# Supplementary Materials

**Fluoride induced leaky gut and bloom of *Erysipelatoclostridium ramosum* mediate the exacerbation of obesity in high-fat-diet fed mice**

**Table S1. The composition of D12492 and D12450J.**

| Product# | D12492 | D12450J |
|---|---|---|
| Protein | 20% kcal | 20% kcal |
| Carbohydrate | 20% kcal | 70% kcal |
| Fat | 60% kcal | 10% kcal |
| Total | 100% | 100% |
| kcal/g | 5.21 | 3.82 |

**Table S2. Primer sequences used in the RT-qPCR experiment.**

| Target gene | Primer Sequence (5´-3´) |
|---|---|
| *Tnf-α* | FW: CTCATGCACCACCATCAAGG |
| | RV: ACCTGACCACTCTCCCTTTG |
| *Il-1β* | FW: AGCTTCAAATCTCGCAGCAG |
| | RV: TCTCCACAGCCACAATGAGT |
| *Il-6* | FW: CTCTGGCGGAGCTATTGAGA |
| | RV: AAGTCTCCTGCGTGGAGAAA |
| *Tlr4* | FW: GCTCTCAGCCATCCACAAAG |
| | RV: GAGTCGGGAAGAGGAAGAGG |
| *Myd88* | FW: TTTCCCAGTATCCTGCGGTT |
| | RV:GCGGAAGAACACAGACAGAC |
| *Zo-1* | FW: TGAGTGCGTTTCTCTCCCTT |
| | RV: CCCTCTGTGTTCCTCATGGT |
| *Occludin* | FW: AGCACTTAACCTGCCTGGAT |
| | RV: AGCCTGTGGAAGCAAGAGAT |
| *Claudin-1* | FW: AGCTGCCTGTTCCATGTACT |
| | RV: CTCCCATTTGTCTGCTGCTC |
| *Muc1* | FW: CACAACTACCAGCTCTGCAC |
| | RV: CTGAGCCTGACGTCACTTTG |
| *Gapdh* | FW: GGACTTACAGAGGTCCGCTT |
| | RV: CTATAGGGCCTGGGTCAGTG |
| *E. ramosum* | FW: ACAATGGATGGTGCAGAGGG |
| | RV: TCAACTCTCTCGTGGTGTGACG |

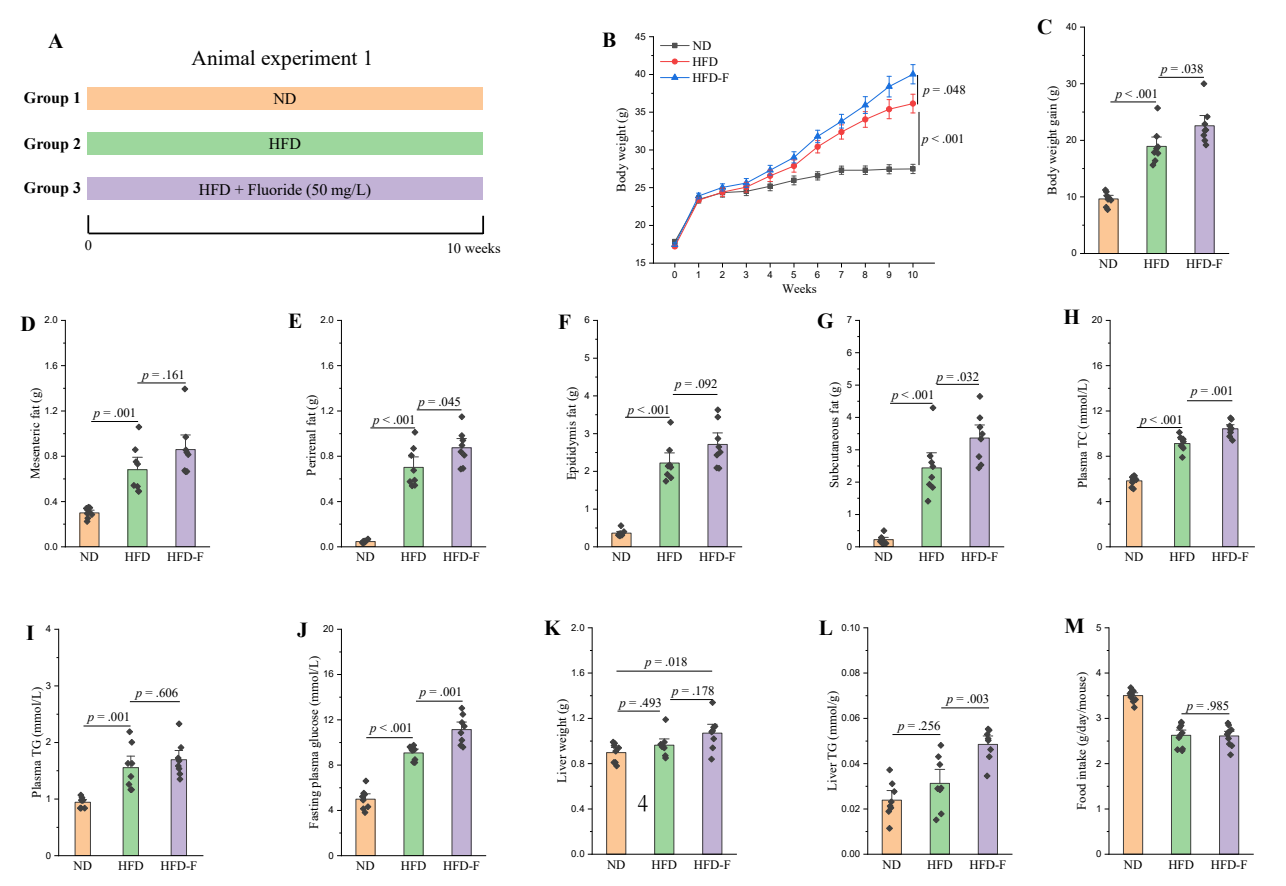

A
Animal experiment 1
Group 1
ND
Group 2
HFD
Group 3
HFD + Fluoride (50 mg/L)
0
10 weeks
B
ND
HFD
HFD-F
Body weight (g)
Weeks
p = .048
p < .001
C
Body weight gain (g)
p < .001
p = .038
D
Mesenteric fat (g)
p = .001
p = .161
E
Perirenal fat (g)
p < .001
p = .045
F
Epididymis fat (g)
p < .001
p = .092
G
Subcutaneous fat (g)
p < .001
p = .032
H
Plasma TC (mmol/L)
p < .001
p = .001
I
Plasma TG (mmol/L)
p = .001
p = .606
J
Fasting plasma glucose (mmol/L)
p < .001
p = .001
K
Liver weight (g)
p = .018
p = .493
p = .178
4
L
Liver TG (mmol/g)
p = .256
p = .003
M
Food intake (g/day/mouse)
p = .985

**Fig. S1. Fluoride exacerbates the obesity in high-fat diet (HFD) mice.** Mice were randomly divided into three groups including normal diet (ND) group, high-fat diet (HFD) group, and HFD plus 50 mg/L of fluoride in drinking water (HFD-F) group (n = 8 per group). **(A)** Scheme of animal experiment 1 over the 10 weeks of dietary intervention. **(B)** Dynamic changes in body weight in mice. **(C)** Body weight gain. **(D)** Mesentery fat. **(E)** Perirenal fat. **(F)** Epididymal fat. **(G)** Subcutaneous fat. **(H-I)** Plasma levels of TC and TG. **(J)** Fasting plasma glucose. **(K)** Liver weight. **(L)** Liver TG. **(M)** Food intake. The results were expressed as means ± SEM. Statistical significance was carried out by one-way analysis of variance (ANOVA) with Tukey test. A value of $p < 0.05$ was considered to be significant.

