Colonic inflammation accompanies an increase of β-catenin signaling and *Lachnospiraceae/Streptococcaceae* bacteria in the hind gut of high-fat diet-fed mice

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Abbreviation used: CRP, C-reactive protein; HF, high-fat; HFD, high-fat diet; IBD, inflammatory bowel disease; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; LF, low-fat; LFD, low-fat diet; rRNA, ribosomal RNA; TNF-α, tumor necrosis factor α.
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Abstract

Consumption of an obesigenic / high-fat (HF) diet is associated with a high colon cancer risk, and may alter the gut microbiota. To test the hypothesis that long-term HF feeding accelerates inflammatory process and changes gut microbiome composition, C57BL/6 mice were fed a HF (45% energy) or low-fat (LF) (10% energy) diet for 36 weeks. At the end of the study, body weights in the HF group were 35% greater than those in the LF group. These changes were associated with dramatic increases in body fat composition, inflammatory cell infiltration, inducible nitric oxide synthase (iNOS) protein concentration and cell proliferation marker (Ki67) in ileum and colon. Similarly, β-catenin expression was increased in colon (but not ileum). Consistent with gut inflammation phenotype, we also found that plasma leptin, IL6, and tumor necrosis factor-α concentrations were also elevated in mice fed the HF diet, indicative of chronic inflammation. Fecal DNA was extracted and the V1-V3 hypervariable region of the microbial 16S rRNA gene was amplified using primers suitable for 454-pyrosequencing. Compared to the LF group, the HF group had high proportions of bacteria from the family *Lachnospiraceae/Streptococcaceae* which is known to be involved in the development of metabolic disorders, diabetes and colon cancer. Taken together, our data demonstrate, for the first time, that long-term HF consumption not only increases inflammatory status but also accompanies an increase of colonic β-catenin signaling and *Lachnospiraceae/Streptococcaceae* bacteria in the hindgut of C57BL/6 mice.

Keywords: colonic inflammation; cancer; high fat; microbiome

1. Introduction

The incidence of inflammatory bowel disease (IBD) is rising in the Western world, as well as in the regions where IBD was previously thought to be uncommon (e.g., China, South Korea) [1]. The precise cause of IBD is unknown. However, the spread of the "Western" diet, high in fat and protein, but low in fruits and vegetables, has been indicated as a promoting factor on the risk of IBD [2,3]. High-fat diet (HFD) related obesity has emerged as one of the leading environmental risk factors for IBD and colon cancer development [3-5] as supported by epidemiological studies as well as controlled experimental studies in mice [6-9]. Consumption of a HFD can lead to accumulation of excess body fat that is associated with adipose tissue dysfunction and a chronic state of low-grade inflammation, which is known to promote IBD and tumor development [10,11]. While the pathways that are active in promoting obesity-related gut inflammation remain to be characterized, it is possible that the process may involve the hind gut microbiota, which can affect gut inflammatory status and the extraction of energy from the diet [12-14].

Although there is a growing body of evidence that implicates chronic inflammation as a link between HFD-induced obesity and IBD risk, little is known about the association of intestinal pathohistological status and altered gut microbiota (dysbiosis) in a long-term HF feeding mouse model of obesity. Adipose tissue manifests proinflammatory transformation during both obesity and IBD, and recent data demonstrate that manipulation of the intestinal microbiota alters host immune cell homeostasis and IBD risk [15,16]. It is understood that intestinal microbiota play an important role in the pathogenesis of IBD. In addition, IBD patients are well known to have a higher risk of developing colon cancer due to chronic inflammation [17,18]. Therefore, these research areas are now well integrated. The

gastrointestinal tract is poised in a state of equilibrium that permits rapid protective responses against pathogens, but curtails damage by hindering long-lasting vigorous inflammatory processes [19]. The present study addressed this issue and tested the hypothesis that a long-term HF feeding (36 wk) promotes certain gut bacteria and intestinal inflammation.

