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Life Sciences 336 (2024) 122328

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Iron chelation and supplementation: A comparison in the management of inflammatory bowel disease using drosophila

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ARTICLE INFO

Keywords:
Inflammatory bowel disease
Iron overload
Iron chelation
Bathophenanthroline disulfonate
Drosophila

ABSTRACT

Aims: Inflammatory Bowel Disease (IBD) is associated with systemic iron deficiency and has been managed with iron supplements which cause adverse side effects. Conversely, some reports highlight iron depletion to ameliorate IBD. The underlying intestinal response and comparative benefit of iron depletion and supplementation in IBD is unknown. The aims of this work were to characterize and compare the effects of iron supplementation and iron depletion in IBD.

Main methods: IBD was induced in *Drosophila melanogaster* using 3 % dextran sodium sulfate (DSS) in diet for 7 days. Using this model, we investigated the impacts of acute iron depletion (using bathophenanthroline disulfonate, BPS) and supplementation (using ferrous sulphate, FS), before and after IBD induction, on gut iron homeostasis, cell death, gut permeability, inflammation, antioxidant defence, antimicrobial response and several fly phenotypes.

Key findings: DSS decreased fly mass ($p < 0.001$), increased gut permeability ($p < 0.001$) and shortened lifespan ($p = 0.035$) compared to control. The DSS-fed flies also showed significantly elevated lipid peroxidation ($p < 0.001$), and the upregulated expression of apoptotic marker- *drice* ($p < 0.001$), tight junction protein – *bbg* ($p < 0.001$), antimicrobial peptide – *dpta* ($p = 0.002$) and proinflammatory cytokine – *upd2* ($p < 0.001$). BPS significantly ($p < 0.05$) increased fly mass and lifespan, decreased gut permeability, decreased lipid peroxidation and decreased levels of *drice*, *bbg*, *dpta* and *upd2* in IBD flies. This iron chelation (using BPS) showed better protection from DSS-induced IBD than iron supplementation (using FS). Preventive and curative interventions, by BPS or FS, also differed in outcomes.

Significance: This may inform precise management strategies aimed at tackling IBD and its recurrence.

1. Introduction

Inflammatory Bowel Disease (IBD) is a broad term used to identify three diseases characterised by gastrointestinal inflammation; Ulcerative Colitis, Crohn's Disease and indeterminate colitis [1,2]. Recent data from the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2019, shows that IBD affects over 4.8million people worldwide [3]. Between 1990 and 2019, the age-standardized prevalence rate decreased in high (EAPC, estimated annual percentage change = −0.34) and high-middle (EAPC = −0.32) income countries but increased in middle (1.25), low-middle (0.52) and low-income countries (0.29) [3,4].

An estimated 60–80 % of IBD patients suffer from systemic iron deficiency (ID) [5]. This has informed the clinical management of IBD with iron supplements. The rationale for use of iron lies in its ability to increase systemic iron levels. The discovery of this symptomatic benefit can be traced to as early as 1935 [6]. Though, this approach resolves the systemic ID, the effect of iron on the gut is conflicted. Iron supplementation is associated with gut related adverse effects such as cramp, diarrhoea, and constipation [7,8]. It is also documented to exacerbate

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https://doi.org/10.1016/j.lfs.2023.122328
Received 23 September 2023; Received in revised form 20 November 2023; Accepted 30 November 2023
Available online 5 December 2023
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IBD [9,10]. Iron supplementation may also trigger accumulation of cellular iron which has been implicated in cell death and DNA damage [11]. More so, iron overload may worsen the immune response (macrophage hyperinflammatory response) which damages tight junctions, issues enterocyte death by apoptosis and enhances gut leakage [12].

On the other hand, some reports show that iron depletion ameliorates IBD. For instance, deferiprone, an iron chelator, is reported to protect the gut from gastric damage and inflammatory colitis in rats [13]. But this is contested by a similar report of Ettreiki et al. [14], where iron supplementation was shown to protect against colitis and gut microbial dysbiosis upon induction of inflammatory colitis in both rats and mice. Till date, however, little is known about how gut cells respond to these apparently contrasting interventions.

The gut forms the natural entry route for nutrients, ions and water. It is also the first barrier against toxins, antigens, bacteria and other pathogens [15]. A compromise to this selective barrier function, the "leaky gut," derives from damage to protective structures like tight junctions and permits the translocation of organismal molecules from the gut lumen into the circulation, triggering exaggerated immune response and aggravating inflammation. Such inflammation due to loss of intestinal integrity accompanies inflammatory bowel disease (IBD). Since 1990, the chemical colitogen called dextran sodium sulfate (DSS) has predominantly been employed for inducing IBD (experimental colitis) in animal models [16,17].

At the anatomical and molecular levels, DSS-induced gut inflammation in *Drosophila melanogaster* (henceforth *Drosophila*) closely mimics the pathophysiological features of inflammatory bowel disease [18]. Using this turnkey model, often termed the leaky gut syndrome (LGS) fly, we investigated the effects of iron depletion and supplementation on gut iron homeostasis, cell death, gut permeability, inflammation, antioxidant defence, antimicrobial response and several fly phenotypes, before and after mucosal inflammation.

2. Materials and methods

2.1. Reagents

Dextran Sodium Sulfate (MW-54 kDa) was purchased from Nacalai Tesque, Japan (M9T9773). Bathophenanthroline disulfonic acid disodium salt hydrate, BPS (MW-590.53 g/mol) was purchased from Sigma Aldrich, Switzerland (CAS no. 52746-49-3). Ferrous Sulphate, $FeSO_4{\cdot}7H_2O$ (MW-278.02 g/mol) was purchased from Kermel, China. Brilliant Cresyl Blue (GURR Searle Diagnostics, England) was used as tracer dye. Designed primers were synthesized by Inqaba Biotech, South Africa.

2.2. Fly culture

Wild type *Drosophila melanogaster* Meigen (Harwich strain) from the Centre for Advanced Medical Research and Training (CAMRET), Usmanu Danfodiyo University, Sokoto was used for this study. Except otherwise stated, flies were raised on our standard laboratory diet (oligidic media composed of 10 g Agar Agar, 100 g Cornflour, 20 g Baker's yeast, 1 g methyl paraben and 1.7 l distilled water) under natural light-dark cycle, controlled temperature (25 ± 3 °C) and humidity (55 ± 5 %).

2.3. Experimental design

Flies were placed and transferred onto treated diets based on grouping (see Fig. 1). LGS, iron depletion and iron overload were induced with DSS [18,20], BPS [21–24] and $FeSO_4$ [25], respectively. Prior to experimentation, we performed preliminary dose selection studies to determine acute concentration and duration required for chelation and supplementation. The iron chelated diets were prepared by thoroughly mixing a 20 mM stock of BPS (an iron (II) chelator) per 10 ml melted aliquot of the standard food to make a final concentration of

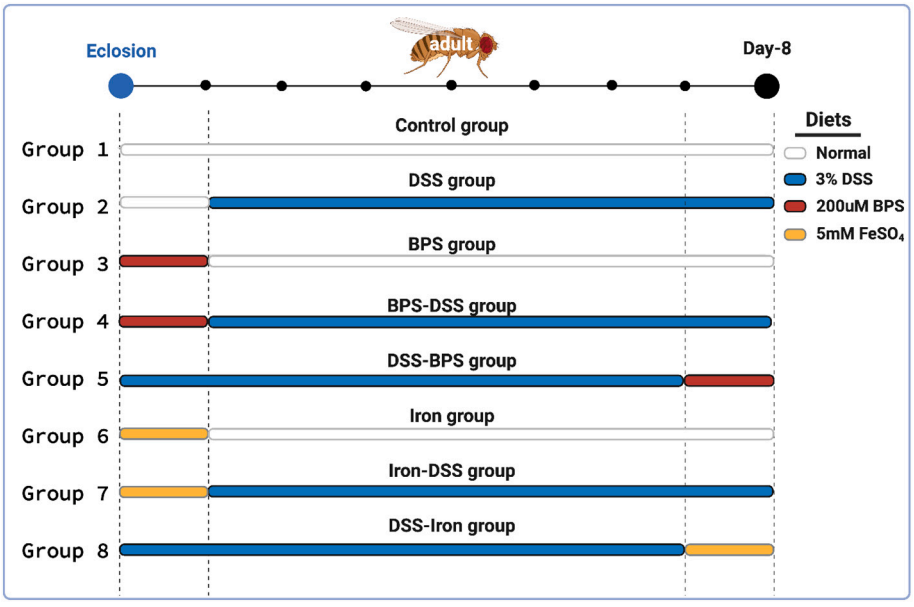


Fig. 1. Experimental outline showing eight study groups raised on different regimens for eight days. Pupae hatch and flies fed on the prescribed treated diets (coloured bars). After exposure, brood were fasted for 2 h before downstream analysis. Group 1 (Control group): flies on standard diet for 8 days post-eclosion, Group 2 (DSS group i.e., DSS only): flies on standard diet for 1 day followed by 3 % DSS diet for 7 days, Group 3 (BPS group also called iron-chelated group i.e., BPS only): flies on 200 μM BPS diet for 1 day followed by standard diet for 7 days, Group 4 (BPS-DSS): flies on 200 μM BPS diet for 1 day followed by 3 % DSS diet for 7 days, Group 5 (DSS-BPS): flies on 3 % DSS diet for 7 days followed by 200 μM BPS diet for 1 day, Group 6 (Iron group also called iron-supplemented group i.e., FeSO4 only): flies on 5 mM FeSO4 for 1 day followed by standard diet for 7 days, Group 7 (Iron-DSS): flies on 5 mM FeSO4 for 1 day followed by 3 % DSS diet for 7 days, Group 8 (DSS-Iron): flies on 3 % DSS diet for 7 days followed by 5 mM FeSO4 for 1 day. BPS-Bathophenanthroline disulfonate, DSS-Dextran sodium sulfate, FeSO4-ferrous sulfate (the gold standard/reference Fe compound for food fortification in humans [19]). Designed using BioRender. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

200 μM BPS. To prepare iron loaded diets, we thoroughly mixed a 100 mM stock of $FeSO_4$ per 10 ml melted aliquot of the standard food to make a final concentration of 5 mM $FeSO_4$. DSS diets (3 % w/v) were made by rigorously mixing melted food with clearly dissolved 15 %(w/v) stock solution. Flies (n = triplicates of 20 each) were weighed at the start and after the experimental period. Equal number of male and female flies were used.