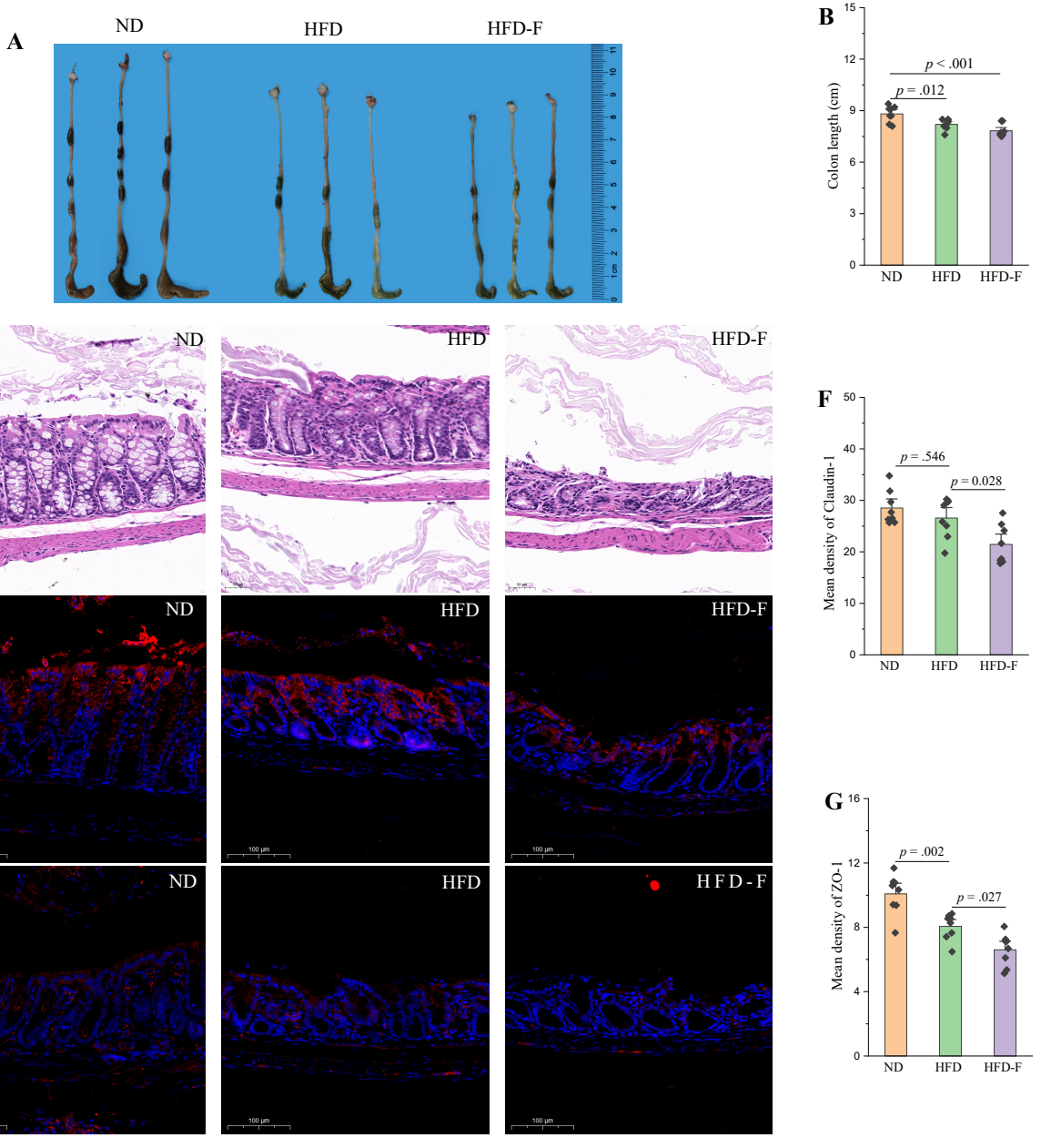
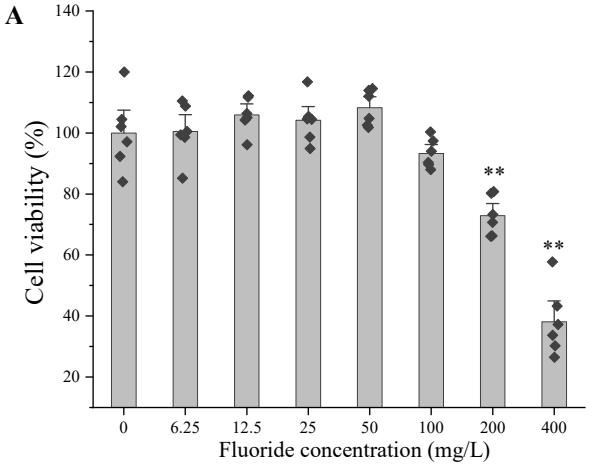

A
ND
HFD
HFD-F
B
Colon length (cm)
p < .001
p = .012
ND
HFD
HFD-F
C
ND
HFD
HFD-F
D
Claudin-1
ND
HFD
HFD-F
E
ZO-1
ND
HFD
HFD-F
F
Mean density of Claudin-1
p = .546
p = 0.028
ND
HFD
HFD-F
G
Mean density of ZO-1
p = .002
p = .027
ND
HFD
HFD-F

**Fig. S2. Fluoride drives the intestinal barrier permeability in HFD mice. (A)** Representative morphology of colon. **(B)** Colon length (n = 8). **(C)** Representative H&E staining of colon, Scale bar = 100 μm. **(D-E)** Representative immunofluorescence images of Claudin-1 and ZO-1 in colon, 20 ×, Scale bar = 100 μm. **(F-G)** Mean density of Claudin-1, MUC1 and ZO-1 in immunofluorescence images was evaluated by ImageJ software (n = 8). The results were expressed as means ± SEM. Statistical significance was carried out by one-way analysis of variance (ANOVA) with Tukey test. A value of $p < 0.05$ was considered to be significant.


A
Cell viability (%)
140
120
100
80
60
40
20
**
**
0
6.25
12.5
25
50
100
200
400
Fluoride concentration (mg/L)



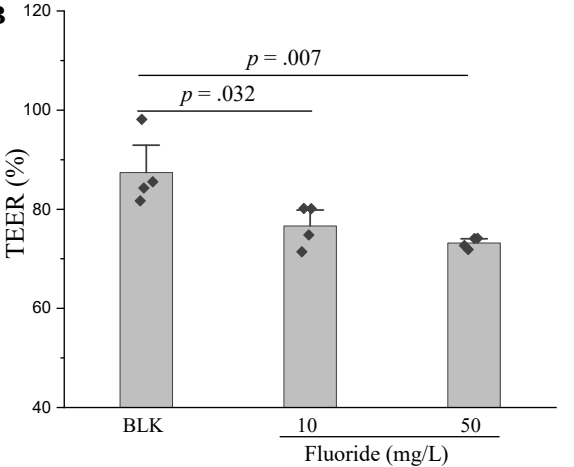

B
TEER (%)
120
100
80
60
40
p = .007
p = .032
BLK
10
50
Fluoride (mg/L)

**Fig. S3. Fluoride drives Caco-2 cell permeability in vitro. (A)** The cytotoxicity of fluoride on the Caco-2 cells evaluated by MTT method (n = 6). **(B)** The effect of fluoride on the Caco-2 cell permeability evaluated by transepithelial electrical resistance (TEER) assay (n = 4). The results were expressed as means ± SEM. Statistical significance was carried out by one-way analysis of variance (ANOVA) with Tukey test. A value of $p < 0.05$ was considered to be significant.

