

# Caffeic Acid Alleviates Chronic Sleep Deprivation-Induced Intestinal Damage by Inhibiting the IMD Pathway in *Drosophila*

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**Background:** Sleep is vital for maintaining the health of the organism. Chronic sleep deprivation (CSD) is a key contributor to significant health risks, including the induction of gastrointestinal disorders. However, the mechanism of CSD caused intestinal damage remains unclear.

**Methods:** *Drosophila melanogaster* as an in vivo model was used to investigate the mechanism of CSD-induced intestinal injury, as well as the ameliorative effect of caffeic acid.

**Results:** CSD resulted in reduced survival and severely affected intestinal homeostasis in flies, as evidenced by disruption of intestinal acid-base homeostasis, increased feeding, increased intestinal permeability and shortened intestinal length. Meanwhile, the expressions of the immune deficiency (IMD) pathway-related genes *PGRP-SBI*, *Dpt*, *AttA*, *AttB* and *Mtk* were significantly up-regulated in the intestine of CSD flies. On the other hand, Caffeic acid supplementation restored intestinal acid-base homeostasis and intake, while improving intestinal barrier permeability and intestinal length, and effectively reducing intestinal damage. In addition, administration of caffeic acid decreased the expressions of *PGRP-SBI*, *Dpt*, *AttA* and *Mtk* genes in the CSD flies gut.

**Discussion:** These results suggested that CSD could disrupt gut homeostasis in adult flies by overactivating the IMD pathway, while Caffeic acid has an obvious protective role on the gut homeostasis.

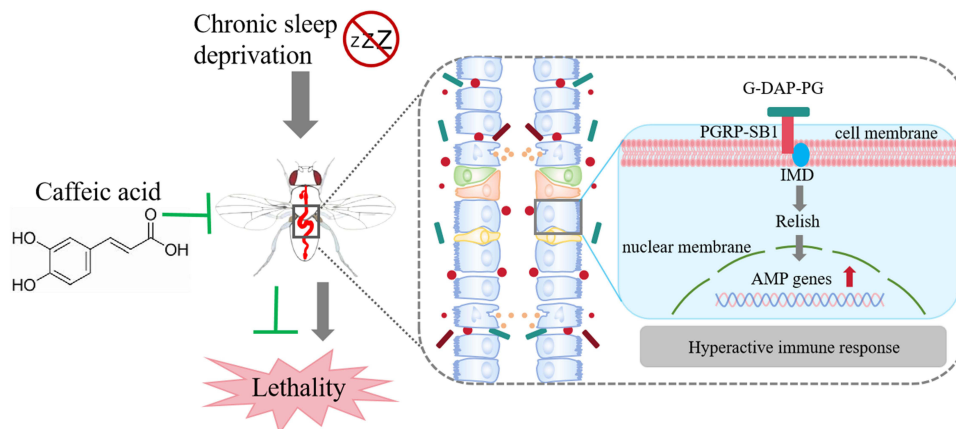
**Keywords:** thermogenetic CSD, intestine, Immune signaling, polyphenolic compounds, *Drosophila melanogaster*

## Introduction

As people's lifestyles change, sleep time has severely decreased, with nearly one-third of adults sleep less than 6 h per day.<sup>1</sup> Chronic sleep deprivation (CSD) has a serious impact on human health and leads to various diseases, such as heart disease, diabetes, and cancer, even leading to death.<sup>2,3</sup> Multiple studies have proved that the intestinal damage caused by CSD is the major factor for premature death.<sup>4-6</sup> However, the molecular mechanism of CSD induced intestinal damage is still unclear. Therefore, it is necessary to dissect the mechanism of intestinal damage caused by CSD and find the effective therapeutic drugs.

CSD can cause serious intestinal damage, such as loss of intestinal mucosal integrity and the occurrence of intestinal inflammation.<sup>7,8</sup> Current research has found that oxidative stress, inflammatory response, and imbalance of gut microbiota are the major causes of intestinal damage caused by CSD.<sup>9,10</sup> After sleep deprivation, the levels of antioxidant enzymes (GSH-PX, SOD, and CAT) in the intestine are down regulated, the expression of inflammatory pathway NF-κB is up-regulated, and the diversity and abundance of gut microbiota are significantly decreased.<sup>6,11</sup> Notably, innate immunity as the first line of defense is crucial for maintaining intestinal homeostasis.<sup>12,13</sup> Immune deficiency (IMD)

## Graphical Abstract



and Toll signaling pathways, two highly conserved recognition and signaling cascades, control the innate immune response in flies.<sup>14</sup> When the intestinal homeostasis is imbalanced, the intestinal pathway and Toll pathway are activated, and increase the proliferation of intestinal stem cells, promoting the regeneration of flies intestinal epithelium and enhancing the systemic immune response.<sup>15,16</sup> Conversely, there is an up-regulation of IMD pathway-associated AMP genes expression in the intestine of aged flies, leading to intestinal homeostasis imbalance and a reduction in fruit fly lifespan.<sup>17</sup> However, the precise contribution of innate immunity to intestinal injury induced by CSD remains elusive.

*Drosophila melanogaster* serves as an exemplary model organism for investigating the intricate interplay among gut injury, sleep and health. The major signaling pathways associated with immunity and tissue regeneration are conserved in both flies and humans. Moreover, flies and humans share strikingly similar sleep patterns governed by their endogenous circadian clocks.<sup>18,19</sup> Although previous studies have demonstrated that oxidative damage led to the gut hastens premature mortality in CSD flies, the underlying molecular mechanism linking gut injury with sleep deprivation remains largely unexplored.<sup>4</sup>

There is a relative dearth of research on treatment strategies for intestinal injury induced by CSD. Currently, antioxidants such as ebselen, glutathione, and methylene blue have been proven to be ineffective in rescuing the survival rate in CSD flies.<sup>4</sup> In recent years, natural small molecules have garnered increasing attention due to their efficacy and low toxicity, exhibiting potential for treating various intestinal disorders.<sup>20–22</sup> Caffeic acid is a polyphenolic compound with notable impact on diverse diseases, particularly in the context of intestinal ailments like ulcerative colitis and colon cancer treatment.<sup>23–25</sup> Specifically, caffeic acid inhibits NF- $\kappa$ B pathway and chemokine biosynthesis, attenuating mucin content and restoring gut microbiota diversity in murine models of colitis, ultimately modulating intestinal inflammation and lesions.<sup>26,27</sup> Additionally, caffeic acid inhibits the proliferation of HCT 15 colon cancer cells, induces apoptosis in these cells, and prevents metastasis by targeting MEK1 and TOPK.<sup>28,29</sup> Nevertheless, the preventive and therapeutic effects of caffeic acid on sleep deprivation-induced intestinal damage remain elusive.