2.4. Gut permeability assay (smurf assay)

Gut permeability was assessed with slight modifications to the method previously described by Rera et al. [26]; Rera et al. [27]. Briefly, flies ($n = 40$ per group) were fasted and placed in empty vials containing 2 by 4 cm filter papers soaked in 500 μl of a dyed fluid medium; 2.5 % (*w/v*) blue dye (Brilliant Cresyl Blue) in 5 % sucrose and 15 % ethanol. After 24 h on paper-diet at 25 °C, smurf positive flies were identified based on the appearance of blue colouration outside the gut. Dead flies were exempted from count because gut integrity is lost after death.

2.5. Indirect colorimetry and the capillary feeder assay

Food intake was measured by the capillary feeder (CAFE) assay and indirect colorimetry [28]. Briefly, 50 ml plastic vials with caps having two openings that hold pipette tips (20–200 μl) were fabricated. Two glass microcapillaries filled with liquid diet (5 % sucrose solution, 1 % red-6 food dye and 15 % ethanol in dH_2O) were suspended on the pipette tips and this set up was made in triplicates for each experimental group. Flies ($n = 20$ per group) were habituated to this chamber for 24 h (with liquid diet) and the experiment was conducted in daylight. After 12 h experimental period, flies were beheaded, rinsed in 1× PBS, transferred into 1.5 ml micro-centrifuge tubes and weighed. Each sample was homogenised in 100 μl of 1× PBS and centrifuged at 9100 ×*g* for 10 min. The supernatant (50 μl) was transferred into a micro-well and absorbance was measured at 442 nm (as readout for the red dye in food) using a UV–vis microplate spectrophotometer (AD Touch Microplate Reader, 11000). Values where normalized to primary blank (PBS only), secondary blank (PBS and normal flies) and sample weight.

2.6. Fly iron content analysis

At the terminal point of the exposure protocol, flies (n ~ 14) were fasted, anaesthetized with ice, weighed and rinsed in distilled water. Flies were then digested by immersion in 1 ml of 65 % HNO_3. We placed the mix in a heating block at 100 °C for 10 min or further; until a homogeneous solution was observed. Following this, the digested samples were diluted to 5 ml with distilled water and iron concentration was measured using microwave plasma atomic emission spectroscopy (MY19479002- MP-AES, Agilent). Iron concentration was normalized to body weight.

2.7. Lifespan assay

Flies ($n = 40$ per treatment group) from the fully exposed brood (mated male ($n = 20$) and females (n = 20) separate) were placed on 10 ml standard diets in 50 ml vials. Flies from the untreated group served as controls. Every three days, dead flies were removed and food vials were replenished with freshly prepared diets. Records of natural deaths and censors (accidental deaths and escapees) were taken until all the flies died. All the groups were blinded prior to assay by assigning random numbers.

2.8. Startle induced negative geotaxis assay

Following exposure to treatments, we assessed locomotor activity with a slightly modified Startle Induced Negative Geotaxis (SING) protocol, earlier described by Coulom and Birman (2004). SING quantifies the climbing behaviour of flies in response to a gentle mechanical stimulus. Briefly, under stable lighting conditions and 1 h after cold anaesthesia, flies were tapped to the bottom of a sterile 23 cm long cylinder (1.5 cm diameter) and allowed to climb for 30 s. At the end of this time, the number that crossed a 20 cm mark and those below a 3 cm mark were counted and the results tabulated. The experiment was conducted for three independent sets of flies ($n = 20$) and repeated thrice with a 1 min resting period in between. Performance index (PI) was calculated using the formular;

$$PI = \tfrac{1}{2}\left(\left(n_{tot} + n_{top} - n_{bot}\right)/n_{tot}\right).$$

Where: n_{top} is number of flies at the top, n_{bot} is number of flies at the bottom and n_{tot} is total number of flies.

2.9. Sample preparation for antioxidant assay

After the experimental period, whole flies (M:F) were harvested from each group (n = 20), weighed and rinsed in freshly prepared and autoclaved ice-cold PBS. Samples were collected in a micro-centrifuge tube and homogenised with a micro-pestle in extraction reagent. The homogenates were centrifuged at 8000 ×*g* for 10 min at 4 °C and the resulting supernatant transferred into sterile micro-centrifuge tubes for the following downstream biochemical assays.

2.9.1. Measurement of catalase activity

Catalase activity was determined using a catalase (CAT) activity assay kit (Beijing Solarbio Science & Technology Co., Ltd., China; Cat No BC0200) based on the peroxidase function of catalase [29] and according to the manufacturer's protocol. The rate of change in sample absorbance at 240 nm was determined spectrophotometrically (AD Touch Microplate Reader, 11000**)** and used to calculate the enzyme activity using the following formulae:

$$\Delta A = A_{immediate} - A_{1min} \quad (1)$$

$$CAT\ Enzyme\ Activity\left(\frac{U}{g}\right) = 678 \times \Delta A \div weight(g) \quad (2)$$

Where: $A_{immediate}$ is the immediate absorbance reading at 240 nm, A_{1min} is absorbance reading after 1 min at 240 nm, and ΔA is the difference between the immediate absorbance and absorbance after 1 min. One unit of enzyme activity is defined as the amount of enzyme that catalyses the degradation of 1 μmol of H_2O_2 in the reaction system per minute of every gram *Drosophila* tissue sample.

2.9.2. Measurement of glutathione peroxidase-like activity

Glutathione peroxidase (GSH-Px/GPx)-like activity was measured using a GSH-Px/GPx assay kit (Beijing Solarbio Science & Technology Co., Ltd., China; Cat No BC1190) in accordance with the manufacturer's instructions. This assay is based on the ability of GSH to react with 5,59 -dithio-bis(2-nitrobenzoate) (DTNB) to form a compound 5-thio-2-nitrobenzoic acid (TNB) with absorption peaks at 412 nm [30,31]. Using test samples (T), controls (C), standards (S), and blanks (B), we determined the absorbance of TNB at 412 nm (using the AD Touch Microplate Reader, 11000**)** and calculated the enzyme activity with the formulae:

$$\Delta A_T = A_C - A_T \quad (3)$$

$$\Delta A_S = A_S - A_B \quad (4)$$

$$Glutathione\ Peroxidase\ Enzyme-like\ Activity\left(\frac{U}{g}weight\right) = 2 \times \Delta A_T \div \Delta A_S \div weight(g) \quad (5)$$

Where: A_C is the absorbance of control sample at 412 nm, A_T is the

absorbance of test sample at 412 nm, A_S is the absorbance of standard sample at 412 nm and A_B is the absorbance of the blank sample at 412 nm. One unit of enzyme activity is defined as the amount of enzyme that catalyses the oxidation of 1 nmol of GSH per minute in the reaction system of every gram Drosophila tissue sample.

2.9.3. Malondialdehyde content assay

Lipid peroxidation was estimated using a malondialdehyde (MDA) content assay kit (Beijing Solarbio Science & Technology Co., Ltd., China; Cat No BC0020) as per the manufacturer's instructions. This was based on the ability of MDA to produce a brown-red complex (3,5,5-three methyl sulfamethoxazole-2,4-two ketone) upon reaction with thiobarbituric acid [32,33]. Using test samples (T), and blanks (B), we determined the absorbance at 532 nm and 600 nm (using the AD Touch Microplate Reader, 11000**)** and calculated the MDA content with the formulae:

$$\Delta A532 = A532(T) - A532(B) \tag{6}$$

$$\Delta A600 = A600(T) - A600(B) \tag{7}$$

$$\Delta A = \Delta A532 - \Delta A600 \tag{8}$$

$$MDA\ content\left(\frac{nmol}{g}weight\right) = 32.258 \times \Delta A \div weight(g) \tag{9}$$

Where: $A532(T)$ is the absorbance of test sample at 532 nm, $A532(B)$ is the absorbance of the blank sample at 532 nm, $A600(T)$ is the absorbance of the test sample at 600 nm and $A600(B)$ is the absorbance of the blank sample at 600 nm.

2.10. Gene expression by reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Total ribonucleic acid (RNA) was extracted from fly GI tract ($n = 10$ flies per sample) using a column-based RNA isolation Kit (Liferiver, LVN01 - Shanghai, China). cDNA synthesis and Real time PCR were conducted using TransScript® Green one-step qRT-PCR Supermix kit (AQ211, TransGen Biotech, China) as per the manufacturer's protocol. We designed primers (listed in Table 1) to target transcripts for key proteins that are associated with iron import into enterocytes (Malvolio, *mvl* - homolog to human divalent metal transporter1, DMT1) [34,35]; iron export (ferritin heavy chain homolog, *fer1hch*) [36,37]; mitochondrial iron utility (*Drosophila* frataxin, *dfh*) [38]; iron trafficking (transferrin, *tsf1*) [39–41]; apoptotic cell death (*Drosophila* ice-like caspase, *drice* - homolog to human effector caspase3) [42]; tight junction integrity (big bang gene, *bbg*) [43]; antimicrobial response (diptericin, *dpta*) [44,45]; and inflammatory response (unpaired-2, *upd2*) [46]. The RT-qPCR was performed on a Rotor-Gene Q 5plex HRM thermal cycler (Qiagen). Following optimization and melt curve analysis, Cq values were normalized to reference gene (AcTC5) and control sample using the comparative $2^{-\Delta\Delta ct}$ method [47]. All experiments were conducted in replicates.

Table 1
Primers used for RT-qPCR.