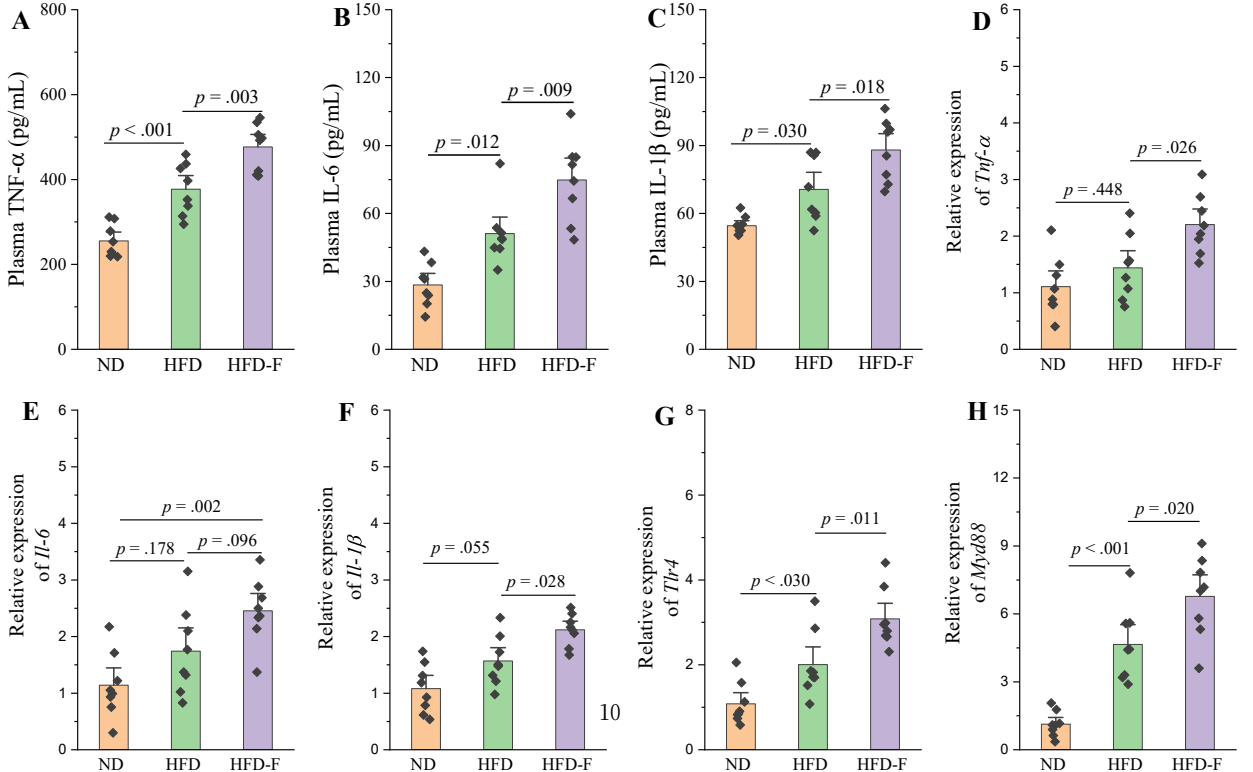

A
Plasma TNF-α (pg/mL)
800
600
400
200
0
p < .001
p = .003
ND
HFD
HFD-F
B
Plasma IL-6 (pg/mL)
150
120
90
60
30
0
p = .012
p = .009
ND
HFD
HFD-F
C
Plasma IL-1β (pg/mL)
150
120
90
60
30
0
p = .030
p = .018
ND
HFD
HFD-F
D
Relative expression of Tnf-α
6
5
4
3
2
1
0
p = .448
p = .026
ND
HFD
HFD-F
E
Relative expression of Il-6
6
5
4
3
2
1
0
p = .002
p = .178
p = .096
ND
HFD
HFD-F
F
Relative expression of Il-1β
6
5
4
3
2
1
0
p = .055
p = .028
ND
HFD
HFD-F
G
Relative expression of Tlr4
8
6
4
2
0
p < .030
p = .011
ND
HFD
HFD-F
H
Relative expression of Myd88
15
12
9
6
3
0
p < .001
p = .020
ND
HFD
HFD-F

**Fig. S4. Fluoride deteriorates the inflammation in HFD mice. (A-C)** The plasma levels of TNF-α, IL-6, and IL-1β (n = 8). **(D-H)** Relative mRNA expression levels of *Tnf-α*, *Il-6*, *Il-1β*, *Myd88* and *Tlr4* in liver (n = 8). The results were expressed as means ± SEM. Statistical significance was carried out by one-way analysis of variance (ANOVA) with Tukey test. A value of $p < 0.05$ was considered to be significant.

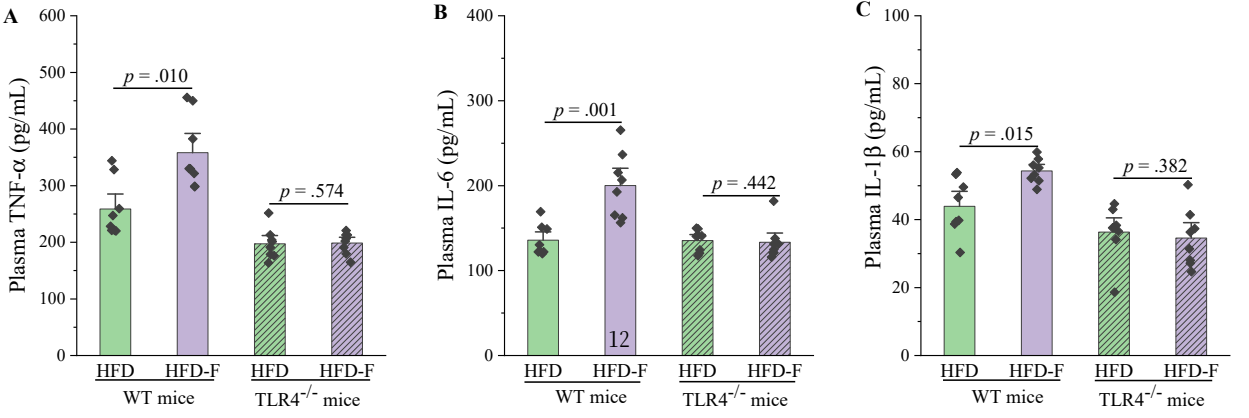
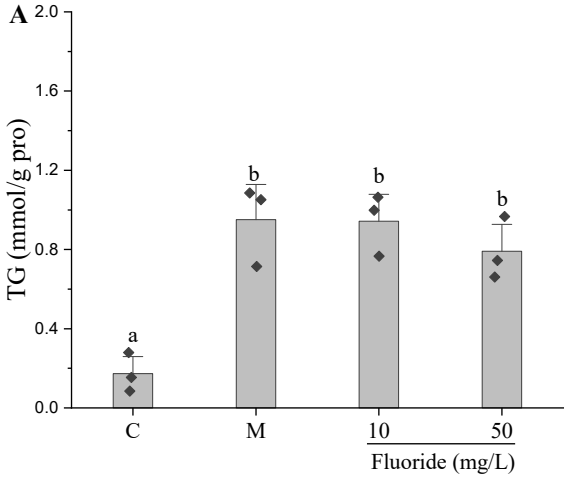
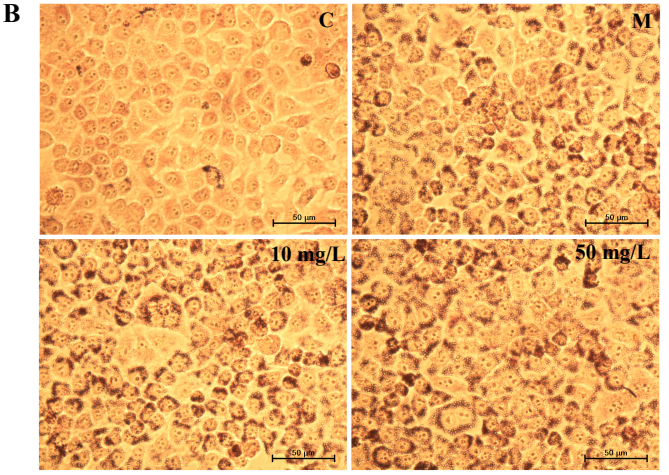
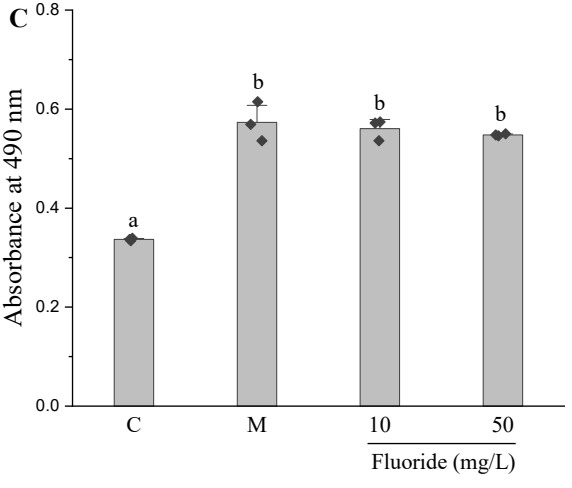
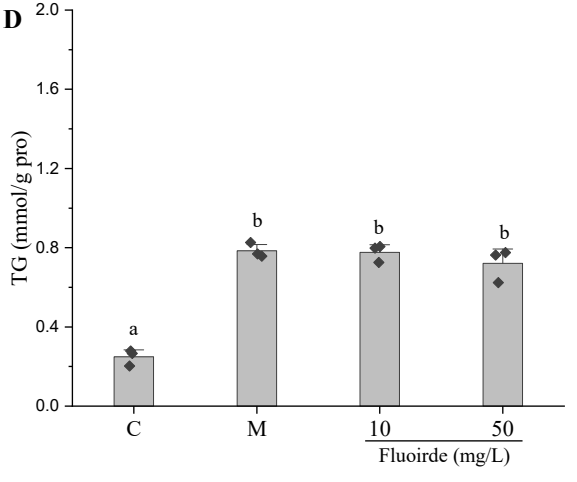

A
Plasma TNF-α (pg/mL)
600
500
400
300
200
100
0
p = .010
p = .574
HFD
HFD-F
HFD
HFD-F
WT mice
TLR4-/- mice
B
Plasma IL-6 (pg/mL)
400
300
200
100
0
p = .001
p = .442
12
HFD
HFD-F
HFD
HFD-F
WT mice
TLR4-/- mice
C
Plasma IL-1β (pg/mL)
100
80
60
40
20
0
p = .015
p = .382
HFD
HFD-F
HFD
HFD-F
WT mice
TLR4-/- mice