In this study, we investigated the underlying mechanism of intestinal injury induced by CSD and explored the therapeutic effect of caffeic acid using adult fly as a model organism. The results demonstrated that CSD led to excessive activation of the intestinal IMD pathway, while highlighting caffeic acid as a potential intervention for mitigating such injuries through inhibition of the IMD pathway. In summary, our study offers novel insights into the mechanisms and therapeutic strategies about intestinal injury induced by CSD.

## Materials And Methods

### Fly Stocks and Husbandry

The following flies<sup>7</sup> were obtained from the Bloomington *Drosophila* stock center (BDSC; Indiana, USA): *w<sup>1118</sup>* (#5905); *60D04-Gal4* (#45356); *11H05-Gal4* (#45016); *UAS-TrpA1* (#26263). *w<sup>1118</sup>* represents the genetic background and is often used

as the appropriate controls. *11H05*- and *60D04-Gal4* target neurons in the brain whose activation leads to loss of sleep in flies. Heat-activated cation channel TrpA1 activates neural circuits in flies under temperature control. Flies were raised at 25 °C, 60% humidity under a 12h light/12h dark cycle unless otherwise stated. Flies were maintained on a standard cornmeal-agar medium.

## Sleep Deprivation

Flies genotypes *11H05>TrpA1* or *60D04>TrpA1* were obtained by crossing *11H05-Gal4* flies or *60D04-Gal4* flies with *UAS-TrpA1* flies using the UAS/Gal4 system and cultured at 18°C. After eclosion, male flies were raised at multi-functional artificial climate chamber at a temperature of 29°C, 12:12 LD for 10 d.

Mechanical sleep deprivation was performed at 25°C using a multifunctional oscillator. After eclosion, *w<sup>1118</sup>* flies were reared at room temperature for 2 d, then flies were placed into tubes containing standard food and fixed between shock-absorbing sponge plates. The multifunctional oscillator speed was 1100 rpm and delivered 10s-long vibrations at random intervals centered around 180s ( $\pm 30$ s) ([Supplementary Figure 1](#)). Relevant tests were performed after 10 d of sleep deprivation.

## Drug Treatment

All chemicals used are analytical grade. Caffeic acid was purchased from Shanghai Yuanye Biotechnology Co., Ltd, Shanghai, China, and purity levels exceeding 98%. Caffeic acid was dissolved in anhydrous ethanol for 20 mm and then was diluted in the standard food to achieve final concentrations of 0.2 mm for further experiments.

## Sleep Monitoring

Adult male flies were individually inserted into 5×65 mm glass tubes, loaded into the *Drosophila* Activity Monitoring Systems (DAMS, Wuhan Yi Hong Technology Co., Ltd., China), and housed under 12:12 h light/dark cycle conditions.<sup>30</sup> Flies were kept in this condition for 5 d, without sleep analysis for the first 2 d that allowed flies to acclimatize to the environmental conditions. Data were analyzed using ShinyR-DAM (<https://karolcichewicz.shinyapps.io/shinyr-dam/>) software to count sleep-wake cycle, total sleep time, daytime sleep time, nighttime sleep time, sleep bout number, sleep bout length, and sleep latency. For each genotype, a minimum of 15 males were tested.

## Lifespan Assay

Adult (2–4 days-old) male and female flies were reared on food with or without 0.2 mm caffeic acid. Fresh food was changed every three days and the number of deaths was recorded daily until all flies died. Three independent replicates were set up for each group with a minimum of 3 bottles per replicate.

## Bromophenol Blue Treatment

The acid–base homeostasis was observed using the bromophenol blue assay as previously described,<sup>31</sup> 200  $\mu$ L of 2% Bromophenol blue sodium was added to a vial containing 1% agar medium, and several small holes were made in the surface of the food with a pipette to ensure adequate absorption. All flies were starved for 6 h followed by feeding of pH indicator Bromophenol Blue (Sigma, B5525) for 4 h. Images were taken immediately after gut dissection. The type of the GI tract of flies fed with Bromophenol blue include ‘Homeostasis’ (CCR area: yellow), CCR is acidic and the rest of the gut is alkaline; ‘Perturbed A’ (CCR area: blue), the acidic region is lost and the whole gut is basic; ‘Perturbed B’ (CCR area: light blue), the strongly acidic region is lost and the rest of the gut becomes less alkaline.<sup>32</sup>

## Food Intake Assay

Flies were starved for 18 h and then transferred to staining medium for 3 h. The staining medium was composed of 2% sodium bromophenol blue, 5% sucrose and 1% agarose. Fly abdominal color was observed under a light microscope and scored.<sup>30</sup> Abdominal score was recorded from 0 to 3, in which 0 (colorless abdomen), 1 (less than 1/3 the length of the abdomen), 2 (between 1/3 and 2/3 the length of the abdomen) and 3 (more than 2/3 the length of the abdomen). The experiment was repeated independently at least 3 times.

## Excretion Measurement

The fly excretion measurement was performed as previous study.<sup>31</sup> In brief, after starvation for 3 h, flies were placed in 2 mL centrifuge tube with small holes in tube wall and dyed medium in tube cover for 18 h. The dyed medium was composed of 5% sucrose, 2% Bromophenol blue sodium, and 1% agarose. The centrifuge tubes were then washed with phosphate buffered saline (PBS). Afterwards, the excretion was determined by measuring the absorbance at 625 nm using an enzyme meter. Three replicates were established for each group.

## “Smurf” Assay

Smurf assay was performed as previously described.<sup>33</sup> Briefly, flies were fed with or without 0.2 mm caffeic acid for 10 d. After starved for 3 h, flies were incubated on staining medium for 18 h. The dyed medium consisted of 2% Bromophenol blue sodium, 5% sucrose and 1% agar. Smurf flies with the blue colour visible outside the digestive tract were counted. Three biological replicates were used for the experiment.

## Intestinal Length

Guts were dissected in cold PBS, and length of the intestines were observed under a light microscope. Length measurement tool is vernier caliper. The experiment was repeated independently more than 10 times.

## RNA Sequencing and Analysis

Total RNA was extracted from tissues using TRIzol reagent according to the instructions. RNA purity was characterized and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Transcriptome libraries were constructed using the VAHTS Universal V5 RNA-seq Library Prep kit according to the instructions. Transcriptome sequencing and analysis were performed by Shanghai Ouyi Biotechnology Co.

## Quantitative RT-PCR Analysis

Intestines were dissected in pre-cooled PBS, and total RNA was extracted from tissues dissected from 60 male or female flies using TRIzol (Invitrogen).<sup>30</sup> Total RNA was processed by Hieff® reverse transcriptase (Shanghai YEASEN, China) to synthesize DNA (cDNA). Quantitative PCR was performed using the GFX 96 Connect™ Optics Module (Bio-Rad Laboratories) following the instructions of the Multiplex PCR Master Mix (Shanghai YEASEN, China). The relative expression of genes was calculated using the  $2^{-\Delta\Delta Ct}$  method and normalized to the relative expression of the house-keeping gene *Rp49*. The experiment was repeated independently at least three times. All primers are shown in Table 1.