Gene product (mRNA)	Strand	Primer (5′-3′)	Product size
Bbg	Forward	TCGTAGAATGAAGCCGATTTCC	116
	Reverse	CCGAGGTTAGTTCCACATAAGC	
DptA	Forward	CAGTACCCACTCAATCTTCAGG	128
	Reverse	CCTCCATTCAGTCCAATCTCG	
Upd2	Forward	TTGACCATAAACGCCTCCTATC	90
	Reverse	GTGAAAGTTGAGACGCTCCT	
Drice	Forward	ACAGCGATAGTGATTGCATCC	108
	Reverse	TGAAGAAGCTCCAGATGTTATCC	
Mvl	Forward	CGGTCAGTTCTCAATGGAAGG	98
	Reverse	GGCAGAAGGTGGGAATAATGG	
Fer1HCH	Forward	CTGGCTGTTCCTGAGATTACC	114
	Reverse	CATGGCCAAGTACTGGTAGG	
Dfh	Forward	CTGCCCTACTGTAGTTAATTCCG	123
	Reverse	GTATATCGAGTGACCACTGTGC	
Tsf1	Forward	CGAATATCTGTGCGAGGATGG	97
	Reverse	CTGCTCGTTGGAGATGTAGC	
ActC5	Forward	GAGCGCGGTTACTCTTTCA	89
	Reverse	TCAAAGTCGAGGGCAACATAG	

Abbreviations: ActC5-Actin; Bbg-big bang gene; dfh-*Drosophila* frataxin; DptA-Diptericin; Drice-death related ICE-like caspase; Fer1HCH-ferritin heavy chain; mvl-malvolio; Tsf1-transferrin; Upd2-unpaired.

2.11. Data analysis

Datasets were qualitatively (histogram and QQ plot) and statistically (Shapiro-Wilk test) evaluated for normality. Following normality tests, all data were interrogated for significance using IBM SPSS software (version 23). Inferential analyses were conducted with one way ANOVA for normal distributions (followed by Tukey post hoc) or Kruskall-Wallis rank sum test for non-normal distributions (followed by multiple pair-wise comparisons). The significance level was set at $p \leq 0.05$. Time to event data were analysed for time, rate and difference using the Kaplan Meier Survival test while Log rank statistic was adopted to compute the significance of survival distributions for the different groups. Data were visualised in R.

3. Results

3.1. Effect of dietary iron availability on weight, food intake, mobility and survival of Drosophila IBD model

Given that increase in weight loss follows gut inflammation [18,48], we explored the consequence of our treatments on fly mass. As shown in Fig. 2a, DSS significantly decreased fly mass compared to the control group ($p < 0.001$). This effect was apparently reversed only in the DSS-BPS group ($p < 0.001$) (see Fig. 2a). Conversely, the greatest loss of fly mass was found when flies were exposed to the DSS-Iron treatment ($p < 0.001$), compared to control. This indicates a beneficial effect of DSS-BPS treatment and highlights a detrimental effect of DSS-Iron treatment on DSS-induced inflammation.

Furthermore, we found a significant increase in fluid diet intake following DSS-induced inflammation ($p < 0.001$) (Fig. 2c). This increase was also present in all treated groups. Notably, the greatest increase in intake was in the DSS-BPS group and this was strikingly consistent with the greatest gain in body weight (see Fig. 2a). In addition, there was significant decrease in locomotor performance following DSS exposure ($p = 0.016$) (Fig. 2c). This decrease was consistent across all groups except the iron-chelated(i.e., BPS) and the BPS-DSS groups. Lastly, our survival record revealed that flies from the DSS-inflamed group (median survival time = 24 days, death rate at day 39 = 100 %) have a significantly shorter lifespan ($p = 0.035$) compared to control flies (median survival time = 30 days, death rate at day 39 = 85 %). Other groups did not show significant change in survival times compared to control (see Table 2).

3.2. BPS administration after DSS assault confers greatest protection from DSS induced oxidative stress

As oxidative stress is largely implicated in the pathophysiology of IBD [49], we determined the implications of our regimens on the redox axis. We observed the greatest increase in catalase activity in the DSS-BPS group (Fig. 3a). This activity was ~99 % higher than in DSS-inflamed flies ($p = 0.007$) and can confer protection against oxidative stress by degrading hydrogen peroxide to water and oxygen [50]. A similar trend was observed in glutathione activity which was 176 % higher in the DSS-BPS group than the DSS-inflamed group ($p = 0.001$). Though, the activities of these antioxidant enzymes in the DSS-inflamed

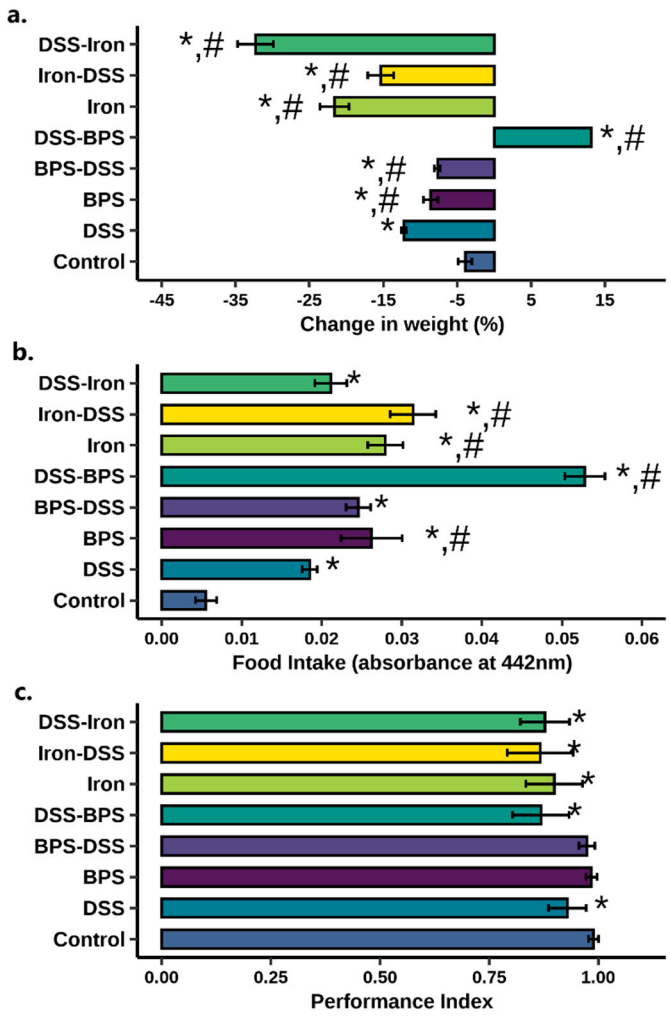


Fig. 2. Effect of treatments on fly a.) bodyweight b.) fluid diet intake c.) locomotor performance. Bars represent mean ± SD. * and # indicate mean values are significantly different at $p \leq 0.05$, compared to control and DSS-inflamed group, respectively (a,b-One way ANOVA with Tukey post hoc, *c*-Kruskal-Wallis Test with pairwise comparisons, d-log rank statistics). Results in (a) are expressed as percentage of the change in weight calculated by subtracting the initial weight of 20 flies at day 0 from their final weight at day 8, and refer to 3 independent experiments and 2 technical replicates. Results in (b) are food intake values calculated by dividing the normalized absorbance values (442 nm) of homogenised live flies (n ~ 20) by their headless weight and normalizing to secondary blank (samples without dye). Results in (b) refer to 3 independent experiments. Results in (c) represent performance index calculated for flies ($n = 20$) and refer to 3 independent experiments and 3 technical replicates. Group 1 (Control group): flies on standard diet for 8 days post-eclosion, Group 2 (DSS group i.e., DSS only): flies on standard diet for 1 day followed by 3 % DSS diet for 7 days, Group 3 (BPS group also called iron-chelated group i.e., BPS only): flies on 200 μM BPS diet for 1 day followed by standard diet for 7 days, Group 4 (BPS-DSS): flies on 200 μM BPS diet for 1 day followed by 3 % DSS diet for 7 days, Group 5 (DSS-BPS): flies on 3 % DSS diet for 7 days followed by 200 μM BPS diet for 1 day, Group 6 (Iron group also called iron-supplemented group i.e., FeSO4 only): flies on 5 mM FeSO4 for 1 day followed by standard diet for 7 days, Group 7 (Iron-DSS): flies on 5 mM FeSO4 for 1 day followed by 3 % DSS diet for 7 days, Group 8 (DSS-Iron): flies on 3 % DSS diet for 7 days followed by 5 mM FeSO4 for 1 day. BPS-Bathophenanthroline disulfonate, DSS-Dextran sodium sulfate, $FeSO_4$-ferrous sulfate.

group were not significantly different from controls ($p > 0.05$), the DSS-inflamed group had significantly greater malondialdehyde levels than the controls ($p = 0.035$). A similarly significant elevation in lipid peroxidation was found with BPS-DSS ($p = 0.003$), Iron ($p < 0.001$), Iron-DSS ($p = 0.035$) and DSS-Iron ($p < 0.001$) treatments, all reflecting a path to tissue damage potentially via ferroptosis (see Fig. 3d). These results suggests that DSS-Iron has a limited antioxidative impact on the oxidative damage induced by DSS. On the other hand, DSS-BPS demonstrated a clearly sufficient antioxidative impact on the oxidative damage induced by DSS.

Table 2
Difference in the median death time across groups.

Group	Median survival time
Control	30.00 ± 1.68
DSS	24.00 ± 2.02[a]
BPS	24.00 ± 2.97
BPS-DSS	30.00 ± 1.62
DSS-BPS	30.00 ± 6.46
Iron	30.00 ± 4.30
Iron-DSS	27.00 ± 3.21
DSS-Iron	30.00 ± 1.51

[a] Indicates significant difference compared to control ($p \leq 0.05$). Median survival time refers to the time (days) it takes for 50 % of flies to have died. Each fly represents one unit of analysis ($n = 40$). Group 1 (Control group): flies on standard diet for 8 days post-eclosion, Group 2 (DSS group i.e., DSS only): flies on standard diet for 1 day followed by 3 % DSS diet for 7 days, Group 3 (BPS group also called iron-chelated group i.e., BPS only): flies on 200 μM BPS diet for 1 day followed by standard diet for 7 days, Group 4 (BPS-DSS): flies on 200 μM BPS diet for 1 day followed by 3 % DSS diet for 7 days, Group 5 (DSS-BPS): flies on 3 % DSS diet for 7 days followed by 200 μM BPS diet for 1 day, Group 6 (Iron group also called iron-supplemented group i.e., FeSO4 only): flies on 5 mM FeSO4 for 1 day followed by standard diet for 7 days, Group 7 (Iron-DSS): flies on 5 mM FeSO4 for 1 day followed by 3 % DSS diet for 7 days, Group 8 (DSS-Iron): flies on 3 % DSS diet for 7 days followed by 5 mM FeSO4 for 1 day. BPS-Bathophenanthroline disulfonate, DSS-Dextran sodium sulfate, FeSO4-ferrous sulfate.