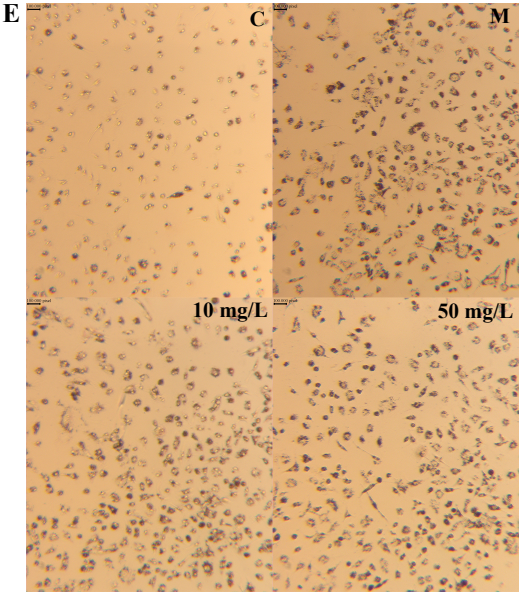
**Fig. S5. Fluoride fails to induce the inflammation in Tlr4$^{-/-}$ mice. (A-C)** The plasma levels of TNF-α, IL-6, and IL-1β. The results were expressed as means ± SEM. Difference in two groups was calculated using the Mann-Whitney test or Kruskal-Wallis test. A value of $p < 0.05$ was considered to be significant.


A
TG (mmol/g pro)
2.0
1.6
1.2
0.8
0.4
0.0
a
b
b
b
C
M
10
50
Fluoride (mg/L)


B
C
M
10 mg/L
50 mg/L


C
Absorbance at 490 nm
0.8
0.6
0.4
0.2
0.0
a
b
b
b
C
M
10
50
Fluoride (mg/L)


D
TG (mmol/g pro)
2.0
1.6
1.2
0.8
0.4
0.0
a
b
b
b
C
M
10
50
Fluoirde (mg/L)


E
C
M
10 mg/L
50 mg/L

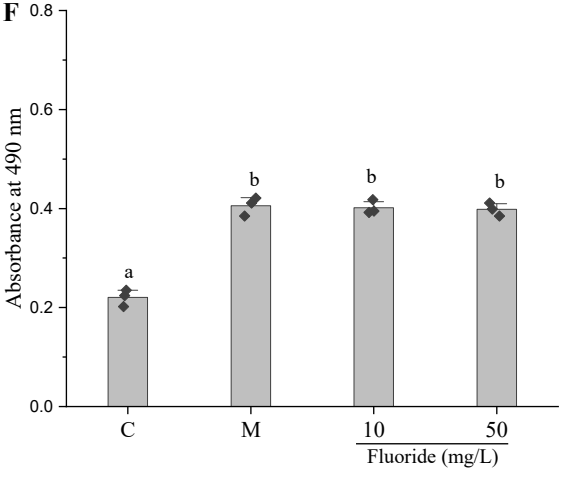

F
Absorbance at 490 nm
0.8
0.6
0.4
0.2
0.0
a
b
b
b
C
M
10
50
Fluoride (mg/L)

**Fig. S6. Fluoride shows limited effect on lipid accumulation using HepG2 cell and primary murine hepatocytes in vitro.** The HepG2 cells or primary murine hepatocytes were cultivated with oleic acid/palmitic acid (OA/PA) and fluoride (10 and 50 mg/L), C: control group without OA/PA or fluoride, M: model group with OA/PA, fluoride: fluoride group with OA/PA and fluoride. **(A)** The effect of fluoride (10 and 50 mg/L) on the level of TG in OA/PA-induced HepG2 cell (n = 3). **(B)** Representative oil red O staining of HepG2 cell. **(C)** The absorbance at 490 nm of oil red solution washed from HepG2 cell by isopropanol (n = 3). **(D)** The effect of fluoride (10 and 50 mg/L) on the level of TG in OA/PA-induced primary murine hepatocytes (n = 3). **(B)** Representative oil red O staining of primary murine hepatocytes. **(C)** The absorbance at 490 nm of oil red solution washed from primary murine hepatocytes by isopropanol (n = 3). The results were expressed as means ± SEM. Statistical significance was carried out by one-way analysis of variance (ANOVA) with Tukey test. A value of $p < 0.05$ was considered to be significant.

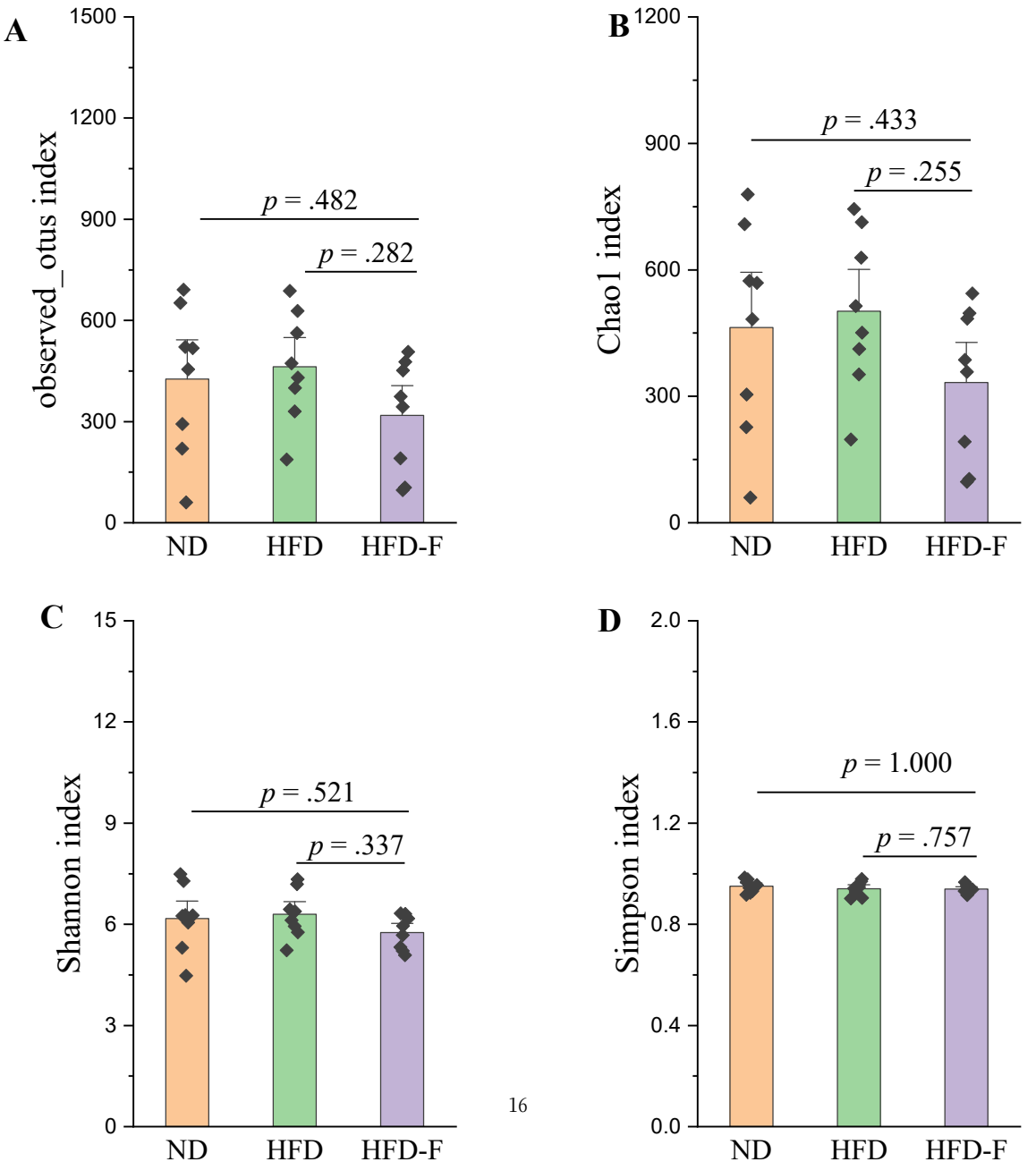

A
observed_otus index
1500
1200
900
600
300
0
p = .482
p = .282
ND
HFD
HFD-F
B
Chao1 index
1200
900
600
300
0
p = .433
p = .255
ND
HFD
HFD-F
C
Shannon index
15
12
9
6
3
0
p = .521
p = .337
ND
HFD
HFD-F
D
Simpson index
2.0
1.6
1.2
0.8
0.4
0.0
p = 1.000
p = .757
ND
HFD
HFD-F

**Fig. S7. Fluoride shows limited effect on α-diversity of gut microbiota. (A)** observed_otus, **(B)** Chao1, **(C)** Shannon, and **(D)** Simpson indexes. The results were expressed as means ± SEM. Statistical significance was carried out by one-way analysis of variance (ANOVA) with Tukey test. A value of $p < 0.05$ was considered to be significant.