2. Materials and Methods

2.1. Animals, diets and treatment

This study was approved by the Animal Care and Use Committee of the Grand Forks Human Nutrition Research Center, and animals were maintained in accordance with NIH guidelines for the care and use of laboratory animals. Male C57BL/6 mice, 5 wk old, were obtained from Charles River Laboratories. Mice were individually housed in Plexiglas™ ventilated cages within a pathogen-free facility that maintained a 12-h light/dark cycle. Mice were given free access to food and deionized water, and were allowed to acclimate in the facility for 2 days before being randomly assigned to two dietary treatment groups (n = 12 each). The feeding experiment was conducted for 36 wk, and treatments consisted of a LF purified diet (10% calories from fat, D12450B, Research Diets) or a HF diet (45% calories from fat, D12451, Research Diets) (Table 1A, B) [20]. Body weight was recorded weekly, and body composition was measured (by MRI scanning, EchoMRI, Houston, TX,) at 9 wk intervals. At the termination of the experiment, mice were feed-deprived for 6 h and then euthanized with a mixture of ketamine and xylazine. Plasma samples were collected and stored at -80ºC for analyses of leptin, interleukin 6 (IL6), tumor necrosis factor-alpha (TNFα), and C-reactive

protein (CRP).

2.2. Ileum and colon histology, and iNOS, Ki67 and β-catenin immunohistochemistry

Ileum and colon segments were fixed in 10% neutral buffered formalin and embedded in paraffin. Five μm sections were mounted on slides and stained with hematoxylin and eosin (H&E). The iNOS, Ki67 and β-catenin expressions were assessed using an immunohistochemistry detection kit (Abcam Inc.). Rabbit polyclonal iNOS, Ki67 and β-catenin antibodies (Abcam Inc.) were diluted 1:100. Each ileum or colon section was scored for the area of infiltration of inflammatory cells, iNOS, Ki67, β-catenin expression, and their respective total section area (mm^2) using a standardized determination of morphology [21,22]. The target areas were captured by Leica MZ6 stereomicroscope and Leica DFC420 C digital camera, and Image Pro Plus Version 6.2 software (North Central Instruments) was used for quantification of digitized images.

2.3. Plasma leptin, IL6, TNFα and CRP

The leptin, IL6 and TNFα concentrations were measured in plasma using ELISA kits (R&D Systems, Inc.). Plasma CRP was assessed by using the CRP (Mouse) assay kit (ANPCO Diagnostics, Salem, NH).

2.4. Detection and quantitation of bacterial composition in fecal samples and 16S sequencing

Fecal pellets were collected from each mouse at wk 18 and wk 36 (two time points) and stored at -80 ^{o}C. DNA was extracted from mouse fecal samples (0.1 grams) using the repeated

bead-beating method [23] and the QIAamp DNA stool Mini Kit (Qiagen, Maryland), and DNA was quantified using a NanoDrop 2000C Spectrophotometer (Thermo Scientific, California). The V1 to V3 region of the bacterial 16S rRNA gene was amplified with universal bacteria primers (IDT, California): 27F [24], (5'-AGAGTTTGATCCTGGCTCAG -3') and 519R [25], (5'-GWATTACCGCGGCKGCTG-3'). PCR procedure was taken as follows [26] : initial denaturing at 98°C for 4 min, then 34 cycles of 98°C for 10 s, 50°C for 30 s, 72° for 2 min, followed by a final extension step of 72°C for 10 min. All PCR results were run on a 1% agarose gel, and bands from each mouse sample were excised from the agarose gel, combined per sample, and purified using the QIAGEN QIAQuick Gel Extraction Kit (QIAGEN, Maryland) according to manufacturer's instructions. The gel-extracted DNA was re-eluted into EB Buffer, and was quantified using the NanoDrop 2000C Spectrophotometer (ThermoScientific, CA) to a minimum required final concentration of 20ng/µl per 20µl sample. The DNA amplicons were frozen and shipped overnight to Molecular Research, LP (MR DNA) for Roche 454 pyrosequencing with Titanium chemistry. Sequences were deposited online in the Sequence Read Archive (SRA) through NCBI (BioProject PRJNA279260).