3.3. Time dependent impact of iron chelation and iron supplementation on iron metabolism in the inflamed gut

To establish the effect of iron availability on its absorption, trafficking and homeostasis in an inflamed gut, we measured the relative abundance of transcripts for key proteins that are associated with iron import into enterocytes (Malvolio, *mvl*- homolog to human divalent metal transporter1, DMT1) [34,35], iron capture, export and transport (ferritin heavy chain homolog, *fer1hch*) [36,37], mitochondrial utility (frataxin, *dfh*) [38], intercellular transport and nutritional immunity (transferrin, *tsf1*) [39–41]. Fig. 4a highlights the relative abundance of *mvl* transcripts across the eight treatment groups. The results show that compared to the control group, the iron-chelated group did not show a significant change ($p = 0.089$) in *mvl* expression. On the other hand, a significant 2-fold increase was observed in the DSS-inflamed (i.e., DSS only) ($p < 0.001$) and the iron-supplemented (i.e., $FeSO_4$ only) ($p < 0.001$) groups. This 2-fold upregulated expression of *mvl* was also observed in the BPS-DSS ($p < 0.001$) and DSS-Iron ($p < 0.001$) groups. Interestingly comparisons to the DSS-inflamed group revealed a significant decrease only following DSS-BPS ($p = 0.002$) and Iron-DSS ($p < 0.001$) treatments. These findings indicate opposing responses in the *mvl* expression for BPS-DSS versus DSS-BPS groups and iron-DSS versus DSS-Iron groups.

Ferritin is majorly expressed in the midgut of *Drosophila* and its transcriptional regulation changes in response to dietary iron [51]. Our study also found that ferritin heavy chain (*fer1hch*) mRNA is downregulated by 1.2-fold following iron chelation (i.e., BPS only) ($p = 0.009$) and upregulated by 1.3-fold following iron supplementation (i.e., $FeSO_4$ only) ($p < 0.001$) (see Fig. 4b). This is a typical response of iron-containing proteins to iron depletion and iron repletion. Also, when

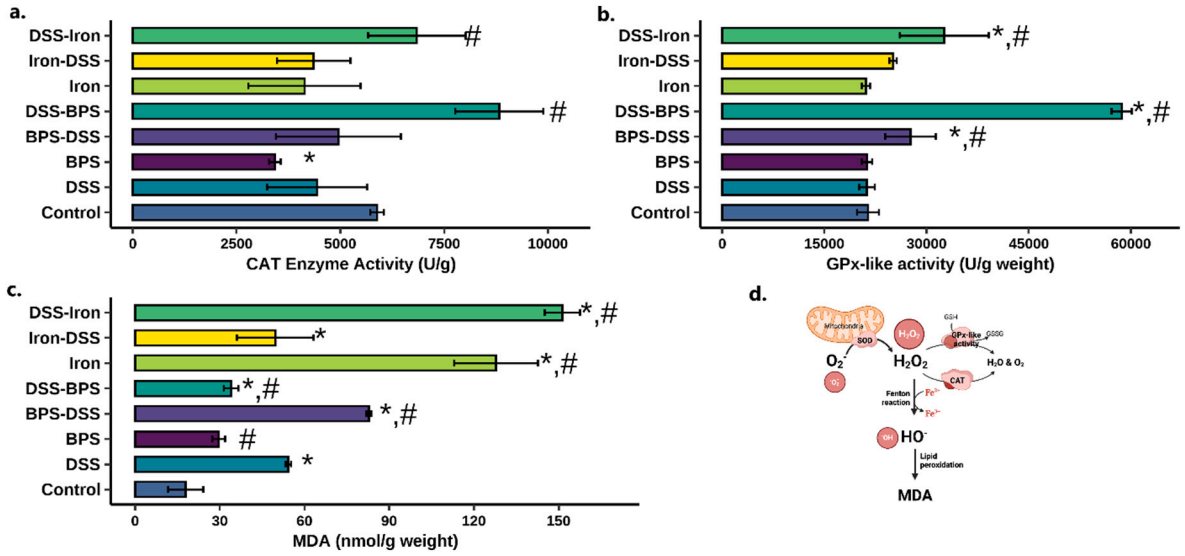


Fig. 3. Effect of treatments on antioxidant status and lipid peroxidation**.** a.) catalase (CAT) b.) glutathione peroxidase (GPx)-like activity c.) malondialdehyde (MDA) d.) redox pathway leading to production of malondialdehyde (MDA). Bars represent mean ± SD. * and # indicate mean values are significantly different at $p \leq 0.05$, compared to control and DSS-treated group respectively (Kruskal-Wallis test with pairwise comparisons). For results in (a), one unit of enzyme activity is defined as the amount of enzyme that catalyses the degradation of 1 μmol of H_2O_2 in the reaction system per minute per gram *Drosophila* tissue sample ($n = 20$) and refer to 2 independent experiments and two technical replicates. For results in (b), one unit of enzyme activity is defined as the amount of enzyme that catalyses the oxidation of 1 nmol of GSH per minute per gram *Drosophila* tissue sample (n = 20) and refer to 2 independent experiments and 2 technical replicates. Results in (c) represent MDA concentration per gram *Drosophila* tissue sample (n = 20) and refer to 2 independent experiments and 2 technical replicates. Group 1 (Control group): flies on standard diet for 8 days post-eclosion, Group 2 (DSS group i.e., DSS only): flies on standard diet for 1 day followed by 3 % DSS diet for 7 days, Group 3 (BPS group also called iron-chelated group i.e., BPS only): flies on 200 μM BPS diet for 1 day followed by standard diet for 7 days, Group 4 (BPS-DSS): flies on 200 μM BPS diet for 1 day followed by 3 % DSS diet for 7 days, Group 5 (DSS-BPS): flies on 3 % DSS diet for 7 days followed by 200 μM BPS diet for 1 day, Group 6 (Iron group also called iron-supplemented group i.e., FeSO4 only): flies on 5 mM FeSO4 for 1 day followed by standard diet for 7 days, Group 7 (Iron-DSS): flies on 5 mM FeSO4 for 1 day followed by 3 % DSS diet for 7 days, Group 8 (DSS-Iron): flies on 3 % DSS diet for 7 days followed by 5 mM FeSO4 for 1 day. BPS-Bathophenanthroline disulfonate, DSS-Dextran sodium sulfate, $FeSO_4$-ferrous sulfate, GPx - Glutathione peroxidase, GSH – reduced glutathione, GSSG – oxidised glutathione.

compared to the DSS-inflamed group, the expression of *fer1hch* mRNA was significantly reduced by 1.5, 1.9 and 1.6-folds due to DSS-BPS ($p < 0.001$), Iron-DSS ($p < 0.001$) and DSS-Iron ($p < 0.001$) treatments, respectively.

Furthermore, we investigated the relative abundance of transferrin (*tsf1*) transcripts. As shown in Fig. 4c, we discovered that iron depletion triggered a 1.5-fold increase in *tsf1* mRNA expression ($p = 0.002$), while iron supplementation triggered about 1.6-fold decrease in the *tsf1* mRNA expression ($p = 0.046$). Also, the expression of *tsf1* mRNA was reduced by 1.6-fold in the DSS-inflamed flies ($p = 0.047$). Both preventive and curative treatments with BPS and iron resulted in significantly increased *tsf1* transcripts but the extent of this increase significantly differed ($p < 0.05$) based on time of treatment, i.e., before DSS (preventive) and after DSS (curative). Like *mvl* expression, this was significantly indicative of opposing responses for preventive and curative treatments.

Notably, the greatest increase in *tsf1* expression was in the Iron-DSS group. This group also showed the greatest level of fly iron content ($p < 0.05$). See Fig. 4e. Surprisingly, iron levels were similarly (like *tsf1*) reduced in the iron-supplemented group ($p < 0.05$) and increased in the iron-chelated group (though insignificantly, $p > 0.05$). Interestingly, DSS led to significantly increased iron levels in fruit flies ($p < 0.05$) compared to controls which was restored only in the DSS-BPS group ($p < 0.05$).

Also, considering the role of frataxin, a mitochondrial protein, in the assembly of ferrous iron to form Fe-S clusters [52], we measured its mRNA expression following treatments. We observed that this utility factor was significantly increased by 1.6, 1.8, and 2-folds due to iron supplementation ($p < 0.001$), DSS ($p < 0.001$) and BPS-DSS ($p < 0.001$) treatments, respectively. Such increase may be indicative of gut cytosolic iron accumulation.

From the data on *mvl*, *fer1hch*, *tsf1* and *dfh* it is immediately apparent that a trend of decrease versus increase (or vice versa) exists between the groups on preventive and curative treatments for both BPS and iron (i.e., $FeSO_4$). This is suggestive that the effect of iron availability on its homeostasis in an inflamed gut may be dependent on timing of administration–before or after.