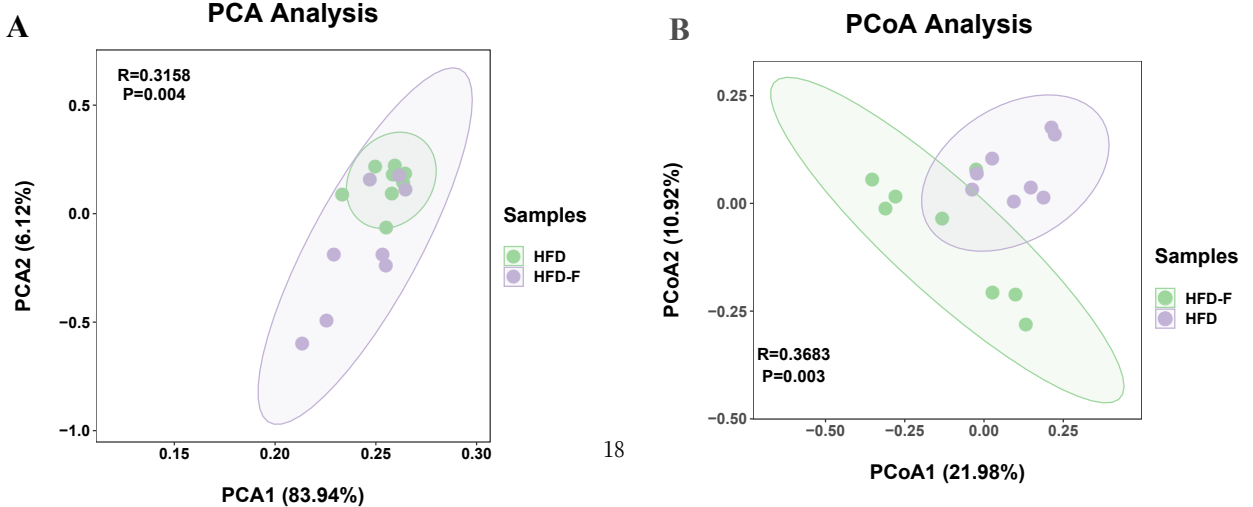
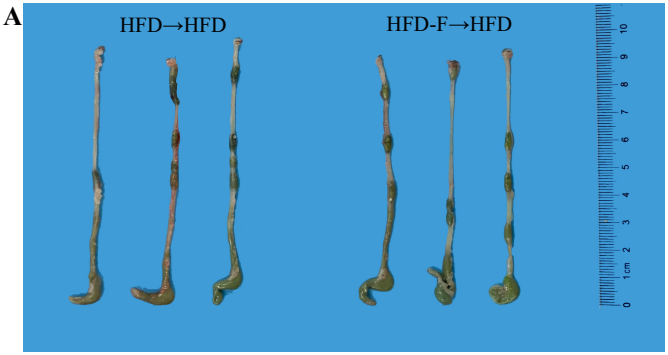
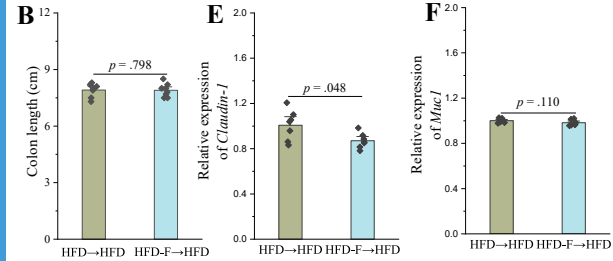
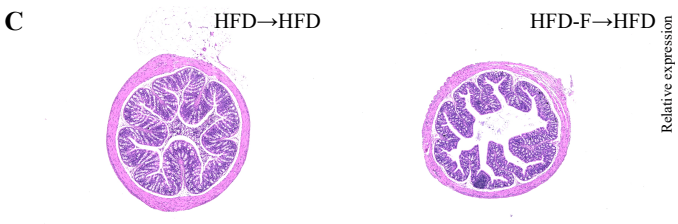
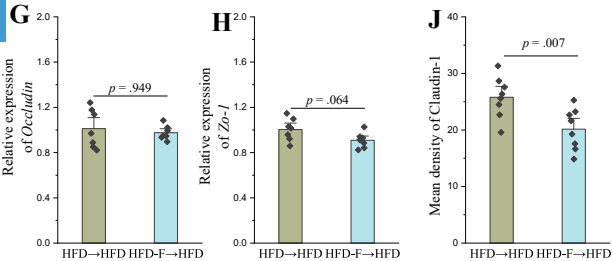

A
PCA Analysis
R=0.3158
P=0.004
PCA2 (6.12%)
PCA1 (83.94%)
Samples
HFD
HFD-F
B
PCoA Analysis
PCoA2 (10.92%)
PCoA1 (21.98%)
R=0.3683
P=0.003
Samples
HFD-F
HFD

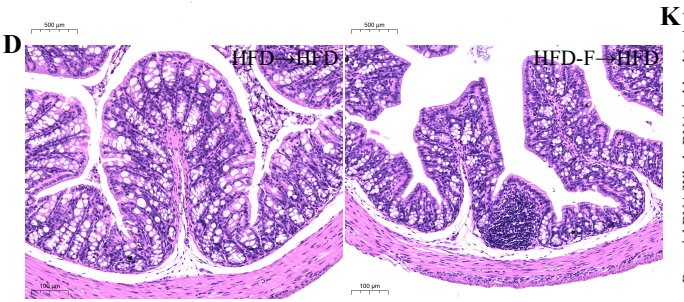
**Fig. S8. Fluoride affects the Beta-diversity of gut microbiota in HFD fed mice including PCA and PCoA**. Gut microbiota was analyzed by 16S rRNA gene sequencing (n = 8 for each group). **(A)** PCA based on the relative abundance of Features of the gut microbiota, **(B)** PCoA of the gut microbiota based on the unweighted unifrac distance matrix.

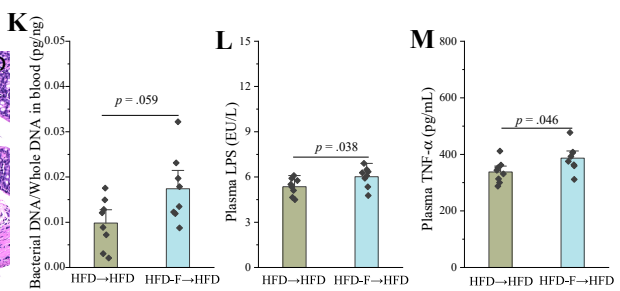

A
HFD→HFD
HFD-F→HFD

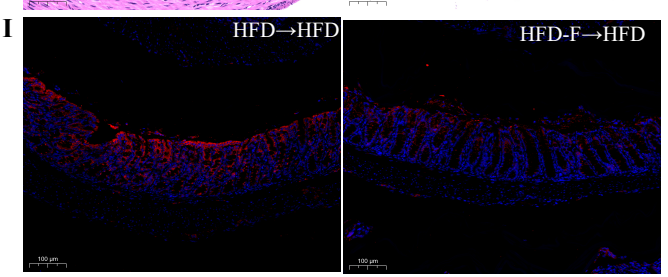

B
Colon length (cm)
p = .798
HFD→HFD HFD-F→HFD
E
Relative expression of Claudin-1
p = .048
HFD→HFD HFD-F→HFD
F
Relative expression of Muc1
p = .110
HFD→HFD HFD-F→HFD

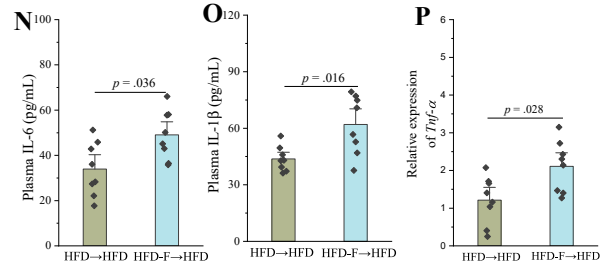

C
HFD→HFD
HFD-F→HFD


G
Relative expression of Occludin
p = .949
HFD→HFD HFD-F→HFD
H
Relative expression of Zo-1
p = .064
HFD→HFD HFD-F→HFD
J
Mean density of Claudin-1
p = .007
HFD→HFD HFD-F→HFD