2.5. Statistical analysis

Results are given as mean ± standard error (SEM). The effects of diet over time on body weight, lean mass and % fat mass were analyzed using repeated measures analysis of variance (ANOVA), followed by Tukey contrasts comparing diets at each time point. Cytokine and immunohistochemistry variables were analyzed using t-tests for unequal variances. JMP V10.0 (SAS Institute, Inc., Cary, NC) was used for all statistical analyses. To analyze the DNA sequencing data and various statistical measures, the open-source computer software program

MOTHUR ver.1.31 [27] was used, following previously described work-flow [26], coupled with JMP V10.0 and analysis of molecular variance (AMOVA). Differences with a p-value < 0.05 were considered statistically significant.

3. Results

3.1. Effects of HF on daily food consumption, body weight and body fat composition

The average daily food intake was (3.49 ± 0.27 g) and (3.36 ± 0.30 g) in the LF and HF groups, respectively. At the end of the 36-wk feeding period, the mean body weight in the HF group (51.9 ± 3.2 g) was greater than those in the LF group (38.5 ± 5.3 g) ($p < 0.0001$). Similarly, the body fat percentage in the HF group was 0.46 fold higher than that in the LF group although the lean body mass in gram in the HF group was 0.16 fold higher than that in the LF group due to the greater overall bodyweight gain (Fig.1).

3.2. Effects of HF on plasma inflammatory cytokines

HF feeding did not significantly affect plasma concentration of CRP. However, the concentrations of plasma leptin, TNFα, IL-6 in the HF group were 2.0, 0.5 and 1.7 fold greater than those in the LF group at the end of the experiment, respectively (Fig. 2).

3.3. Effects of HF on inflammatory cell infiltration, iNOS, Ki67, and β-catenin expression in colon and ileum

At the end of 36-wk feeding period, histological examination of colon sections revealed that the areas of inflammatory cells, iNOS, Ki67 and β-catenin expression in the HF group were 2.6-, 0.5-, 1.1- and 0.3-fold greater than that in the LF group, respectively (Fig. 3). Similarly, ileum histological sections showed that the areas of inflammatory cells, iNOS and Ki67 expression in the HF group were 7.0-, 0.7-, 0.7-fold greater than that in the LF group, respectively (Fig. 4), but β-catenin expression did not differ between the LF and HF groups.

3.4. Effects of HF on gut microbial diversity and composition

Bacterial diversity measures such as abundance-based coverage estimators (ACE), CHAO, Good's Coverage, and Shannon-Weiner index, as well as shared operational taxonomic units (OTUs) and sequences within groups are provided in Table 2. The values of above diversity indexes from 4 different groups did not differ (Table 2). However, the number of total OTUs in HF group was higher than that of LF group ($P < 0.05$) (Table 2). When comparing the HF group with the LF group at the end of 36-wk feeding period, 164 OTUs were shared across the two diets, representing 2,181 shared sequences; the HF group had 809 non-shared OTUs, representing 1,056 non-shared sequences, while the LF had 569 non-shared OTUs representing 703 non-shared sequences (Fig. 5).

In the present study, the phyla *Bacteroidetes, Deferribacteres,* and *Firmicutes* were the three major bacterial taxa identified in the hindgut of mice in the present study. There was a marked increase of *Firmicutes* bacteria in the HF group ($p<0.05$); 72.9% (at 18 wk) and 59.1% (at 36 wk) in total bacteria in the HF group, compared with that of 30.1% (at 18 wk) and 27.4% (at 36 wk) in total bacteria in the LF group (Fig. 6A), respectively. Consistent with this

observation, analysis at the family-level showed that the abundance of bacteria belonging to the families *Lachnospiraceae* and *Streptococcaceae* (phylum *Firmicutes*) was increased because of the HF feeding. *Lachnospiraceae* bacteria represented 31.9% (at 18 wk) and 30.8% (at 36 wk) of total bacteria in the HF group, compared with that of 14.8% (at 18 wk) and 11.0% (at 36 wk) in total bacteria in the LF group (Fig. 6B), respectively; *Streptococcaceae* bacteria represented 2.3% (at 18 wk) and 5.4% (at 36 wk) of total bacteria in the HF group, compared with that of 0.8% (at 18 wk) and 0.1% (at 36 wk) of total bacteria in the LF group (Fig. 6B), respectively. Further analysis at genus-level, showed a marked increase of *Lactococcus* genus in the HF group, 2.3% (at 18 wk) and 5.4% (at 36 wk) of total bacteria in the HF group, compared with that of 0.8% (at 18 wk) and 0.1% (at 36 wk) of total bacteria in the LF group (Fig. 6C).