## Statistical Analysis

Statistical analysis was performed using Prism 9.3.0 (GraphPad software). Three or more experimental groups were analyzed using one-way ANOVA followed by Tukey’s multiple-comparison test. Differences between groups were tested using unpaired two-tailed Student’s *t*-test and Fisher’s exact test. The Log rank test was used to determine the statistical significance of survival rates between groups. The significance level was indicated as \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001.

**Table 1** List of the Primer Sequences Used in the Gene Expression Analysis

Gene	Forward	Reverse
<i>PGRP-SB1</i>	CCGCAATTCAGCGATATTGG	GGGAGTTGATCCCTGGAG
<i>AttA</i>	ACAAGCATCCTAATCGTGCC	TCAGATCCAAACGAGCATCAG
<i>AttB</i>	GGGTAATATTTAACCGAAGT	GTGCTAATCTCTGGTCATC
<i>Dpt</i>	GCTGCGCAATCGCTTCTACT	TGGTGGAGTGGGCTTCATG
<i>Mtk</i>	GCAACTTAATCTTGGAGCGA	CGGTCTTGGTTGGTTAGGAT
<i>Rp49</i>	CTTCATCCGCCACCAGTC	GCACCAGGAAGCTTCTTGAATC

## Results

### Thermogenetic Sleep Deprivation Leads to Intestinal Homeostasis Imbalance in Adult Flies

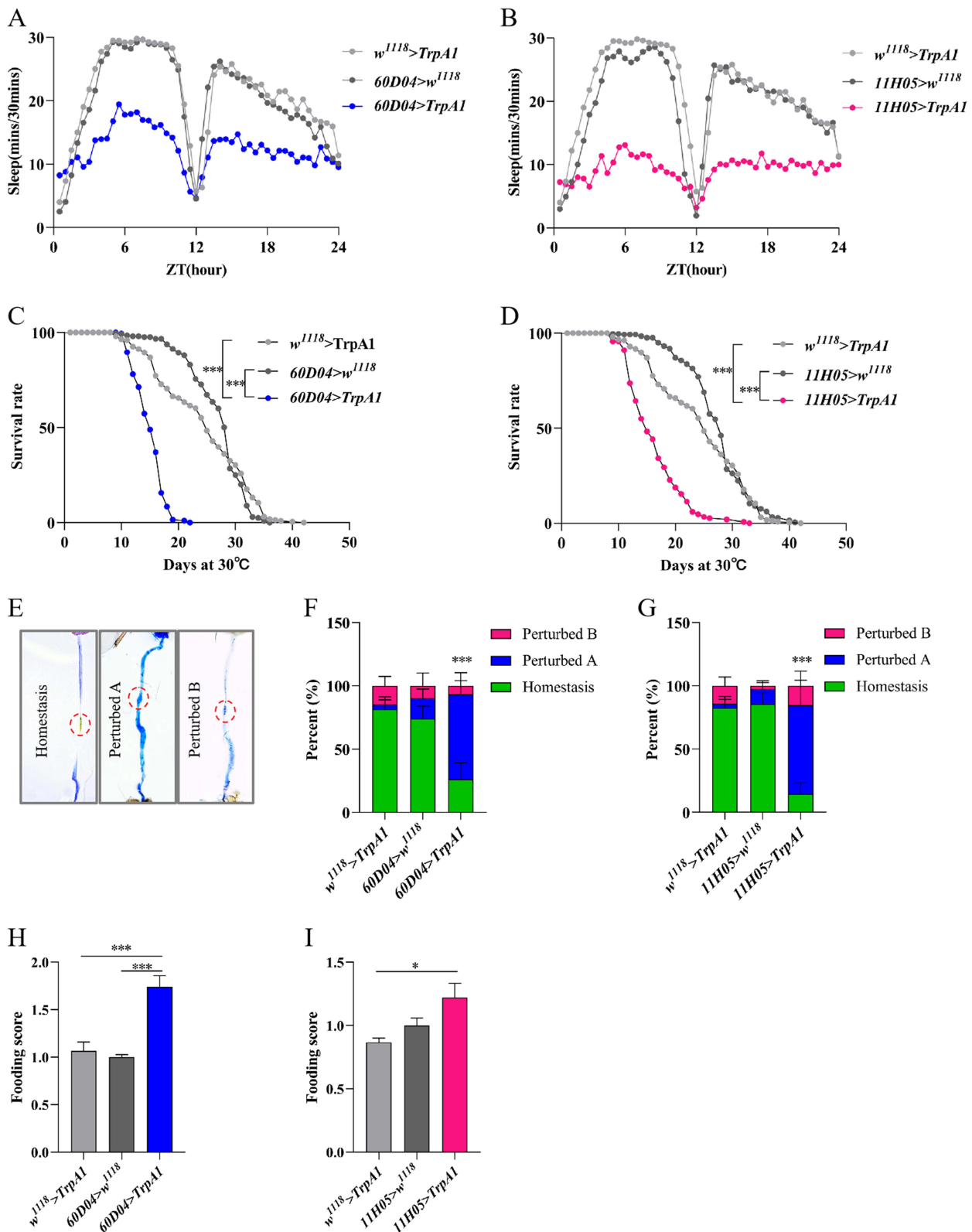
CSD have severe detrimental effects on the body, potentially leading to mortality.<sup>4</sup> To examine the effect of CSD on the gut, we used two specific genotypes *60D04>TrpA1* and *11H05>TrpA1* as SD model. The results showed that sleep was disrupted in both thermogenetic SD flies compared to the control flies (Figure 1A and B). Total sleep time, day sleep time, night sleep time, and sleep bout length in both thermogenetic SD flies at day 10 from continuous CSD were significantly decreased compared to control flies (Supplementary Figure S2A–H), while thermogenetic SD flies had increased sleep bout number and sleep latency (Supplementary Figure S2I–L). In addition, thermogenetic SD flies had considerably reduced survival rate compared to control flies, in which a much greater death rate was discovered when thermogenetic SD flies were cultured in 29°C environment after 10 d (Figure 1C and D).<sup>4</sup> Therefore, the present study analyzed the intestinal homeostasis of flies at day 10 from continuous CSD. The gut tract in control flies exhibited acid-base homeostasis and maintained the integrity of the intestinal barrier to ensure proper physiological functioning.<sup>34,35</sup> Thermogenetic CSD flies showed dramatically impaired intestinal digestive function as demonstrated by disruption of intestinal acid-base balance (Figure 1E–G) and increased food intake (Figure 1H and I). Nevertheless, thermogenetic CSD flies had the similar excretion compared to the control flies (Supplementary Figure S3A and B). An analysis of the intestinal length of flies also revealed no significant difference in intestinal length (Supplementary Figure S3C and D). Overall, thermogenetic CSD could impair gut homeostasis, resulting in premature death.