D
HFD→HFD
HFD-F→HFD


K
Bacterial DNA/Whole DNA in blood (pg/ng)
p = .059
HFD→HFD HFD-F→HFD
L
Plasma LPS (EU/L)
p = .038
HFD→HFD HFD-F→HFD
M
Plasma TNF-α (pg/mL)
p = .046
HFD→HFD HFD-F→HFD


I
HFD→HFD
HFD-F→HFD


N
Plasma IL-6 (pg/mL)
p = .036
HFD→HFD HFD-F→HFD
O
Plasma IL-1β (pg/mL)
p = .016
HFD→HFD HFD-F→HFD
P
Relative expression of Tnf-α
p = .028
HFD→HFD HFD-F→HFD

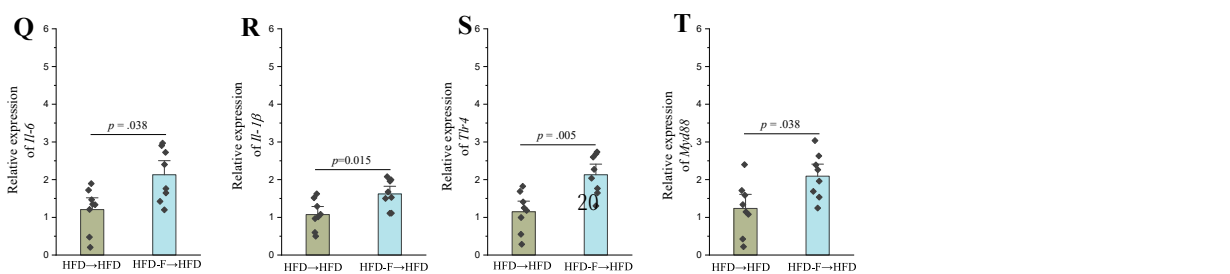

Q
Relative expression of Il-6
p = .038
HFD→HFD HFD-F→HFD
R
Relative expression of Il-1β
p=0.015
HFD→HFD HFD-F→HFD
S
Relative expression of Tlr4
p = .005
HFD→HFD HFD-F→HFD
T
Relative expression of Myd88
p = .038
HFD→HFD HFD-F→HFD

**Fig. S9. Fluoride-induced gut microbiota impairs gut barrier function and promotes the inflammation. (A)** Representative morphology of colon. **(B)** Colon length (n = 8). **(C-D)** Representative H&E staining of colon (10× and 40×, respectively). **(E-H)** Relative mRNA expression levels of *Claudin-1*, *Muc1*, *Occludin* and *Zo-1* in colon (n = 7). **(I)** Representative immunofluorescence images of Claudin-1 in colon, 20 ×, Scale bar = 100 μm. **(J)** Mean density of Claudin-1 in immunofluorescence images was evaluated by ImageJ software (n = 8). **(K)** Bacterial DNA/Whole DNA in blood was measured by RT-qPCR (n = 8). **(L)** Plasma LPS (n = 8). **(M-O)** The plasma levels of TNF-α, IL-6, and IL-1β (n = 8). **(P-T)** Relative mRNA expression levels of *Tnf-α*, *Il-6*, *Il-1β*, *Myd88* and *Tlr4* in liver (n = 8). The results were expressed as means ± SEM. Difference in two groups was calculated using the Mann-Whitney test or Kruskal-Wallis test. A value of $p < 0.05$ was considered to be significant.

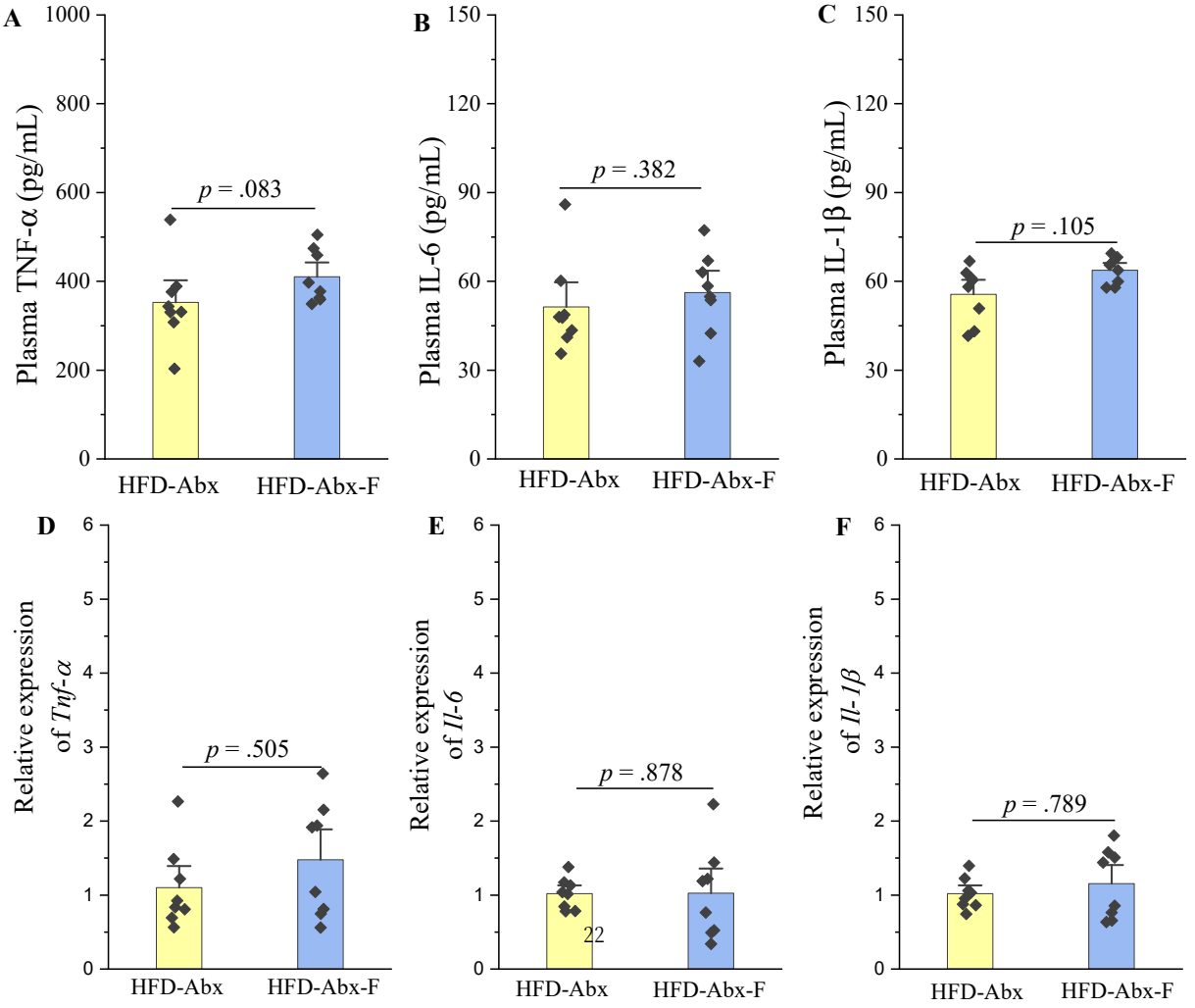

A
Plasma TNF-α (pg/mL)
1000
800
600
400
200
0
p = .083
HFD-Abx
HFD-Abx-F
B
Plasma IL-6 (pg/mL)
150
120
90
60
30
0
p = .382
HFD-Abx
HFD-Abx-F
C
Plasma IL-1β (pg/mL)
150
120
90
60
30
0
p = .105
HFD-Abx
HFD-Abx-F
D
Relative expression of Tnf-α
6
5
4
3
2
1
0
p = .505
HFD-Abx
HFD-Abx-F
E
Relative expression of Il-6
6
5
4
3
2
1
0
p = .878
22
HFD-Abx
HFD-Abx-F
F
Relative expression of Il-1β
6
5
4
3
2
1
0
p = .789
HFD-Abx
HFD-Abx-F

**Fig. S10. Fluoride fails to induce the inflammation after depletion of the gut microbiota by Abx. (A-C)** The plasma levels of TNF-α, IL-6, and IL-1β (n = 8). **(D-F)** Relative mRNA expression levels of *Tnf-α*, *Il-6*, and *Il-1β* in liver (n = 8). The results were expressed as means ± SEM. Difference in two groups was calculated using the Mann-Whitney test or Kruskal-Wallis test. A value of $p < 0.05$ was considered to be significant.