3.4. Evidence of resistance to cell death and epithelial permeability in the DSS-BPS group

To test if individual treatments result in tissue damage, we measured markers of apoptotic cell death (*drice* – human effector caspase3 homolog), junctional integrity (*bbg*) and gut permeability to macromolecules (smurf assay). Notably, when compared to control, no significant change was observed with *drice* mRNA levels in the BPS($p = 0.396$) and DSS-BPS ($p = 0.937$) groups (see Fig. 5a). However, we observed that *drice* mRNA was significantly increased by 1.3, 1.5, 1.6 and 2-folds due to DSS ($p < 0.001$), BPS-DSS ($p < 0.001$), iron supplementation ($p < 0.001$), and DSS-Iron treatments($p < 0.001$), respectively. To determine if these increases were significant enough to cause epithelial permeability, we further measured the relative abundance of transcripts for big-bang gene (*bbg*). In the adult *Drosophila* gut, *bbg* encodes several integral membrane protein isoforms required for the integrity of epithelial septate junctions [43]. As shown in Fig. 5b, we observed significantly upregulated expression of *bbg* transcripts following DSS (1.7fold, $p < 0.001$), BPS (1.5fold, $p < 0.001$), BPS-DSS (2.2fold, $p < 0.001$) and iron supplemented treatments (2fold, $p < 0.001$) compared to controls. Upregulation in *bbg* may suggest compensatory response to breach in the intestinal barrier. Such epithelial barrier dysfunction can be proven by the spread of a blue food dye throughout the fly body, a state termed the "smurf" phenotype [27]. Using the smurf assay, we observed that these same groups (DSS, BPS, BPS-DSS and iron-supplemented) also showed 38 % ($p < 0.001$), 5 % ($p = 0.103$), 12 % ($p = 0.008$) and 5 % ($p = 0.141$) greater permeability defect (positive

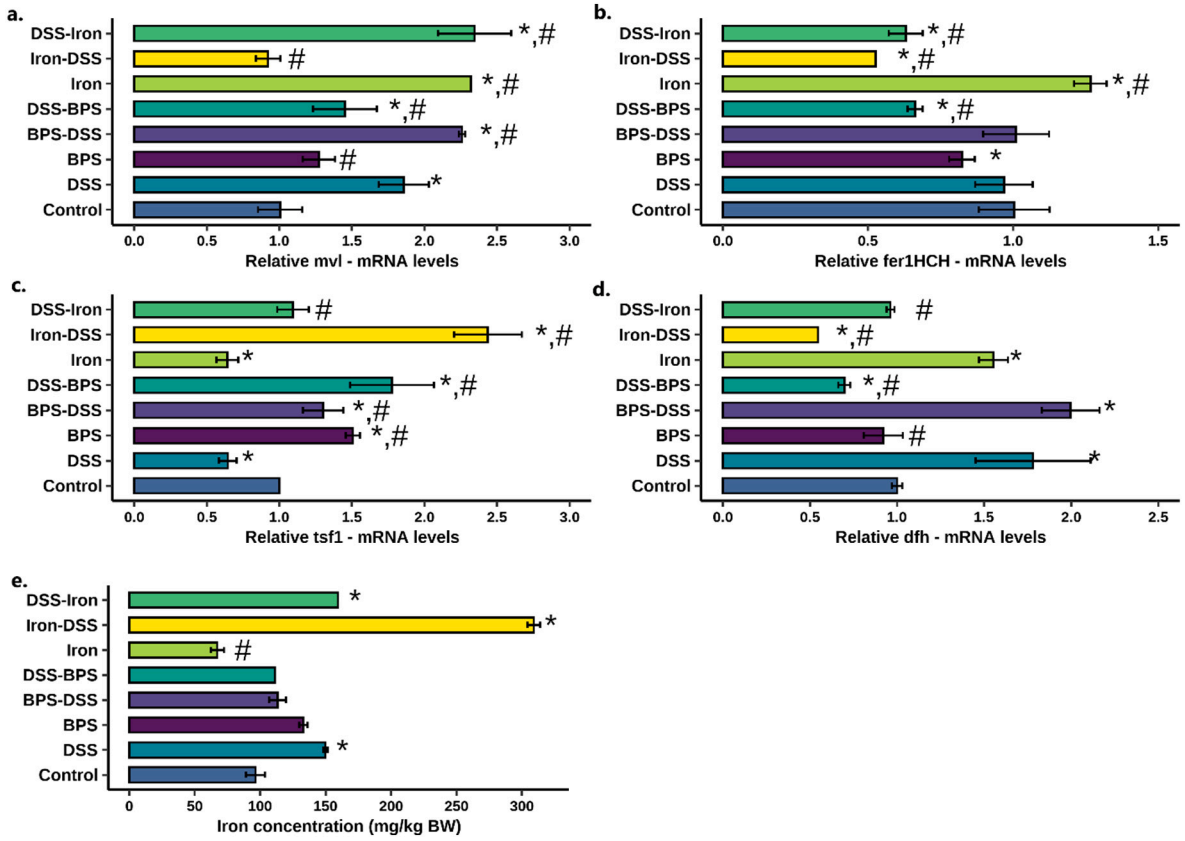


Fig. 4. Effect of interventions on the relative expression of gut a.) malvolio b.) ferritin heavy chain homolog c.) transferrin, d.) mitochondrial frataxin across fly groups and e.) whole fly iron levels 8 days post eclosion. Bars represent mean ± SD. * and # indicate mean values are significantly different at $p \leq 0.05$, compared to control and DSS-inflamed group, respectively (One way ANOVA with Tukey post hoc*)*. For each gene expression dataset, isolated gut RNA from a group of 10flies represent a unit of analysis. Results represent relative mRNA expression normalized to Actc5 using the ΔΔCt method and refer to 2 independent experiments and 2 technical replicates. Results from (e) represent iron levels calculated by dividing measured values (MPAES) with average weight of flies (n ~ 14) and refer to 2 independent experiments and 2 technical replicates. Group 1 (Control group): flies on standard diet for 8 days post-eclosion, Group 2 (DSS group i.e., DSS only): flies on standard diet for 1 day followed by 3 % DSS diet for 7 days, Group 3 (BPS group also called iron-chelated group i.e., BPS only): flies on 200 μM BPS diet for 1 day followed by standard diet for 7 days, Group 4 (BPS-DSS): flies on 200 μM BPS diet for 1 day followed by 3 % DSS diet for 7 days, Group 5 (DSS-BPS): flies on 3 % DSS diet for 7 days followed by 200 μM BPS diet for 1 day, Group 6 (Iron group also called iron-supplemented group i.e., FeSO4 only): flies on 5 mM FeSO4 for 1 day followed by standard diet for 7 days, Group 7 (Iron-DSS): flies on 5 mM FeSO4 for 1 day followed by 3 % DSS diet for 7 days, Group 8 (DSS-Iron): flies on 3 % DSS diet for 7 days followed by 5 mM FeSO4 for 1 day. BPS-Bathophenanthroline disulfonate, DSS-Dextran sodium sulfate, $FeSO_4$-ferrous sulfate.

smurf phenotypes) than controls, respectively (see Fig. 5c). Overall, the most striking result to emerge from these data is that the DSS-BPS treatment was able to completely reverse the effect of DSS on *drice*, *bbg* expression and the smurf transition (see Fig. 5). This further supports the beneficial effect of BPS on DSS induced inflammation. Similar to our data on iron metabolism, differing responses also existed between the groups on preventive and curative treatments.

3.5. DSS-induced antimicrobial response is greatly lowered by curative than preventive therapy

Similar to the mammalian tight junctions, *Drosophila* septate junctions form part of the gut immune barrier which limits paracellular entry and dampens the activation of the imd immune pathway by endogenous flora [43]. Thus, when gut integrity is lost or disrupted like in IBD, immune cells of the lamina propria are exposed to dietary antigens, the resident microbiota or, its products and the imd pathway is strongly activated. The gut also becomes permeable to blue dye, hence the "smurf" transition (Fig. 5d). Smurf flies (with intestinal barrier dysfunction) demonstrate increased expression of genes for antimicrobial peptides (AMPs) due to increased internal microbial load [27]. Given that AMP expression inversely correlates with intestinal integrity, we next measured the expression of diptericin (an AMP) in the gut.

The results showed that compared to the control group, the iron-chelated, DSS-BPS and DSS-Iron groups had significantly down-regulated expression of diptericin-A (*dpta)* ($p < 0.001$, $p < 0.001$, $p < 0.001$, respectively; Fig. 6). In contrast, significant 1.2, 1.6, and 1.2-fold upregulation of *dpta* expression were observed in the DSS-inflamed ($p = 0.002$), BPS-DSS ($p \leq 0.001$) and the iron-supplemented ($p = 0.038$) groups, respectively. In addition, when comparing to the DSS-inflamed group, an apparent decrease in expression was present following DSS-BPS ($p < 0.001$), Iron-DSS ($p = 0.001$) and DSS-Iron ($p < 0.001$) treatments. Collectively, these results suggests that both iron repletion and iron depletion may benefit the inflamed subject to an extent depending on time of administration, before or after inflammatory insult.

3.6. A pro-inflammatory response follows DSS-Iron while an anti-inflammatory response follows DSS-BPS treatments

The JAK-STAT pathway is conserved and homologous to the dome-hopscotch-STAT pathway in fruit flies. While many cytokines can activate the JAK/STAT pathway in mammals, only three ligands called unpaired (upd1, upd2, and upd3) are known to bind to the receptor (Dome) in *Drosophila*. These three ligands impact JAK/STAT transcriptional activity and are released in response to injury or infection [53]. Excessive cytokines trigger an inappropriate activation of the JAK/STAT