### Mechanical Sleep Deprivation Causes Intestinal Damage in Adult Flies

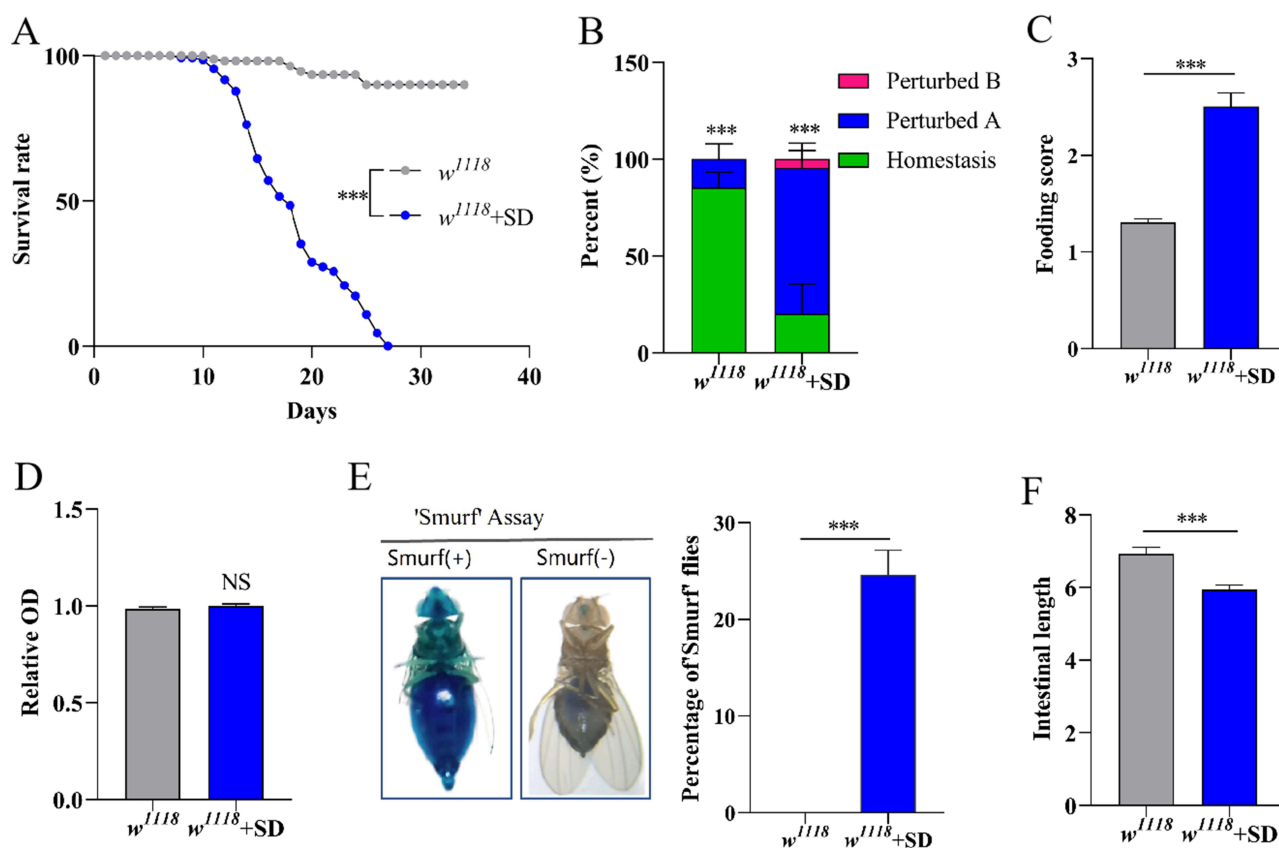
The sleep patterns of flies can be adversely affected by several environmental stimuli, such as vibration, sound, and light.<sup>36</sup> Furthermore, mechanical shocks produced by a mechanical sleep deprivation apparatus were used to induce sleep deprivation in flies. Mechanical CSD was also found to dramatically diminish survival time in flies (Figure 2A). Mechanical CSD flies had disrupted intestinal acid-base homeostasis (Figure 2B) and increased food intake (Figure 2C) compared to control flies, but without impairing excretory function (Figure 2D). The integrity of the intestinal barrier was disrupted in adult male flies after mechanical CSD stimulation (Figure 2E). A considerable shorten of gut length in mechanical CSD flies was found compared to control flies (Figure 2F). Therefore, these results indicated that both forms of CSD could negatively affect gut function.

### Analysis of CSD-Induced Genetic Changes in Flies

To determine the biological processes of CSD-induced intestinal damage, we performed RNA sequencing (RNA-seq) in the thermogenetic genotype *60D04>TrpA1* flies, and two control *w<sup>1118</sup>>TrpA1* and *60D04>w<sup>1118</sup>* flies. Principal component analysis (PCA) showed that the principal components of three samples drove the differences among the samples, and all samples exhibited strong unique clustering in genotypes (Figure 3A). Next, the overlapping differential expressed genes were analyzed in these two pairwise comparisons (1) *60D04>TrpA1* vs *w<sup>1118</sup>>TrpA1*; (2) *60D04>TrpA1* vs *60D04>w<sup>1118</sup>*. Compared to both control group flies, *60D04>TrpA1* flies had 31 up-regulated genes and 66 down-regulated genes (Figure 3B). Functional gene ontology (GO) analysis on the differential expressed genes revealed that various biological processes significantly altered in thermogenetic CSD flies. GO analysis showed a clear distinction between terms enriched in up-regulated gene categories (innate immunity, defense against bacteria, and humoral immunity) and down-regulated gene categories (metabolism and proteolysis) specific profiles (Figure 3C). The differential expression of genes was examined by the enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (Figure 3D). The most focused projects were mainly related to Toll and Imd signaling pathways, metabolic pathways, Hippo signaling pathways, and autophagy signaling pathways. Thus, the identified differential genes were mainly enriched in immune, apoptosis, and autophagy-related items.