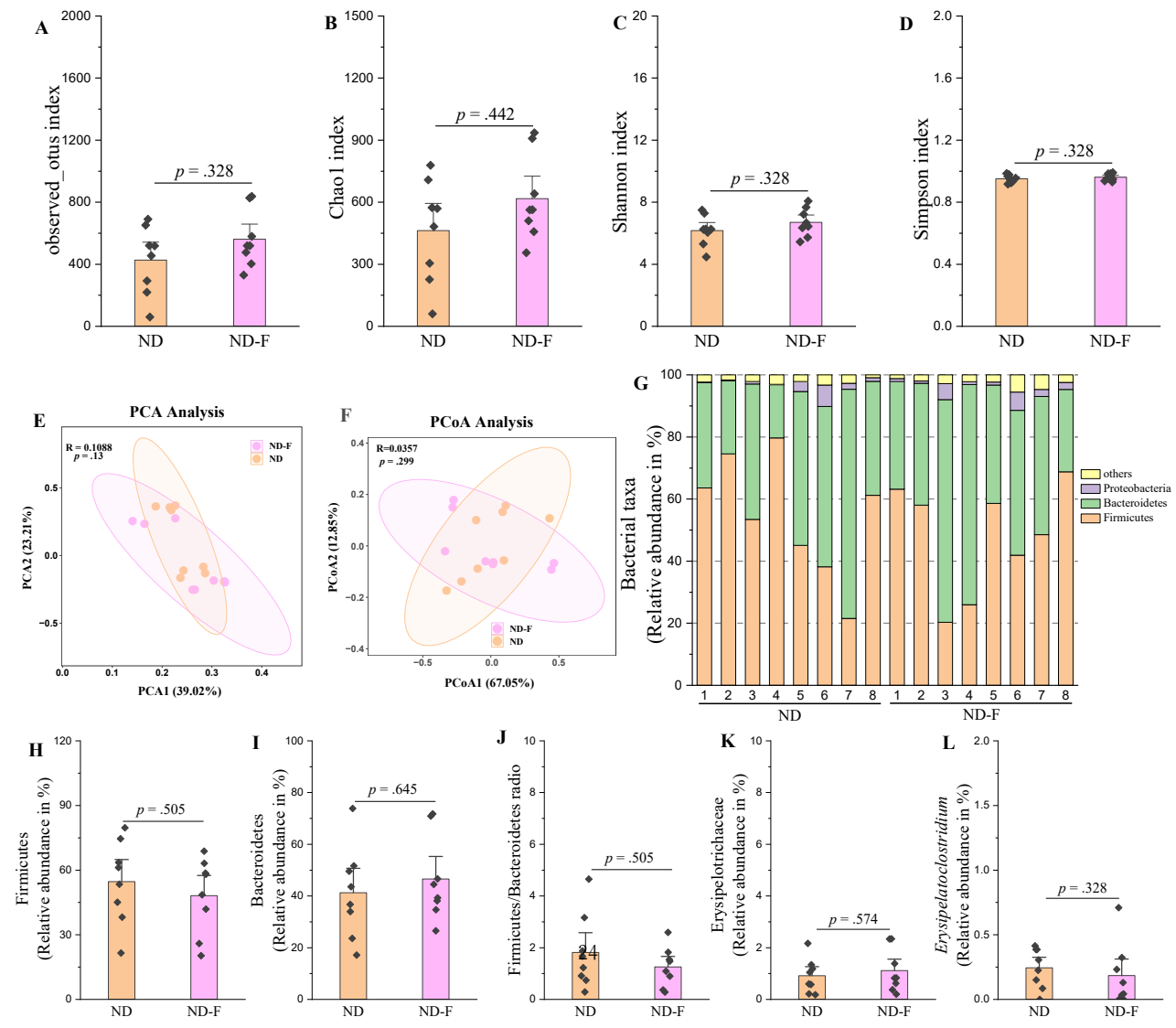

A
observed_otus index
p = .328
ND
ND-F
B
Chao1 index
p = .442
C
Shannon index
p = .328
D
Simpson index
p = .328
E
PCA Analysis
R = 0.1088
p = .13
ND-F
ND
PCA2 (23.21%)
PCA1 (39.02%)
F
PCoA Analysis
R=0.0357
p = .299
PCoA2 (12.85%)
PCoA1 (67.05%)
G
Bacterial taxa
(Relative abundance in %)
others
Proteobacteria
Bacteroidetes
Firmicutes
H
Firmicutes
(Relative abundance in %)
p = .505
I
Bacteroidetes
(Relative abundance in %)
p = .645
J
Firmicutes/Bacteroidetes radio
p = .505
K
Erysipelotrichaceae
(Relative abundance in %)
p = .574
L
Erysipelatoclostridium
(Relative abundance in %)
p = .328

**Fig. S11. Fluoride showed limited effect on the gut microbiota of ND mice.** Gut microbiota was analyzed by 16S rRNA gene sequencing (n = 8 for each group). **(A-D)** α-diversity of gut microbiota evaluated by **(A)** observed_otus, **(B)** Chao1, **(C)** Shannon, and **(D)** Simpson indexes. **(E)** principal component analysis (PCA) based on the relative abundance of Features of the gut microbiota, **(F)** Principal coordinates analysis (PCoA) of the gut microbiota based on the weighted unifrac distance matrix, **(G)** Bacterial taxonomic profiling at the phylum level of gut microbiota, the relative abundances of **(H)** Firmicutes, **(I)** Bacteroidetes, and **(J)** the ratio of Firmicutes to Bacteroidetes. The relative abundances of **(K)** Erysipelotrichaceae, and **(L)** *Erysipelatoclostridium*. The results were expressed as means ± SEM. Difference in two groups was calculated using the Mann-Whitney test or Kruskal-Wallis test. A value of $p < 0.05$ was considered to be significant.

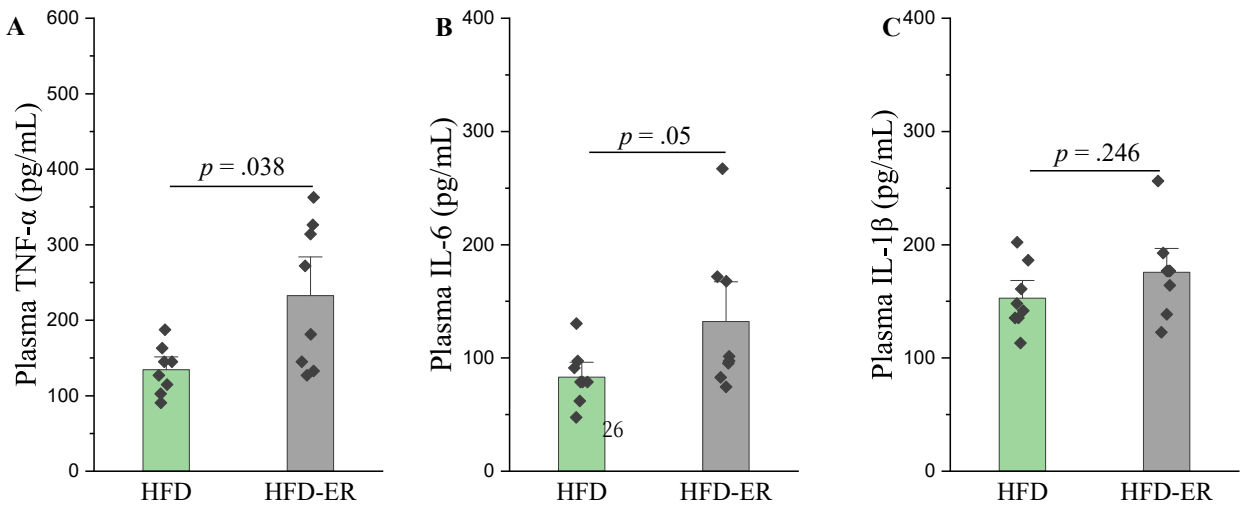

A
Plasma TNF-α (pg/mL)
600
500
400
300
200
100
0
p = .038
HFD
HFD-ER
B
Plasma IL-6 (pg/mL)
400
300
200
100
0
p = .05
26
HFD
HFD-ER
C
Plasma IL-1β (pg/mL)
400
300
200
100
0
p = .246
HFD
HFD-ER

**Fig. S12. *E. ramosum* aggravates the inflammation in HFD mice. (A-C)** The plasma levels of TNF-α, IL-6, and IL-1β (n = 8). The results were expressed as means ± SEM. Difference in two groups was calculated using the Mann-Whitney test or Kruskal-Wallis test. A value of $p < 0.05$ was considered to be significant.