4. Discussion

Undoubtedly, a variety of factors contribute to the etiology of IBD and colon cancer. There is compelling evidence to include diets and the composition of the gut microbiota as key risk factors [3,18,28]. A high-risk Western-type diet for experimental animal diet needs to include multiple risk factors which include high in fat and sugar but low in fiber, vitamin D and calcium [29]. However, a diet high in fat (e.g., lard from bacon consumption) has long been considered as a risk factor for IBD and colon cancer [29,30]. Therefore, although lard contains certain amount of linoleic acid, the diet in this study is a widely accepted high (lard) fat diet for animal models to address diet-induced obesity issue which is partially related to a Western diet [31,32].

The current study undertook to examine the hypothesis that long-term HF feeding mediates dysbiosis and increases the inflammatory status of the hind gut. In the present study,

we used a diet high in both total fat and n6:n3 fatty acid ratio (Table 1A, B), to produce outcomes similar to those observed in obese humans [33,34], namely, increased adiposity (Fig. 1), and production of proinflammatory cytokines (Fig. 2).

Although leptin regulates food intake, its proinflammatory properties are to exert proliferative, anti-apoptotic activities and the activation of monocytes/macrophages [35,36]. TNF-α is secreted in colonocytes and hepatic tissues in response to stimuli from the gut and circulation, respectively [37]. IL-6 is a pleiotropic cytokine that contributes to enhanced T cell survival and apoptosis resistance at the inflamed site [38]. CRP is a sensitive system marker of inflammation, in particular, acute inflammatory events and tissue damage caused by infections [39]. The fact that long-term HF feeding increased plasma concentrations of proinflammatory cytokines, leptin, TNF-α, and IL6, but not CRP, suggest that long-term HF feeding induces a low grade chronic inflammation.

Emerging data demonstrate a promoting effect of a HFD on the risk of IBD [2,3], but little is known about the comparative pathobiology of the hindgut (ileum & colon) and its association with dysbiosis. To determine whether long term HF feeding mediated hindgut inflammation, we examined the ileum and colon with immunohistochemistry analysis. First, in healthy colonic tissues, few immune cells can be found in the mucosa next to the basal membrane of the epithelial layer but, at the inflamed tissue, the immune cells are greatly increased in the lamina propria of intestine and these cells secret proinflammatory cytokines and other related mediators [40,41]. In the present study, long-term HF feeding caused an increase of proinflammatory cells in both ileum and colon (Fig. 3, 4), suggesting that ileum and colon were inflamed to a certain degree. Second, the regulation of proinflammatory cytokines

released by iNOS may contribute to the pathogenesis of the inflammatory process. It is known that iNOS/nitric oxide plays an integral role during intestinal inflammation, and the expression of iNOS was significantly increased in inflamed colon [42,43]. However, whether iNOS is induced in the hindgut in HFD mediated obesity mice is still unclear. That we detected an increase of iNOS protein expression in both ileum and colon provided the detailed distribution of iNOS in ileum and colon with HFD induced obese mice. Third, intestinal inflammation is invariably associated with increased epithelial proliferation. In the colon, it is difficult to examine changes in cell proliferation, but using cell proliferation marker Ki67, epithelial proliferation has been suggested to be increased in inflammatory colon [44,45]. Therefore, the increase of Ki67 expression in both ileum and colon in HFD group provides further insights into HFD induced inflammatory gut. Lastly, β-catenin is another key regulator of colonic inflammation [46], and elevated level of β-catenin expression is linked with IBD and colon cancer [19]. Our data showed that HF feeding increased β-catenin level in colon but not ileum. This new observation is consistent with the fact that HFD induced obesity is a higher cancer-risk factor in colon than in ileum [4,47].