**Figure 1** Thermogenetic CSD causes a decrease in intestinal function in flies. **(A)** Sleep traces in *60D04>TrpA1* flies and the control flies during a 24-h day (n=18-21). **(B)** Sleep traces in *11H05>TrpA1* flies and the control flies (n=21). **(C)** Survival curve of *60D04>TrpA1* flies and controls flies at 29°C (n=220-240). **(D)** Survival curve of *11H05>TrpA1* and controls flies at 29°C (n=220-240). **(E)** Representative images of intestinal acid-base homeostasis. **(F and G)** Quantification of the percentage of intestinal acid-base homeostasis of the flies (n=27-36). The copper cells region(CCR) is indicated by a circle. **(H and I)** Food intake of different genetic flies (n=24-30). Data are presented as means±S.E.M. \*p< 0.05 and \*\*\*p< 0.001.



**Figure 2** Mechanical CSD disrupts gut homeostasis in flies. **(A)** Survival rate of flies with mechanical CSD (n=110-120). **(B)** Quantification of the percentage of gut acid-base homeostasis of flies (n=27-36). **(C)** Food intake of flies (n=24-30). **(D)** Determination of excretion in flies (n=21-30). **(E)** Proportion of "Smurfs" in flies (n=21-30). **(F)** The relative length of the gut (n=15-20). \*\*\* $p < 0.001$  indicates significant difference between  $w^{1118}$  and  $w^{1118}+SD$ .

## CSD Activates the IMD Signaling in the Intestine

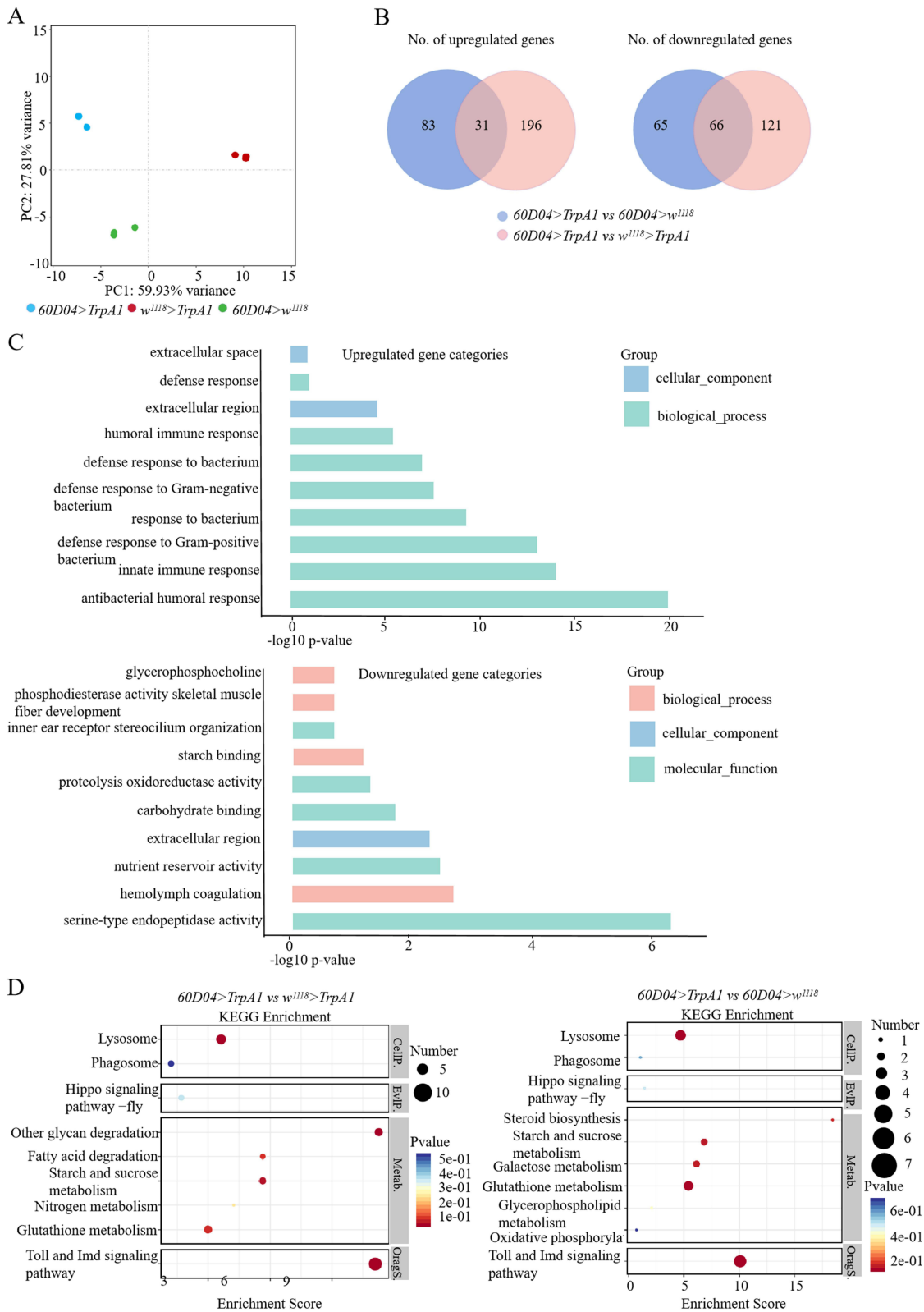
To explore the effect of CSD on the innate immunity, the transcript levels of IMD pathway-related genes *PGRP-SB1*, *AttA*, *AttB*, *Dpt* and *Mtk* were analyzed. CSD resulted in significantly up-regulated expression of these genes based on the transcriptome result (Figure 4A). To further confirm whether CSD could activate the IMD pathway, qRT-PCR was utilized to investigate the expression of these genes. The expression levels of these immune genes were dramatically enhanced in the intestine of thermogenetic CSD flies (Figure 4B and C). Therefore, IMD pathway in the intestine could be activated by CSD.

