in sugar-feeding insects of phylogenetically distant genera and orders.[31]

Asaia has previously been recognised as a natural effector for mosquito (in *Anopheles stephensi* and *An. gambiae*) immune priming.[32] The presence of *Asaia* in *D. melanogaster* may therefore be underlying the immune modulatory effects observed following DFS intake, although further studies would be necessary to establish causality.

In addition, a significant increase in the abundance of *Alloprevotella* has been previously reported in young healthy people receiving a short-term dietary fiber intervention (4 days).[33]

However, we also observed the presence of pathogenic microbes (Table 1) in our flies and, if on one side it is well known that the gut microbiota of *Drosophila* is greatly influenced by microbes in the environment, especially food,[34,35] DFS mainly impacts the absolute quantity of gut microbes rather than the overall gut microbiota composition, however reducing the pathogenic species and increasing the beneficial ones.

It is well known that, in the midguts of *Drosophila melanogaster*, the proliferative capability of intestinal stem cells (ISCs) is very high;[36] likewise the integrity of the intestinal epithelium is strictly influenced by gut microbes, both transient pathogens and commensal bacteria. Gut microbes, together with stem cell activity (leading to gut epithelium renewal), and the gut immune responses give rise to a complex network that is the basis of intestinal homeostasis. Indeed, to resist an infection, the gut epithelium needs both to mount an efficient immune response to eliminate the hazard and replace the injured enterocytes, destroyed by bacteria or the same immune responses, through ISC proliferation.[37]

In old flies, immune senescence alters the healthy microbiota, which, in turn, triggers aberrant epithelium renewal and barrier dysfunction.[36]

In our study, the presence of dietary fiber may have contributed to the selection of the healthy gut microbiota (*g. Asaia* and *Alloprevotella*) that outcompetes the pathogenic bacteria for available resources, stimulating exploitative competition or
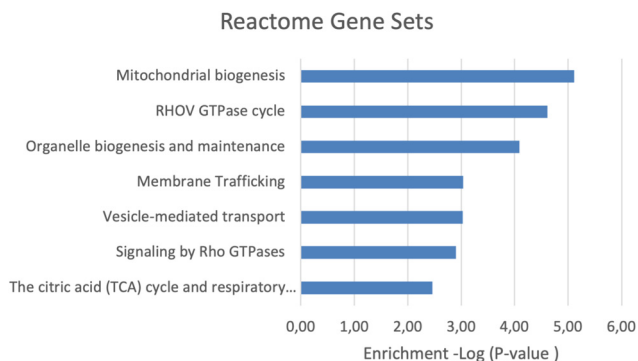
have created an unfavourable gut environment for the unde-sired bacteria.[3,14]

Indeed, 15-day old flies supplemented with DFS, besides the described modulation of the microbiota, also showed a downregulation of *Duox* together with a different modulation of AMPs. This finding is probably the expression of targeted immunosurveillance in the gut environment through the action of AMPs in shaping the microbiota.[38]

In aged flies, the primary innate immune response of *Duox* tends to physiologically decrease,[3] while in the present study, a significantly increased expression of *Duox* and *AttA* was observed following supplementation.

It is plausible that dietary fibers may therefore have pro-moted higher immune responses, thus impacting the homeo-stasis of the intestine and the longevity of flies. Indeed, *AttA* acts not only as an immune effector conferring defence upon systemic infections[39] but also regulates multiple aspects of host metabolism, thus adding an additional role of *AttA* in gut homeostasis.[40]

Consistent with our observations, Si *et al.* (2018)[41] found several AMP-encoded genes upregulated on days 30 or 50 in flies lifelong supplemented with glucomannan hydrolysate. Likewise, Westfall *et al.* (2018)[3] observed an increase in AMP expression that was dramatically benefitted by individual pro-biotic, prebiotic and synbiotic formulation supplementations in aging flies. These authors also found an increase in the expression of *Duox* during ageing in the groups that received different types of supplementations.

In *D. melanogaster*, the control of the expression of AMPs in the body is due to two major signalling pathways: immune deficiency (Imd) and Toll. The Imd pathway acts all along the gut, and gut microbes, by stimulating NF-κB-regulated immune responses of the immune deficiency pathway, induce the production of AMPs, in particular *AttA* and *AttD*.[38]

Physiologically, old flies show higher levels of Imd pathway activity, consistent with their increased bacterial loads.[36,42] Our data also confirm increasing trends in AMP expression in CTRL groups as their age increases, but an even higher AMP expression was also observed in the supplemented flies that may have controlled, more efficiently, this overload of com-mensal bacteria.[42] As hypothesised by Buchon (2013), one of the key roles of the immune response in the gut is to maintain a tight microbial balance to avoid an exacerbated proliferation of intestinal stem cells.[37]

A higher AMP expression in ageing flies correlates with an increase in oxidative stress[43] or could be influenced by chan-ging hormonal signaling,[44] but it is noteworthy that AMP expression is also influenced by the insulin/IGF-1 signalling pathway[45] which, alone, could explain the impact of fiber sup-plementation on AMP overexpression in ageing *Drosophila*.