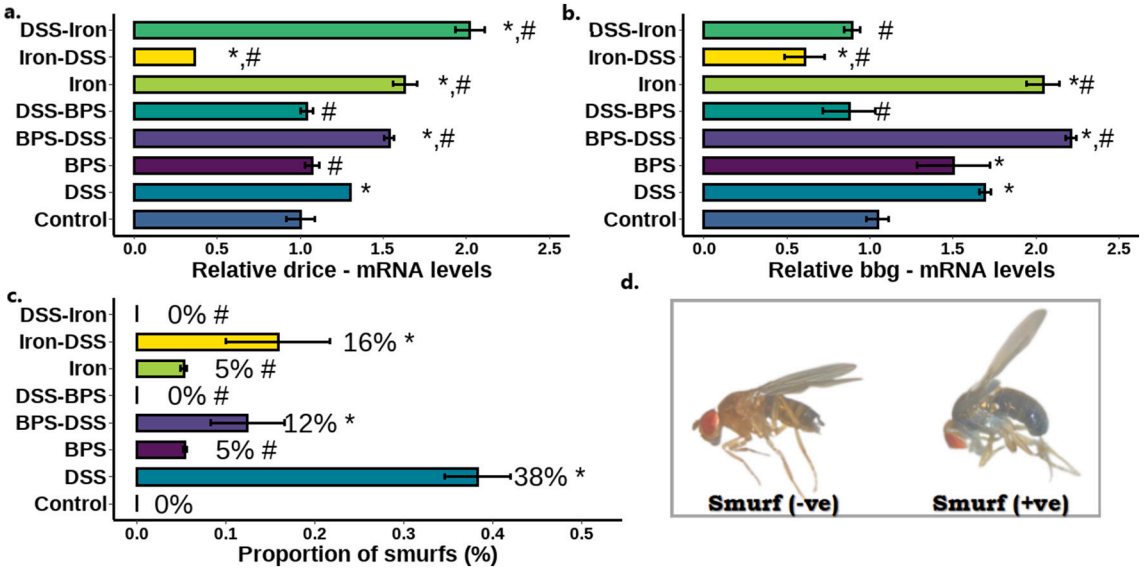


Fig. 5. Transcriptional response of a death effector caspase – *drice* (a.) and big bang gene *(bbg)* (b.) to treatments. Effect of treatments on the smurf transition (c. and d.). For control flies (**left**) blue dye remained in the gut ($smurf^{-ve}$), while in DSS-fed fly (**right**), the gut lost barrier integrity and blue dye escaped to the body ($smurf^{+ve}$) (d.). Bars represent mean ± SD. * and # indicate mean values are significantly different at $p \leq 0.05$, compared to control and DSS-inflamed group, respectively (a & b - One way ANOVA with Tukey post hoc, *c* - Kruskal-Wallis test with pairwise comparisons). For each gene expression dataset (a & b), isolated gut RNA from a group of 10flies represent a unit of analysis. Results (a & b) represent relative mRNA expression normalized to ActC5 using the ΔΔCt method and refer to 2 independent experiments and 2 technical replicates. For the result in (c), flies ($n = 40$) placed for 24 h on paper-diet were counted twice for dye colouration outside gut (definitive smurfs) and this count was converted to percentage of total live flies. Each percentage represents a unit of analysis and the results refer to 2 independent experiments and 2 technical replicates. Group 1 (Control group): flies on standard diet for 8 days post-eclosion, Group 2 (DSS group i.e., DSS only): flies on standard diet for 1 day followed by 3 % DSS diet for 7 days, Group 3 (BPS group also called iron-chelated group i.e., BPS only): flies on 200 μM BPS diet for 1 day followed by standard diet for 7 days, Group 4 (BPS-DSS): flies on 200 μM BPS diet for 1 day followed by 3 % DSS diet for 7 days, Group 5 (DSS-BPS): flies on 3 % DSS diet for 7 days followed by 200 μM BPS diet for 1 day, Group 6 (Iron group also called iron-supplemented group i.e., FeSO4 only): flies on 5 mM FeSO4 for 1 day followed by standard diet for 7 days, Group 7 (Iron-DSS): flies on 5 mM FeSO4 for 1 day followed by 3 % DSS diet for 7 days, Group 8 (DSS-Iron): flies on 3 % DSS diet for 7 days followed by 5 mM FeSO4 for 1 day. BPS- Bathophenanthroline disulfonate, DSS-Dextran sodium sulfate, $FeSO_4$-ferrous sulfate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pathway in IBD [54].

To determine the impact of our treatments on cytokine flux and the activation of inflammatory pathways in the gut, we assayed for *upd2* expression. Surprisingly, only the DSS-inflamed (1.2fold, $p < 0.001$) and the DSS-Iron (1.2fold, $p = 0.008$) treatments upregulated the expression of this proinflammatory cytokine. This rather interesting outcome may explain the aggravated inflammation that follows iron supplementation in IBD. On the contrary, *upd2* was downregulated with BPS, BPS-DSS and DSS-BPS treatments (Fig. 7), confirming the anti-inflammatory effect of BPS.

4. Discussion

Drosophila is an extensively characterised experimental model system with conserved enteric features as humans [55,56]. This and more has supported its use for studying and characterising pathways involved in gut immune response, tissue regeneration, micronutrition and homeostasis, that particularly associate with human diseases such as IBD [57]. It has also been useful for studying metal physiology [58–60]. *Drosophila* shares extensive homology with humans in iron metabolic pathways and offers much to study over its short life cycle [59,61–64]. By chelating dietary iron in *Drosophila*, iron depletion has been established to serve in immune defence (antimicrobial activity) against pathogens (nutritional immunity) [39,65], to protect against Parkinsonism [66–69], to ameliorate Friedrich's ataxia [70], to mediate antifungal effect [71], to stimulate mitophagy [72] and crystal cell differentiation [24].

We herein establish that acute iron chelation with BPS reverses multiple signatures of DSS-induced leaky gut syndrome in *Drosophila*. Observations to support this view are apparent when the DSS-BPS group (group 5) is compared with the DSS-inflamed group (group 2) (Fig. 8). These observations include; 1) only BPS reverses the effect of DSS on body mass (see Fig. 2a); 2) chelating iron (with BPS) in the DSS-treated (LGS) fly i) increases the antioxidant enzyme capacity (see Fig. 3a and b) and ii) inhibits DSS induced lipid peroxidation (see Fig. 3c); 3) BPS chelation also downregulates DSS-induced expression of *mvl* and *dfh* transcripts in fly gut (see Fig. 4a and d); 4) the transcriptional downregulation of *tsf1* by DSS is reversed by subsequent iron chelation (see Fig. 4c); 5) BPS treatment restores DSS-altered body iron levels to normal (Fig. 4e). 6) BPS chelation transcriptionally i) reverts the impact of DSS on tight junctional protein (Fig. 5b), and ii) abrogates DSS induced pro-apoptosis (see Fig. 5a); 7) BPS mitigates DSS induced gut permeability (see Fig. 5c); 8) iron chelation by BPS also reverts the increased antimicrobial response following DSS (see Fig. 6), and 9) effectively inhibits the production of an inflammatory cytokine (see Fig. 7).

BPS is a well-established membrane-impermeable agent for the exogeneous chelation of iron (fe^{2+}) from diets; preventing iron uptake by enterocytes [21,22,73]. Its ability to chelate iron is reflected in the reduced expression of *mvl* transcripts compared to the DSS-inflamed and iron-supplemented groups (see Fig. 4a). Aside its impact on iron metabolism, our data shows that BPS can act as a potent antioxidant. This is because BPS greatly increased the catalase and glutathione peroxidase-like activity by 99 % and 176 % higher in the DSS-BPS flies than the DSS-inflamed flies which can confer protection against oxidative stress by degrading hydrogen peroxide to water and oxygen [50]. See Fig. 3d. However, the activities of these antioxidant enzymes in the DSS-inflamed group were consistently not different from that of the control group. This was surprising, given that a shorter duration (5 days) of 3 % DSS had been shown to increase antioxidant enzyme activity in

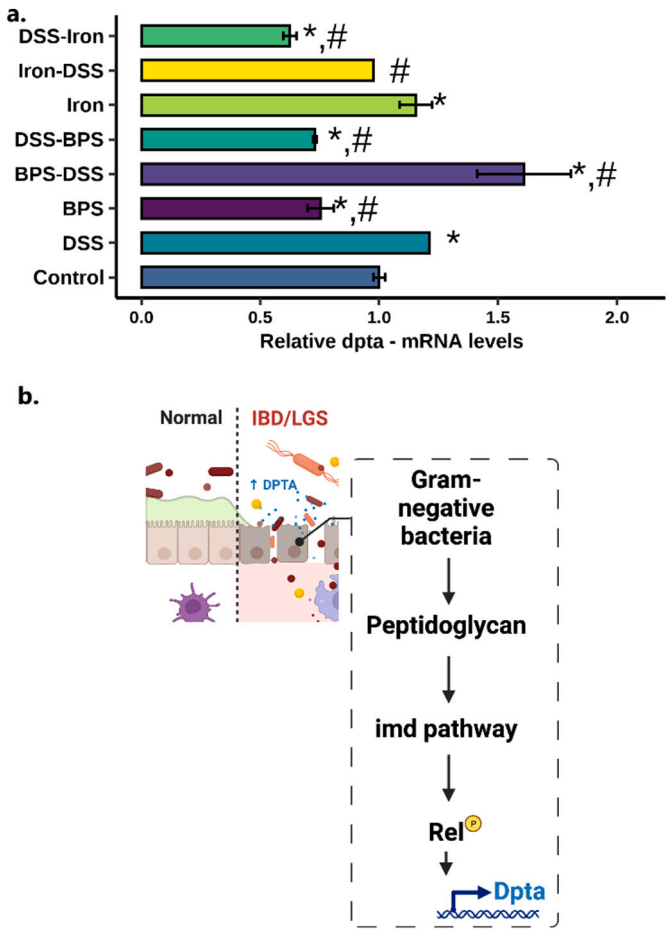


Fig. 6. Effect of treatments on the expression of a gut antimicrobial peptide (a). antimicrobial response to invasion by gram (−ve) bacteria (b). Bars represent mean ± SD. * and # indicate mean values are significantly different at $p \leq 0.05$, compared to control and DSS-inflamed group, respectively (One way ANOVA with Tukey post hoc). For *dpta* gene expression dataset (a), isolated gut RNA from a group of 10 flies represented a unit of analysis. Results represent relative mRNA expression normalized to ActC5 using the ΔΔCt method and refer to 2 independent experiments and 2 technical replicates. b - highlights the typically increased production of diptericin, *dpta* (an antimicrobial peptide) following exposure to gram-negative bacteria in *Drosophila* with inflammatory bowel disease (IBD) i.e., leaky gut syndrome (LGS). The loss of protective physical barriers (peritrophic matrix/mucous layers and epithelial junctions) of IBD allows proximity and entry of gram-negative bacteria. Consequently, peptidoglycans (PGNs) in bacterial walls directly bind and activate transmembrane receptors on epithelial cells. This activation recruits the immune deficiency (Imd) pathway and signals the cleavage of Relish (Rel) into a transcription factor called RHD (N-terminal Rel homology domain). This transcription factor relocates to the nucleus and triggers increased expression of *dpta*. Group 1 (Control group): flies on standard diet for 8 days post-eclosion, Group 2 (DSS group i.e., DSS only): flies on standard diet for 1 day followed by 3 % DSS diet for 7 days, Group 3 (BPS group also called iron-chelated group i.e., BPS only): flies on 200 μM BPS diet for 1 day followed by standard diet for 7 days, Group 4 (BPS-DSS): flies on 200 μM BPS diet for 1 day followed by 3 % DSS diet for 7 days, Group 5 (DSS-BPS): flies on 3 % DSS diet for 7 days followed by 200 μM BPS diet for 1 day, Group 6 (Iron group also called iron-supplemented group i.e., FeSO4 only): flies on 5 mM FeSO4 for 1 day followed by standard diet for 7 days, Group 7 (Iron-DSS): flies on 5 mM FeSO4 for 1 day followed by 3 % DSS diet for 7 days, Group 8 (DSS-Iron): flies on 3 % DSS diet for 7 days followed by 5 mM FeSO4 for 1 day. BPS- Bathophenanthroline disulfonate, DSS-Dextran sodium sulfate, $FeSO_4$-ferrous sulfate. $n = 40$ flies per group.