The other important aspect of gut inflammatory process is the composition of the gut microbiota, which has emerged as an important factor regulating host health and the onset of IBD and colon cancer [48]. Although there are studies on HFD and gut microbiota [49,50], much remains to be determined at lower taxonomic levels (e.g., family, genus) which vary greatly because of diets, feeding time and species of animal hosts. Little is known about the effect of long-term HF consumption on colonic inflammation and microbiota in a mouse model. To gain further insight into pathophysiology, we then characterized the association between the

increase of immune cell infiltration, iNOS, Ki67, β-catenin in this study and the respective gut microbiota composition.

The HFD increased the statistical diversity of gut bacteria because total OTUs were increased in the HF group, although Shannon diversity was not statistically different between diets (Table 2, and Fig. 5). This suggests that HF group had a higher diversity of sequences but not of taxonomic diversity. In other words, species or strain level diversity was increased in HF group, but the overall genetic distance of the HF group was not significantly elevated. However, certain bacterial abundance did change due to HF feeding. We found that HF feeding/obesity greatly increased the abundance of *Firmicutes* bacteria (Fig. 6), which is consistent with the previous report [12]. The longer HF feeding (36 wk vs. 18 wk) did not further increase the abundance of *Firmicutes* bacteria, and body fat percentage also showed a similar pattern. This observation suggests that the relative increase of the abundance of *Firmicutes* bacteria was closely related to the percentage of body fat mass in this HF feeding model.

Lachnospiraceae bacteria (phylum *Firmicutes*, class *Clostridia*) are in the intestinal tract, but relatively rare elsewhere, the relative abundance of these bacteria was increased by early life subtherapeutic antibiotic treatments in an obese mouse model. Furthermore, *Lachnospiraceae* bacteria have also been linked to obesity [51,52]. In addition, a metagenomic study indicated that the taxonomic family *Lachnospiraceae* may be associated with type 2 diabetes (T2D) in humans and mouse models [53,54]. However, the effect of the HF feeding on the relative abundance of *Lachnospiraceae* has been elusive, and little data on long-term HF consumption exists in mouse models. Our present data clearly showed that HF feeding greatly increased the abundance of *Lachnospiraceae*, which is positively correlated with the

observation that HF feeding also increased inflammatory status in this study. The other important finding is that HF feeding also greatly increased the relative abundance of *Streptococcaceae*, specifically bacteria belonging to the genus *Lactococcus* (100% prevalence). This observation suggests new avenues in understanding HF feeding/obesity related IBD and colon cancer because *Streptococcaceae* has been associated with metabolic syndrome and colon cancer [55-57].

The present study is one of the first comprehensive reports in which we simultaneously addressed the impact of HFD on IBD, colon cancer risk, and microbiota in a long-term HF feeding animal experiment. Collectively, these results demonstrate that a long-term HF feeding causes obesity-related inflammatory ileum and colon, and increases β-catenin (colon cancer risk signaling) expression in colon, which is accompanied by an increase of *Lachnospiraceae* and *Streptococcaceae* bacteria in the hindgut of C57BL/6 mice.

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Figure legends

Figure 1, Effect of HF feeding on (A) body weight gain (g); (B) % body fat mass; (C) body lean mass (g). Values are means ± SEM, n = 12. Different from LF: *p < 0.05.

Figure 2, Effect of HF feeding on plasma (A) leptin, (B) TNFα, (C) IL6, (D) CRP concentrations. Values are means ± SEM, n = 12. Different from LF: * $p < 0.005$.

Figure 3, Colon: comparing with the total cross-section area of the colon, effect of HF feeding on (A), the area of inflammatory cells in percentage; (B), the area of iNOS protein expression in percentage; (C), the area of Ki67 protein expression in percentage; (D), the area of β-catenin protein expression in percentage. Values are means ± SEM, n = 10 to 11. Different from LF: * $p < 0.05$, ** $p < 0.005$.

Figure 4, Ileum: comparing with the total cross-section area of the ileum, effect of HF feeding on (A), the area of inflammatory cells in percentage; (B), the area of iNOS protein expression in percentage; (C), the area of Ki67 protein expression in percentage; (D), the area of β-catenin protein expression in percentage. Values are means ± SEM, n = 9 to 11. Different from LF: * $p < 0.05$, ** $p < 0.005$.