In the midgut of flies, however, there exists a second main inducible antimicrobial effector that is represented by reactive oxidative species (ROS) whose production is induced by *Duox*, a member of the NADPH oxidase family.[36]

Besides direct antimicrobial activity, ROS can also act as signalling messengers in tissue repair and wound healing.[46] In

insects, it has been demonstrated that *Duox*-dependent ROS production could be induced by both pathogenic and com-mensal bacteria and it is involved in multiple aspects of gut homeostasis. For instance, *Duox* participates in ISC activation or in the redox-dependent regulation of signalling pathways and cross-linking of the peritrophic matrix.[37,47]

However, healthy ageing is favoured by optimal NADPH oxidase activity, with ROS production being helpful not only in eliminating pathogens but also in managing the bacteria.[48] The immune senescence that characterises ageing could cause the commensal expansion that, by promoting an increased concentration of ROS, determines excessive ISC proliferation with the loss of tissue homeostasis.[49]

Broderick *et al.*,[34] in 2014, showed that differences in feeding, by modulating the microbiota, induce different gene expressions. Even if 53% of the upregulated genes were involved in the Imd pathway, there were also other modulated genes involved in signalling and metabolism, thus unveiling an unexpected connection between the microbiota and host functions.

It has already been demonstrated that an imbalance of the gut microbiota, through the GBA, can impair the brain func-tion. Indeed, the increased permeability of the gut barrier could permit the entry of antigens, reducing the availability of useful metabolites for the brain such as SCFAs and neuro-transmitters and increasing (neuro)inflammation.[50] However, diet has always proved to play a key role in maintaining the health of the intestinal barrier.[51]

Fiber-rich diets are being studied as a possible approach for modulating the microbiota in order to reduce inflammation, induce neuroprotection and improve brain functions.[3,52]

In our study, the brain proteome of 45-day-old flies shows twenty-five differently expressed proteins between M0.25% and CTRL flies and these proteins belong to different metabolic pro-cesses such as mitochondrial biogenesis, the RhoV GTPase cycle, organelle biogenesis and maintenance, membrane trafficking, vesicle-mediated transport, signalling by Rho GTPase and the citric acid cycle and respiratory electron transport.

Among the signalling pathways and/or metabolic processes that were modified by fiber supplementation, the modulation of brain mitochondrial biogenesis (8 of the 29 modulated pro-teins were involved) in M0.25% supplemented flies *vs.* CTRL seems interesting given the importance of mitochondria in the ageing process.[53] Indeed, an impairment of functional and anatomical integrity of the mitochondria represents a hall-mark of aging in *Drosophila*. In addition, this dysfunction rep-resents an important factor in the increased risk of disease and death associated with ageing, and it is accompanied by elevated ROS production.[3,54,55]

Westfall *et al.* (2018)[3] observed that a new probiotic and symbiotic formulation extended longevity in *D. melanogaster* through mechanisms of gut–brain communication that reduced, *inter alia*, loss of mitochondrial complex integrity.

Interestingly, mitochondrial biogenesis has been shown to induce synaptic plasticity in neurons.[56] In our study, fiber sup-

plementation modulated not only the aforementioned process (mitochondrial biogenesis) but also membrane trafficking (5 proteins out of 25 proteins involved) and signalling by Rho GTPase (4 proteins out of 25 proteins involved), all involved in synaptic plasticity maintenance. The promising 'theory of microbiome-driven synaptic plasticity' postulated that through neurotransmitters, neuronal electrophysiology, and immune mediators which interact with both the central and enteric nervous systems and signalling cascades, the microbiome–gut–brain axis can induce synaptic plasticity and long-term changes to the physical and functional neuronal structures.[57,58]

Finally, in our study, fiber supplementation also modulated some brain proteins involved in the RhoV GTPase cycle (12/25 proteins). RhoV, important during development for maintaining E-cadherin at adherens junctions,[59] activates the JNK and p38 MAPK cascades, essential for cell survival and immune response regulation.[60] The modulation of immune responses in the intestines of M0.25% flies may relate to the central Rhov GTPase cycle modulation. This cycle, controlling tight junctions, could affect brain cell communications, membrane trafficking and vesicle-mediated transport, favouring neuroplasticity in flies fed with DFS. More research on the roles and interactions of RhoV GTPase is essential, given its potential for synaptic plasticity and cell adhesion in various cell types, including neurons and enterocytes.

5. Conclusions

In the present study, lifelong dietary fiber supplementation improved the half-life of both sexes of *D. melanogaster* and promoted longevity in female flies, by simultaneously affecting the gut microbiota, local gut immunity and brain proteome. Even if most of the work in this field has been performed in animal models, and further experiments, especially those with gnotobiotic or axenic flies, are needed to confirm the effect of DFS, our data show that a diet rich in fiber could modulate the gut environment, with positive effects on the brain proteome revealing a combinatorial effect on various triggers of ageing including the basic signalling pathway and mitochondria biogenesis.

However, it was found that the modulation of the RHOV GTPase cycle in the brain is an element of innovation and in our opinion, its involvement during aging could serve as a potential target of the microbiota–GBA communication axis which could be an area of exploration for future research studies.

Author contributions

All authors contributed to this work significantly. Conceptualization: D.B. and C.A.; methodology: D.B., D.V., L.G., C.D., and C.A.; software: D.B., L.G., M.R., A.C., and L.Z.; formal analysis: D.B., D.V., A.C., C.D., I.C., M.R., and L.Z.; resources: D.B., D.V., and L.G.; data curation: L.G, A.C., D.V., C.D. C.A., L.Z., and D.B.; writing – original draft preparation: D.B.; writing – review & editing: L.G., C.A., D.V., E.V., C.D., G. F., and S.H. All authors have read and agreed to the published version of the manuscript.

Data availability

The datasets supporting this article have been uploaded as part of the ESI.† Other data that support the findings of this study will be made available upon request to the corresponding author.

Conflicts of interest

There are no conflicts to declare.

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