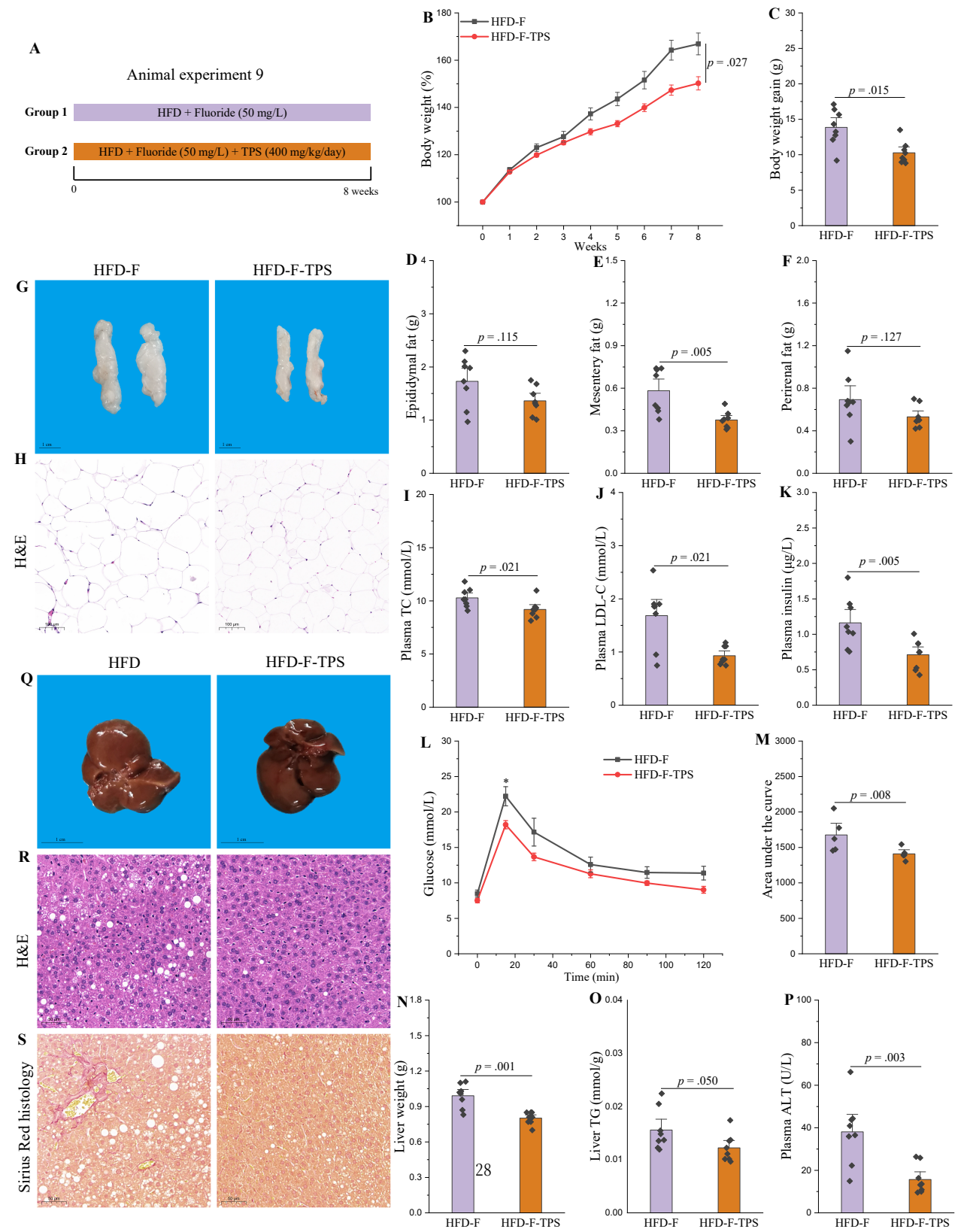

A
Animal experiment 9
Group 1
HFD + Fluoride (50 mg/L)
Group 2
HFD + Fluoride (50 mg/L) + TPS (400 mg/kg/day)
0
8 weeks
B
HFD-F
HFD-F-TPS
p = .027
Body weight (%)
Weeks
C
p = .015
Body weight gain (g)
HFD-F
HFD-F-TPS
G
H
H&E
D
p = .115
Epididymal fat (g)
E
p = .005
Mesentery fat (g)
F
p = .127
Perirenal fat (g)
I
p = .021
Plasma TC (mmol/L)
J
p = .021
Plasma LDL-C (mmol/L)
K
p = .005
Plasma insulin (μg/L)
HFD
HFD-F-TPS
Q
R
H&E
S
Sirius Red histology
L
HFD-F
HFD-F-TPS
Glucose (mmol/L)
Time (min)
M
p = .008
Area under the curve
N
p = .001
Liver weight (g)
28
O
p = .050
Liver TG (mmol/g)
P
p = .003
Plasma ALT (U/L)

**Fig. S13. TPS abolish the fluoride-induced obesity in HFD mice. (A)** Scheme of animal experiment 9 over the 8 weeks. Mice were randomly divided into two groups including HFD plus 50 mg/L of fluoride in drinking water (HFD-F) group, and HFD plus 50 mg/L of fluoride in drinking water and 400 mg/kg/day of TPS from Fuzhuan brick by intragastric gavage (HFD-F-TPS) group (n = 8 per group). **(B)** Dynamic changes in body weight in mice. **(C)** Body weight gain. **(D)** Epididymal fat. **(E)** Mesentery fat. **(F)** Perirenalt fat. **(G-H)** Representative morphology and H&E staining of Epididymal fat. **(I-K)** Plasma levels of TC, LDL-C and insulin. **(L)** OGTT was carried out at week 7, mice were fasted overnight and gavaged with a dosage of glucose with 1.5 mg/g body weight (n = 5 per group). **(M)** AUC for OGTT. **(N)** Liver weight. **(O)** Liver TG. **(P)** Plasma ALT. **(Q-S)** Representative morphology, H&E staining and Sirius Red histology of liver. The results were expressed as means ± SEM. Difference in two groups was calculated using the Mann-Whitney test or Kruskal-Wallis test. A value of $p < 0.05$ was considered to be significant.

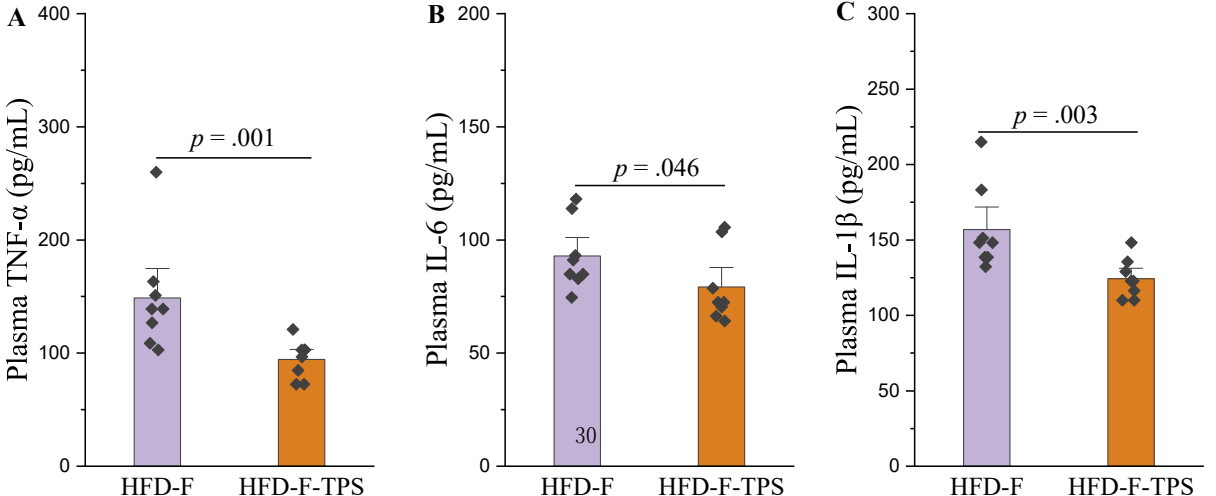

A
Plasma TNF-α (pg/mL)
400
300
200
100
0
p = .001
HFD-F
HFD-F-TPS
B
Plasma IL-6 (pg/mL)
200
150
100
50
0
p = .046
30
HFD-F
HFD-F-TPS
C
Plasma IL-1β (pg/mL)
300
250
200
150
100
50
0
p = .003
HFD-F
HFD-F-TPS

**Fig. S14. TPS abolish the fluoride-induced inflammation in HFD mice. (A-C)** The plasma levels of TNF-α, IL-6, and IL-1β (n = 8). The results were expressed as means ± SEM. Difference in two groups was calculated using the Mann-Whitney test or Kruskal-Wallis test. A value of $p < 0.05$ was considered to be significant.