Figure 5, Venn diagram compares HFD (red) with LFD (blue) at 36 wks in terms of shared and non-shared OTUs and sequences.

Figure 6, Effects of HF feeding on the abundance of (A) *Firmicutes*; (B) *Lachnospiraceae*; (C) *Streptococcaceae / Lactococcus*. Values are means ± SEM, n = 6 at each time point. Different from LF: * $p < 0.05$.

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Table 1A Composition of experimental diets

Ingredient	Low-Fat (LF), gm kcal		High-Fat (HF), gm kcal	
Casein, lactic	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	315	1260	72.8	291
MaltoDextrin	35	140	100	400
Sucrose	350	1400	172.8	691
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	20	180	177.5	1598
Trace Element Mix[b]	10	0	10	0
Dicalcium phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, $1H_2O$	16.5	0	16.5	0
Vitamin Mix [c]	10	40	10	40
Choline Bitartrate	2	0	2	0
Total	1055.05	4057	858.15	4057
Calculated content				
Total energy, kcal/kg	3700		4600	
Total fat, g/kg	42.7		235.3	
Fat calories, %	10		45	
n6:n3 fatty acid ratio	8.3		12.5	

[a]Research Diets, Inc., New Brunswick, NJ.
[b] Amounts per 10 g of premix: 0.5 g Mg, 0.3 g S, 1.0 g Na, 1.6 g Cl, 6.0 mg Cu, 0.2 mg I, 45.0 mg Fe, 59mg Mn, 0.2 mg Se and 29 mg Zn.
[C] Amount per 10 g of premix:: 4000 IU vitamin A palmitate, 1000 IU cholecalciferol, 50 IU vitamin E acetate, 0.5 mg menadione sodium bisulfate, 0.2 mg biotin, 10 mg cyanocobalamin, 2 mg folic acid, 30 mg nicotinic acid, 16 mg calcium pantothenate, 7 mg pyridoxine-HCL, 6 mg riboflavin, 6 mg thiamin HCl.

Table 1B

Table 1B Composition of fats in experimental diet

Ingredient (gm)	Low-Fat (LF),	High-Fat (HF),
Lard	20	177.5
Soybean Oil	25	25
Total	45	202.5
10:0, Capric	0.0	0.1
12:0, Lauric	0.0	0.2
14:0, Myristic	0.2	2.0
14:1n-7, Myristoleic	0	0
16:0, Palmitic	6.5	36.9
16:1n-7, Palmitoleic	0.3	2.4
18:0, Stearic	3.1	19.8
18:1n-9, Oleic	12.6	64.4
18:2n-6, Linoleic	18.3	56.7
18:3n-3,alpha- Linolenic	2.2	4.3
18:4n-3, Stearidonic	0	0
20, Arachidic	0.0	0.3
20:1n-9 Eicosenoic	0.1	1.1
20:2 n-6 Eicosadienoic	0.2	1.4
20:3n-3 Eicosatrienoic	0.0	0.2
20:4n-6, Arachidonic	0.1	0.5
22:5n-3, Docosapentaenoic	0.0	0.2
22:6n-3, Docosahexaenoic	0	0
Total	43.7	191.3

Table 2 Mean statistical measures of bacterial diversity, per group

	LFD	**LFD**	**HFD**	**HFD**
	18 weeks	**36 weeks**	**18 weeks**	**36 weeks**
Total Seq	13685 (2731)	10373 (2343)	10021 (3165)	7108 (1225)
Total OTUs*	152 (5)	153 (4.23)	269 (4.94)	205 (2.97)
ACE	1519 (55)	1442 (296)	1442 (169)	1318 (117)
CHAO	582 (58)	574 (97.4)	572 (55)	553 (22.6)
Good's Coverage	0.48 (0.026)	0.5 (0.03)	0.49 (0.026)	0.48 (0.016)
Shannon-Weiner	4.18 (0.12)	4.25 (0.08)	4.19 (0.126)	4.26 (0.074)

* Number of total OTUs in HF group was higher than that of LF group ($p < 0.05$). Standard error mean (SEM) is given in parentheses. Total sequences are those which passed quality assurance steps.