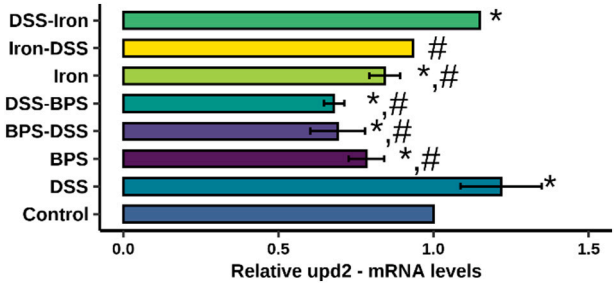


Fig. 7. Effect of treatments on the expression of upd2. Bars represent mean ± SD. * and # indicate mean values are significantly different at $p \leq 0.05$, compared to control and DSS-inflamed group, respectively (One way ANOVA with Tukey post hoc). BPS-Bathophenanthroline disulfonate, DSS-Dextran sodium sulfate, $FeSO_4$-ferrous sulfate. $n = 40$ flies per group. For this gene expression dataset, isolated gut RNA from a group of 10flies represents a unit of analysis. Result represents relative mRNA expression levels for upd2 normalized to ActC5 by the ΔΔCt method and refer to 2 independent experiments and 2 technical replicates. Group 1 (Control group): flies on standard diet for 8 days post-eclosion, Group 2 (DSS group i.e., DSS only): flies on standard diet for 1 day followed by 3 % DSS diet for 7 days, Group 3 (BPS group also called iron-chelated group i.e., BPS only): flies on 200 μM BPS diet for 1 day followed by standard diet for 7 days, Group 4 (BPS-DSS): flies on 200 μM BPS diet for 1 day followed by 3 % DSS diet for 7 days, Group 5 (DSS-BPS): flies on 3 % DSS diet for 7 days followed by 200 μM BPS diet for 1 day, Group 6 (Iron group also called iron-supplemented group i.e., FeSO4 only): flies on 5 mM FeSO4 for 1 day followed by standard diet for 7 days, Group 7 (Iron-DSS): flies on 5 mM FeSO4 for 1 day followed by 3 % DSS diet for 7 days, Group 8 (DSS-Iron): flies on 3 % DSS diet for 7 days followed by 5 mM FeSO4 for 1 day. BPS- Bathophenanthroline disulfonate, DSS-Dextran sodium sulfate, $FeSO_4$-ferrous sulfate. n = 40 flies per group.

Drosophila gut [18]. We thus hypothesised that these findings were due to a loss in ability of the enzyme systems to compensate for oxidative damage following 7 days of DSS possibly due to GSH exhaustion and/or accelerated turnover by the Fenton reaction (Fig. 3d). Notably, iron deposition, GSH exhaustion, GPx inactivation and lipid peroxidation are fundamental hallmarks of ferroptosis. To test this hypothesis, we compared the levels of malondialdehyde (a marker of lipid peroxidation due to oxidative attack) between the control and DSS-inflamed groups (Fig. 3c). Indeed, the malondialdehyde level was significantly greater in the DSS-inflamed flies than in controls. This further confirmed the oxidative effect of DSS. Also, the reduced MDA levels of the DSS-BPS group, clearly demonstrated the ability of BPS to protect gut membranes from damage by inhibiting DSS-induced lipid peroxidation.

Like the DSS-BPS group, our other attempt at curative therapy with iron (i.e., DSS-Iron treatment) also led to increased antioxidant enzyme activity (both catalase and GPx-like activity). But this response was insufficient to prevent the oxidative induction of lipid peroxidation i.e., as shown by high MDA levels (Fig. 3c and d). This insufficiency was perhaps due to GSH exhaustion. Notably, this group of DSS-Iron flies also showed increased expression of the effector caspase for apoptosis (Fig. 5). Based on the H_2O_2 theory behind IBD (reviewed in [74]), such death signals are sufficient to induce damages to the basement membranes, deplete tight junctions and increase barrier permeability [75]. But surprisingly, we observed transcriptional decrease in tight junctional protein (*bbg*) expression, antimicrobial peptide (*dpta*) expression and a mitigated smurf transition in the DSS-Iron group. We attribute these outcomes to be a consequence of aggravated cell death and cell renewal in the fly gut. This was reasoned because DSS-Iron triggered the greatest increase in lipid peroxidation (MDA) and transcription of pro-apoptotic *drice* (cell death factors) even compared to DSS alone, and was able to increase the expression levels of *upd2* (an activator of cell

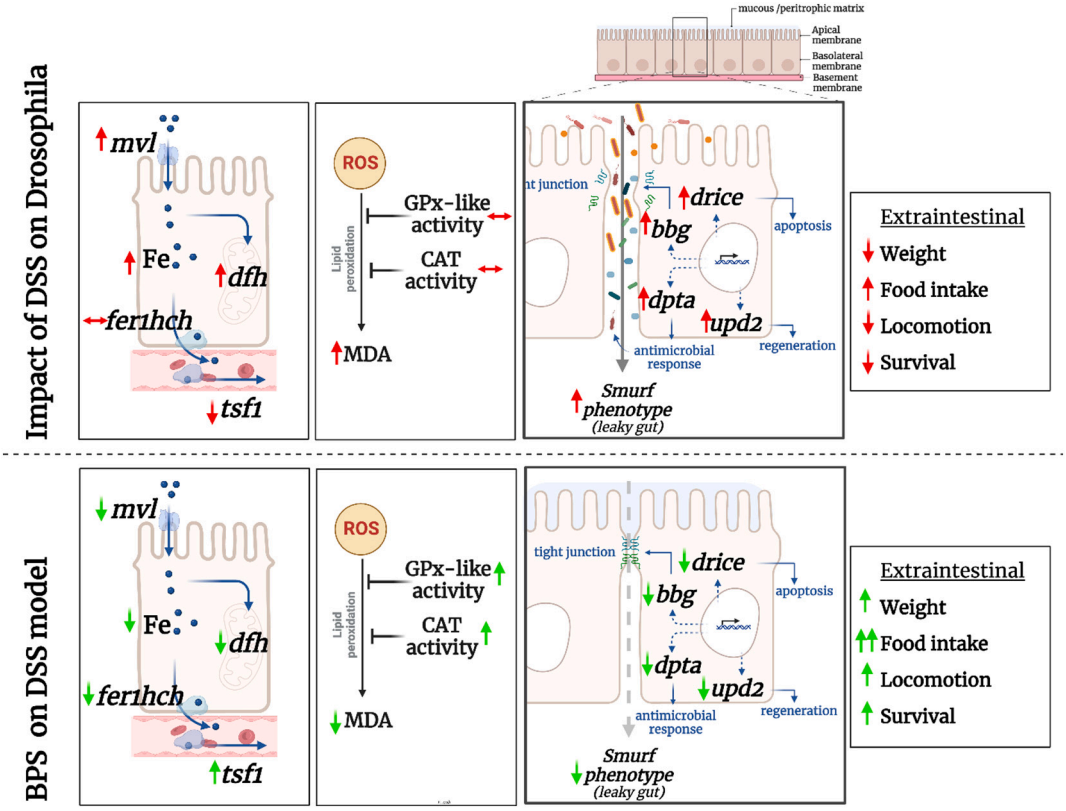


Fig. 8. Mechanistic effects of acute iron chelation using BPS on signatures of DSS-induced leaky gut syndrome in *Drosophila.* Upper half = Group 2 (DSS group i.e., DSS only): flies on standard diet for 1 day followed by 3 % DSS diet for 7 days (Red arrows; ↓ indicates significant decrease ($p > 0.05$) compared to control group, ↑ indicates significant increased ($p < 0.05$) compared to control group, ↔ indicates no significant change ($p < 0.05$) compared to control group). Lower half = Group 5 (DSS-BPS): flies on 3 % DSS diet for 7 days followed by 200 μM BPS diet for 1 day (Green arrows; ↓ indicates significant decrease ($p > 0.05$) compared to DSS group, ↑ indicates significant increased ($p < 0.05$) compared to DSS group, ↔ indicates no significant change ($p < 0.05$) compared to DSS group). bbg, big bang gene; CAT, catalase; dfh, frataxin; dpta, diptericin A; Drice, Drosophila ice like caspase; Fe, iron; fer1hch, ferritin heavy chain 1; GPx, glutathione peroxidase; MDA, malondialdehyde; mvl, Malvolio; ROS, reactive oxygen species; tsf1, transferrin 1; upd2, unpaired 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

renewal expressed in enteroblasts). Upd2 is an IL-6-like cytokine that mediates inflammatory response. Notably, IL-6 is a biomarker that is increased in ulcerative colitis patients [76]. Upd2 is upregulated in response to apoptosis and injury (such as with DSS), and signals through the JAK-STAT pathway to promote intestinal stem cell proliferation and differentiation [46]. More so, the lowered *dpta* expression of this group, could be consequent to the extensive damage and repair, which may prevent macromolecular entry; hence reduced *dpta* levels and perhaps greatly reduced body weight. Further research on the stage of gut damage or repair will prove useful.