## Caffeic Acid Rescues Mechanical CSD-Induced Death and Intestinal Damage in Adult Flies

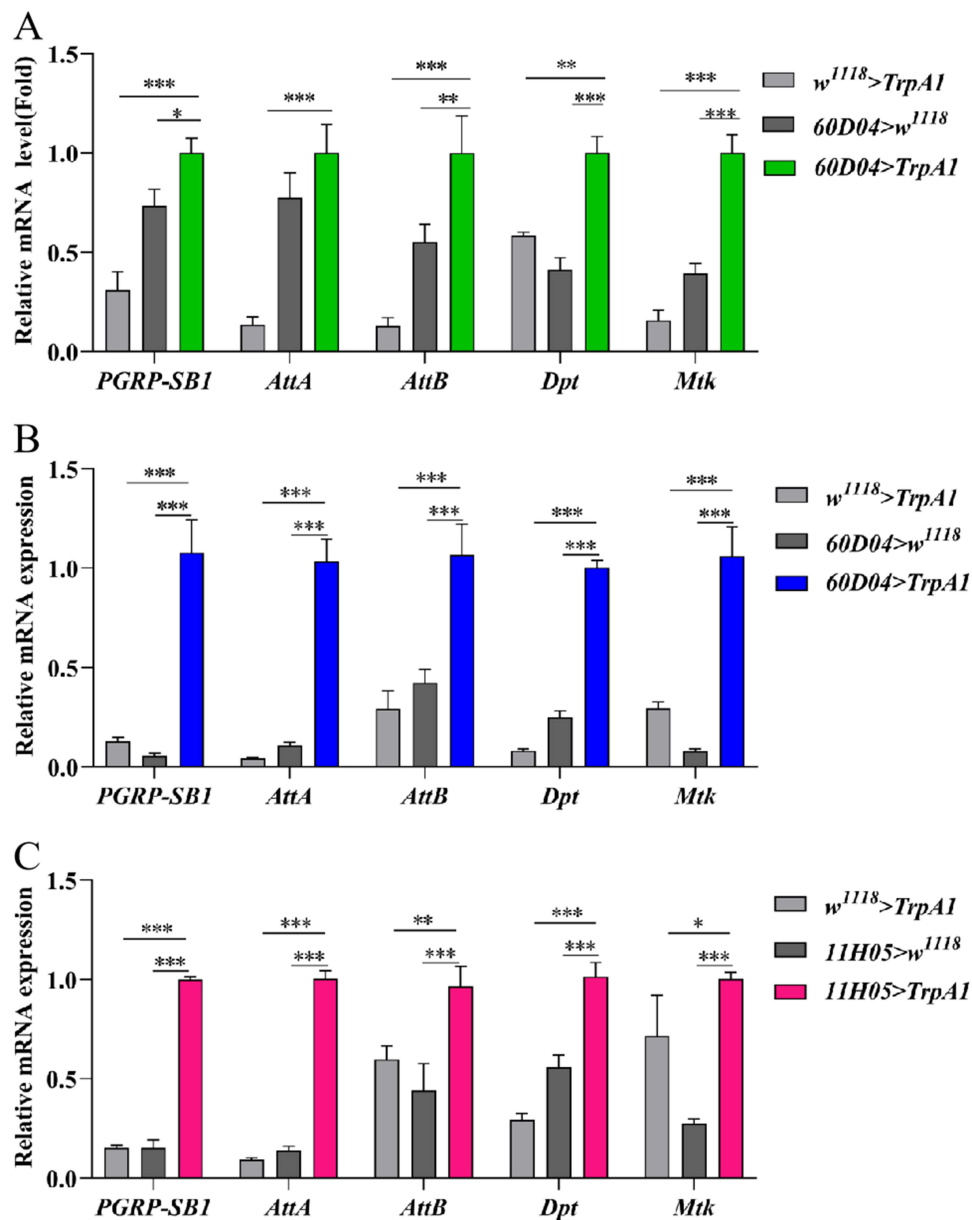
Next, we determined the protective role of caffeic acid against mechanical CSD-induced injury. Administration of 0.2 mm caffeic acid remarkably extended the survival rate of mechanical SD flies (Figure 5A). Meanwhile, caffeic acid supplementation could rescue the intestinal acid-base homeostasis and reduce the food consumption in mechanical CSD flies (Figure 5B and C). In addition, the disrupted intestinal barrier was significantly improved in mechanical CSD flies (Figure 5D). Overall, these results demonstrated that caffeic acid could increase the survival duration and alleviate the disrupted intestine function caused by mechanical CSD.

## Caffeic Acid Alleviates Thermogenetic CSD Induced Intestinal Damage via the Inhibition of the IMD Pathway

Flies survival and intestinal function were evaluated in thermogenetic CSD flies after feeding 0.2 mm caffeic acid. Administration of caffeic acid significantly extended the survival rate of the thermogenetic CSD flies (Figure 6A and B).