Figure 1

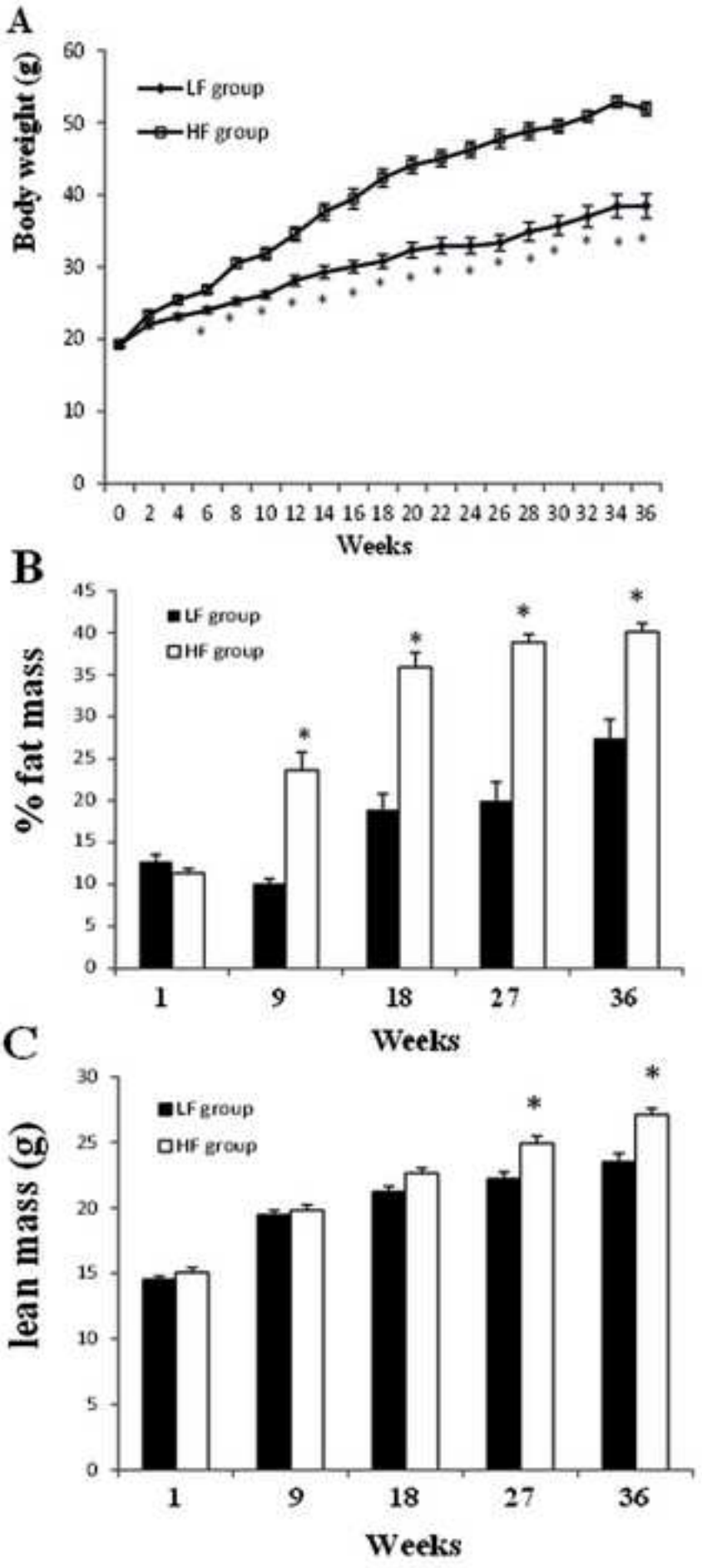

A
Body weight (g)
LF group
HF group
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Weeks
B
% fat mass
LF group
HF group
1 9 18 27 36
Weeks
C
lean mass (g)
LF group
HF group
1 9 18 27 36
Weeks