Taken together, the most striking results to emerge is that the DSS-BPS was able to completely reverse the effect of DSS on *drice* expression, *bbg* expression and the smurf transition (see Fig. 5). This shows that damage (*drice*, *bbg*) and leakage (smurf, *dpta*) were ameliorated by DSS-BPS. However, we could only show that repair occurred in this group based on comparisons of its *upd2* expression to that of the DSS group. Upd2 transcripts significantly increased with DSS damage and this was significantly brought down by DSS-BPS. This was possibly a decreased need for repair which indirectly demonstrates that BPS can reverse damage or injury. Thus, the transcriptional profile for *drice*, *bbg*, *upd2* and *dpta* highlight BPS as an anti-apoptotic agent – mitigating cell death, a barrier protective factor – preventing gut barrier defect, an anti-inflammatory factor - suppressing the production of a cytokine and the anti-microbial response.

Looking at extra-intestinal impacts, Fig. 2b highlights increased fluid diet intake in all treated groups. The increase following DSS treatments is consistent with previous findings of increased water intake following experimental colitis in rodents [77,78] and may have persisted irrespective of treatment. Previous report also show that iron chelation increases intake [79]. This explains the greatest increase in intake found in flies administered BPS after DSS (group 5). Consistently, this group had the greatest gain in body weight (see Fig. 2a). In contrast, the DSS-Iron group had the greatest loss in body weight.

Fatique is an extra-intestinal, debilitating symptom of IBD that births decreased activity in patients [80]. This is demonstrated by a significant decrease in locomotor performance following DSS exposure (Fig. 2c). This decrease was consistent across all groups except the iron-chelated and the BPS-DSS groups. Possibly, increased body weight may also account for the decreased locomotor performance in the DSS-BPS group (Fig. 2c).

We also discovered a differential response of LGS flies to iron chelation and iron supplementation, depending on the timing of administration – before (preventive) or after (curative). This was particularly evident with iron chelation which, in comparison to the DSS-inflamed group, came in the form of opposing outcomes (*mvl*, *dfh*, MDA, *drice*, *bbg*, *dpta*, fly mass and locomotion) or one sided (maximal or minimal) response (*fer1hch*, *tsf1*, CAT activity, GPx-like activity, smurf, fluid food intake and survival time). Similar alternation in outcomes that exists between the BPS-DSS versus DSS-BPS groups was also found in Iron-DSS versus DSS-Iron groups for certain markers (*mvl*, *tsf1*, iron, MDA, *drice*, smurf, *upd2*, *dpta*, fluid food intake, CAT and GPx-like activity). The cause of this outcome dependency on timing of intervention is unknown. We do, however, speculate that this may be consequent to exposure at a critical window of "developmental programming" (i.e., the 1st day of

adult fly life for the BPS-DSS and Iron-DSS groups). Developmental programming defines the occurrence of adaptive phenotypic changes in later life due to environmental exposure to certain factors, called 'stressors' or 'cues', at critical periods of early life. For many organisms, the most significant critical periods or periods of plasticity are intrauterine and postnatal life [81]. These critical periods are potent windows for developing some targeted studies and possible preventive measures. Programming may be beneficial in the sense of a "predictive adaptive response (PAR)" which appropriately adjusts and primes the subject to cope with future adversity such as nutrient scarcity. However, PAR may also be detrimental when it mismatches with the future condition resulting in stress and disorder. We believe the PAR is present in the preventive groups of BPS or iron (i.e., BPS-DSS and Iron-DSS) because gut maturation isn't complete at the first day post eclosion [55]. Study of hormonal action clearly supports the existence of such early-adult-specific critical period (first 3 days post eclosion) in drosophila life [82]. Regardless, what is clear from our finding is that the effect of iron availability on an inflamed gut is dependent on timing of intervention. This may inform management strategies aimed at tackling IBD recurrence.

In *Drosophila*, iron trafficking from the gut may occur via two major routes, a secretory route and a *tsf1* route. We find that DSS triggers decrease in *tsf1* expression (Fig. 4c). This could lead to increased iron accumulation in the gut as demonstrated by *tsf1* knockdown in *Drosophila* [41]. DSS also increased *dfh* expression (Fig. 4d), which is also indicative of gut cytosolic iron accumulation [70]. This supports the theory that local iron excess accompanies IBD and promotes the oxidative damage as well as growth of specific pathogenic bacteria that require large intake of iron [83–85]. Thus, the phenotypic intestinal damage and gut dysbiosis that accompanies the disease. Notably, we found that DSS upregulates the expression of *dpta*, an antimicrobial peptide, in the fly gut. Such increase in *dpta* expression is consistent with increase in gram-negative aerobes such as *Bacteroidaceae* and *Clostridium* spp. that are peculiar to colitis [17,86,87]. This increase also occurs with iron supplementation and is expected given that iron is an essential micronutrient for the growth of most pathogenic bacteria [88]. However, when DSS-inflamed flies where subjected to 200 μM BPS (DSS-BPS), there was a significant decrease. This was indicative of the antimicrobial effects of BPS on the inflamed gut.

Comparing BPS and iron groups, we observed increased and decreased iron levels, respectively. It was unusual to find iron levels decreasing after iron supplementation and then increased after iron chelation. This we speculate to be due to developmental programming because both interventions were administered only for 1 day after eclosion after which flies returned to control diet. The PAR may be occurring as a result of exposure during this critical period of early life (1st day after eclosion). Alternatively, it is possible that early iron loading (an essential micronutrient for the growth of most pathogenic bacteria) may have induced the growth of bacteria that compete for iron. This is supported by the upregulated expression of *dpta* in this group; an antimicrobial peptide that is consistent with increased microbes. Notably, DSS increased iron levels but DSS-BPS was able to restore the whole fly iron levels to that of controls. This was indicative that BPS treatment restores iron homeostasis in the body of flies fed DSS.

Finally, comparing BPS-DSS to DSS-BPS, we find that the DSS-BPS results in 14 beneficial outcomes (*mvl*, *dfh*, *tsf1*, iron, CAT, GPx-like activity, MDA, *drice*, *bbg*, smurf, *dpta*, *upd2*, fly mass and survival) while the BPS-DSS elicits only 6 (*tsf1*, iron, GPx-like activity, smurf, *upd2* and survival). On the other hand, both iron-Iron-DSS and DSS-Iron treatments result in 8 beneficial outcomes (Iron-DSS; *mvl*, *dfh*, *tsf1*, *drice*, *bbg*, smurf, *dpta* and survival. DSS-Iron*dfh*, *tsf1*, CAT, GPx-like activity, *bbg*, smurf, *dpta* and survival).

Overall, our data highlights the beneficial effect of DSS-BPS on DSS induced gut inflammation. The significance of responses identified in this study for the prevention or treatment of IBD need to be further examined in higher mammals that are closer biological representatives to humans. Secondly, although our findings suggest that BPS can ameliorate damage (*drice*, *bbg*) and leakage (smurf, *dpta*) induced by DSS, we were only able to demonstrate regeneration occurred based on comparisons of upd2 expression. This indirect indicator was not sufficient to examine cell renewal and differentiation. Further research is needed to determine the stage of gut damage or repair. Also, the gut microbiome is seeded from birth and can be altered by environmental exposure. Its presence and diversity is vital for immune surveillance and is strongly affected in IBD and by iron availability. Though not covered in this study, it will be interesting to elucidate the influence microbial presence and diversity has on and how it is influenced by iron intake in IBD.

5. Summary and conclusion

In this study, a Drosophila model of IBD was developed using DSS and iron chelation and supplementation were used to determine the most effective curative or preventive measure. The results showed that the curative use of iron chelation reversed the impact of DSS on 14 parameters (*mvl*, *dfh*, *tsf1*, iron, CAT, GPx, MDA, *drice*, *bbg*, smurf, *dpta*, *upd2*, fly mass and survival) while the preventive use of iron chelation countered the impact of DSS on 6 parameters (*tsf1*, iron, GPx, smurf, *upd2* and survival). On the other hand, the curative use of iron supplementation was able to reverse the effect of DSS on 8 parameters (*mvl*, *dfh*, *tsf1*, *drice*, *bbg*, smurf, *dpta* and survival) while the preventive use of iron supplementation countered the impact of DSS on 8 parameters (*dfh*, *tsf1*, CAT, GPx, *bbg*, smurf, *dpta* and survival). In aggregate, these results demonstrate that iron chelation confers the most beneficial antioxidative, anti-microbial, anti-inflammatory and iron homeostatic effects on experimental colitis in fruit flies. We also discover differential outcomes in the responses of LGS flies to preventive versus curative treatments. It would be interesting to examine these impacts of iron-rich or -low diets using mammals with experimental colitis. These findings may offer better understanding of the functional effects of iron availability during IBD and may pave the way toward more precise therapies.

Funding

This study was supported by the Centre for Advanced Medical Research and Training (CAMRET), Usmanu Danfodiyo University Sokoto, Nigeria; as part of its postgraduate scholarship to D.U [CAMRET/2019/MSc/SCH003]. D.U is also a beneficiary of Grant-In-Aid of Research (GIAR) from Sigma Xi [G03152021120259462].

Data statement

Data is available upon request from the corresponding author.

CRediT authorship contribution statement

Dawoud Usman: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Murtala Bello Abubakar:** Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. **Kasimu Ghandi Ibrahim:** Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Mustapha Umar Imam:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None.

Acknowledgements

The authors would like to thank H. Badmos (Cagans lab, Glasgow, UK) and the biomedical science research and training centre (BioRTC, Yobe) for technical expertise related to *Drosophila* dissection. We thank B. Abubakar (Pharmacology and Toxicology, UDUS) for providing the DSS for the study at no cost. We appreciate all members of the CAMRET postgraduate forum for valuable discussions, insights and continued support throughout the duration of this project. We also thank the anonymous reviewers for critical reading of the manuscript. All authors have approved the submission of this manuscript and declared no conflict of interest.

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