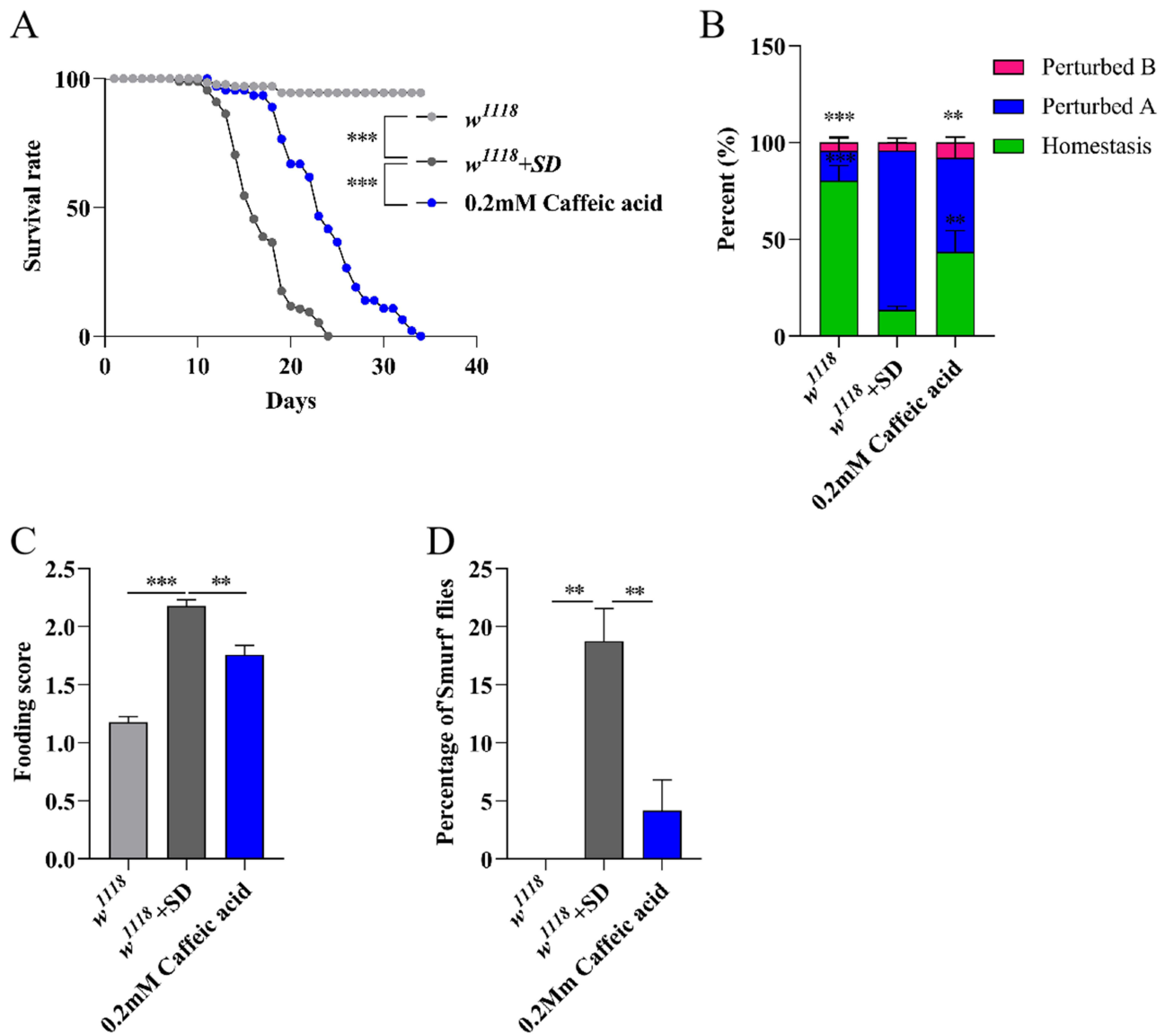


**Figure 3** Thermogenetic CSD causes upregulation of immune response-related genes. **(A)** Principal component analysis (PCA) plot showing the variance among the genotypes used in RNA-seq. **(B)** Venn diagram showing the overlap of differentially regulated genes in  $60D04 > TrpA1$  versus control  $w^{1118} > TrpA1$ ,  $60D04 > TrpA1$  versus control  $60D04 > w^{1118}$  in RNA-seq analysis. The numbers of up- and down-regulated genes shown in the Venn diagrams are based on >1.5 fold-change. **(C)** GO analysis of biological processes of up-regulated (up) and down-regulated (down) genes based on their  $-\log_{10}$  of FDR. **(D)** KEGG enrichment analysis of differentially regulated genes in RNA-seq analysis of  $60D04 > TrpA1$  versus control  $w^{1118} > TrpA1$ ,  $60D04 > TrpA1$  versus control  $60D04 > w^{1118}$ .



**Figure 4** CSD activates the intestinal IMD signaling pathway. **(A)** Gene expression of innate immune-related genes in thermogenetic CSD flies (n=3). **(B and C)** mRNA levels of IMD pathway-related genes in thermogenetic CSD flies (n=3). Data are presented as means±S.E.M. \**p*< 0.05, \*\**p*< 0.01, \*\*\**p*< 0.001.

Meanwhile, in both thermogenetic CSD flies, caffeic acid supplementation remarkably rescued the disrupted intestinal acid-base balance (Figure 6C and D). Additionally, it was seen that the food intake was also reduced in thermogenetic CSD flies after fed with 0.2 mm caffeic acid (Figure 6E and F). These results further indicated that caffeic acid could alleviate CSD-induced intestinal injury. Next, we detected whether caffeic acid could regulate IMD signaling pathway in the intestine of CSD flies. The results showed that the expressions of IMD pathway-related genes *PGRP-SB1*, *Dpt*, *AttA*, *AttB* and *Mtk* were significantly reduced in the intestine of thermogenetic CSD flies after fed with 0.2 mm caffeic acid for 10 d (Figure 6G). Therefore, these findings suggested that caffeic acid has ability to ameliorate CSD-induced intestinal damage by reducing IMD pathway overactivation.

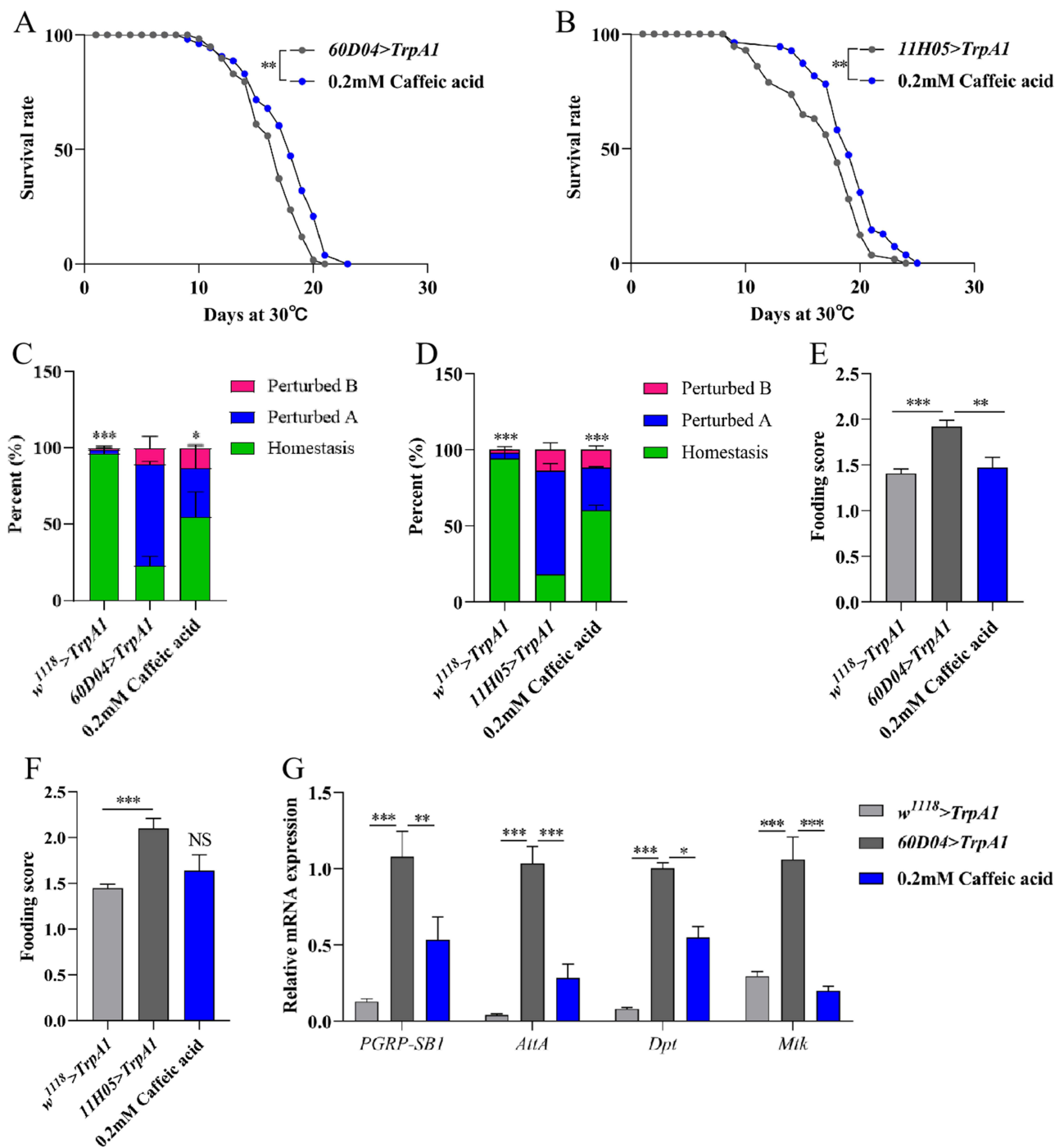


**Figure 5** Caffeic acid prolongs survival time and protects intestinal homeostasis in mechanically CSD flies. **(A)** caffeic acid improves survival of flies with mechanical CSD (n=110-120). **(B)** Caffeic acid restores acid-base homeostasis in the intestinal tract of flies with mechanical CSD (n=27-36). **(C)** Reduced food intake in mechanical CSD flies after fed with caffeic acid (n=24-30). **(D)** Mechanical CSD flies fed with caffeic acid reduce proportion of “Smurf” flies (n=21-30). Compared to *w<sup>1118</sup>+SD*, \*\**p*< 0.01 and \*\*\**p*< 0.001 indicate significant difference.

## Discussion

In recent years, there are a lot of discussion regarding how CSD affects the gut. Increased oxidative stress and catabolism in the gut have been documented in rodent models and clinical trials.<sup>4,37</sup> However, the complicated link between sleep and gut immunity is still largely unknown. To investigate the mechanism of CSD damage in the intestinal tract and to clarify the potential protective effect of caffeic acid, this study utilized adult flies as a model organism for experimental evaluation. Here, we found that CSD could damage gut homeostasis and intestinal function in flies. Furthermore, IMD signaling pathway in the gut was overactivated during the course of CSD. Finally, caffeic acid partially rescued gut function in CSD flies by blocking the IMD pathway.

Previous study has found that CSD could lead to early death mainly via oxidative stress damage in the gut of animals, suggesting that gut homeostasis is linked to organism health.<sup>4</sup> In this study, the thermogenetic sleep deprivation and mechanical CSD fly models were constructed to detect the damaging effect of CSD on the gut. Intestinal function was disrupted in both CSD flies species, as evidenced by an imbalance in intestinal acid-base homeostasis and an increase in



**Figure 6** Caffeic acid improves survival and maintains intestinal homeostasis in thermogenetic CSD flies by inhibiting the IMD pathway. **(A and B)** Caffeic acid improves survival in CSD flies (n=110–120). **(C and D)** Caffeic acid restores acid-base homeostasis in the fly gut (n=27-36). **(E and F)** Caffeic acid reduces food intake in CSD flies (n=24-30). **(G)** Caffeic acid reduces mRNA levels of genes related to the intestinal IMD pathway in CSD flies (n=3). \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001.

food intake. We found that mechanical CSD had a more significant injury on gut function compared to thermogenetic CSD, which may be because mechanical stimulation could engage the homeostat and also induce the serious body injury except gut. It is important to note that mechanical CSD could lead to an increase in fly intestinal permeability and a significant shortening of intestinal length. This suggested that physical shocks may cause additional damage to flies. Previous studies found that *sleepless* (*sss<sup>Δ</sup>*)<sup>38</sup> mutant flies with sleep deprivation had an imbalance in intestinal acid-base homeostasis, increased food intake, and disruption of the intestinal barrier, compared to control flies.<sup>38</sup> Another study

found that CSD led to increased intestinal permeability and decreased colon length in mice.<sup>10</sup> These studies confirm that sleep deprivation severely disrupts intestinal homeostasis, laying the foundation for the next steps in exploring its molecular mechanisms.

It has been proved that CSD could induce intestinal injury by promoting the development of intestinal oxidative stress and activation of the NF- $\kappa$ B pathway in mice and adult flies.<sup>6,7,39,40</sup> Here, the results of transcriptomics testing revealed that CSD-induced intestinal damage may be associated with biological processes including innate immunity. Analysis of KEGG enrichment of up-regulated genes revealed that CSD induced hyperactivation of the IMD pathway in flies. It has been shown that over-activation of the IMD signaling pathway in flies leads to an imbalance in intestinal homeostasis, which further exacerbates intestinal damage and even death.<sup>41</sup> The *Drosophila* gene encodes 13 PGRPs, in which the expression of *PGRP-SB1* is highly induced upon infection, far more than any other PGRP.<sup>42</sup> PGRP-SB1 massively activates the IMD signaling pathway by recognizing the diaminopimelic acid peptidoglycan (DAP) of Gram-negative bacterial cell walls.<sup>43</sup> We found that CSD led to massive expression of *PGRP-SB1*, *Dpt*, *AttA*, *AttB* and *Mtk* in flies by using transcriptomics and RT-PCR. The results of our preliminary experiments found that CSD led to the accumulation of ROS in the fly gut. The expression of genes involved in the immune response could increase in response to oxidative stress.<sup>44</sup> Thus, CSD-induced oxidative stress in the gut may underlie the hyperactivation of the IMD pathway.

Caffeic acid is a polyphenolic compound extensively present in various plants.<sup>26,45</sup> Numerous pharmacological activities of caffeic acid have been reported, including antioxidative, anti-inflammatory, and antibacterial activities, and it has also the potential to attenuate intestinal injury.<sup>24,46,47</sup> It was found that caffeic acid could ameliorate DSS-induced ulcerative colitis in mice, significantly reducing intestinal mucosal inflammation.<sup>48</sup> Here, administration of caffeic acid could significantly reduce mortality of CSD flies. Meanwhile, it ameliorated CSD-induced intestinal damage, including restoration of intestinal acid-base homeostasis, decreased food intake, and decreased intestinal permeability. Previous studies have proved that caffeic acid ameliorated DSS-induced intestinal injury by affecting the intestinal microbiota and activating the Nrf-2/HO-1 pathway to reduce intestinal oxidative stress and inflammatory responses in mice.<sup>49</sup> Here, we found that caffeic acid restored intestinal function and homeostasis by inhibiting the IMD pathway in the fly gut, suggesting that caffeic acid has a protective effect against CSD-induced intestinal immune injury. However, the possibility that caffeic acid ameliorates CSD intestinal damage by affecting intestinal oxidative stress and gut microbiota lacks demonstration and warrants further consideration in subsequent studies.

This study reveals that CSD induces hyperactivation of the IMD pathway in the gut, providing a new perspective on the relationship between CSD and the gut. The results showed that CSD could disrupt the intestine, including disruption of intestinal acid-base homeostasis, increased uptake, increased intestinal permeability, and shortened intestinal length. Consumption of a diet rich in caffeic acid has a highly significant beneficial effect on CSD-induced intestinal injury. In conclusion, our findings suggest that the IMD pathway is a promising avenue for the treatment of CSD gut injury. Caffeic acid significantly reduced mortality and ameliorated CSD-induced intestinal injury in flies with CSD by inhibiting the IMD pathway. In summary, caffeic acid is a new and promising compound for ameliorating CSD-induced intestinal damage.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

The authors declare that they have no conflicts of interest in this work.

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