Figure 2

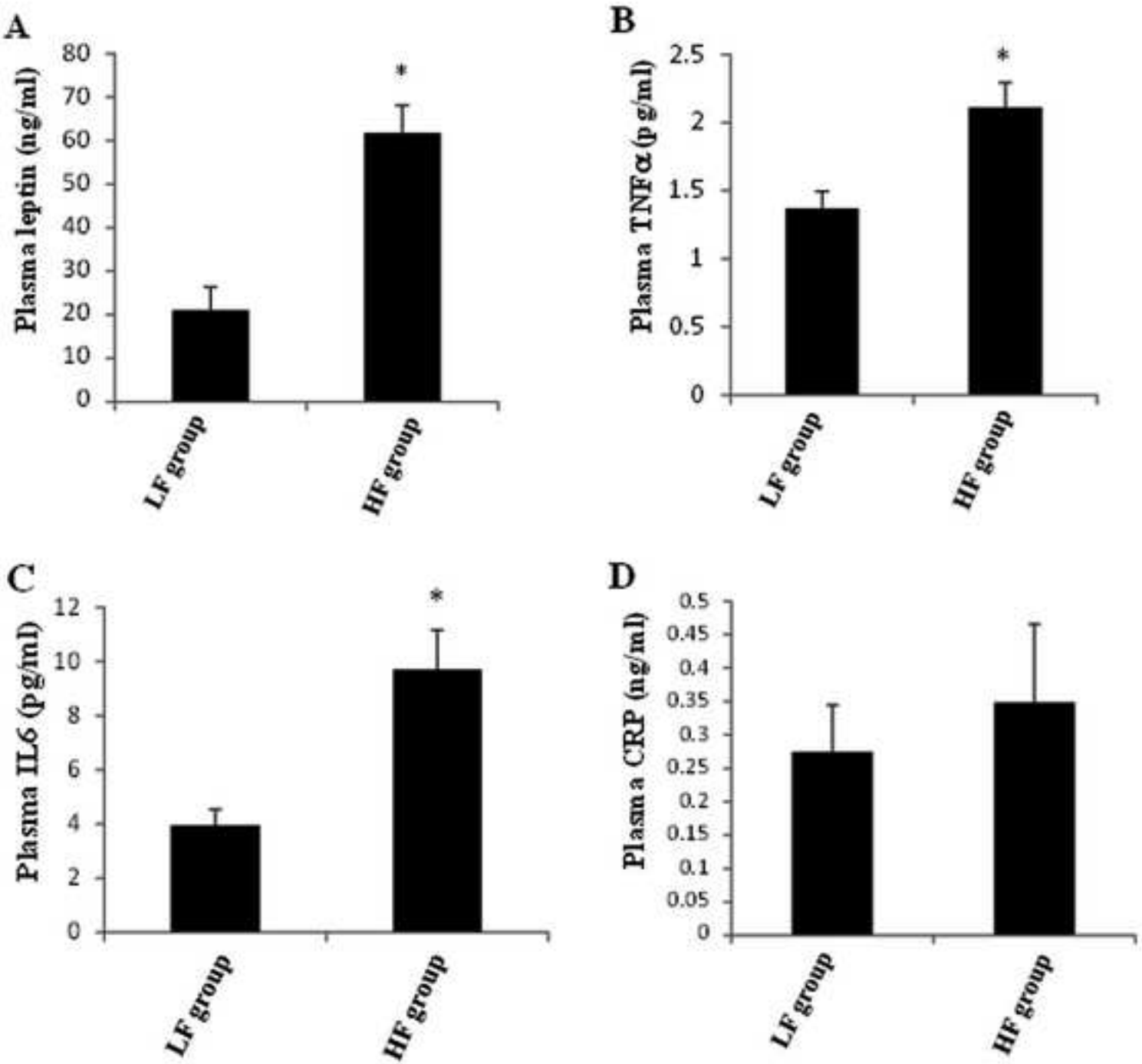

A
Plasma leptin (ng/ml)
80
70
60
50
40
30
20
10
0
*
LF group
HF group
B
Plasma TNFα (pg/ml)
2.5
2
1.5
1
0.5
0
*
LF group
HF group
C
Plasma IL6 (pg/ml)
12
10
8
6
4
2
0
*
LF group
HF group
D
Plasma CRP (ng/ml)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
LF group
HF group

Figure 3

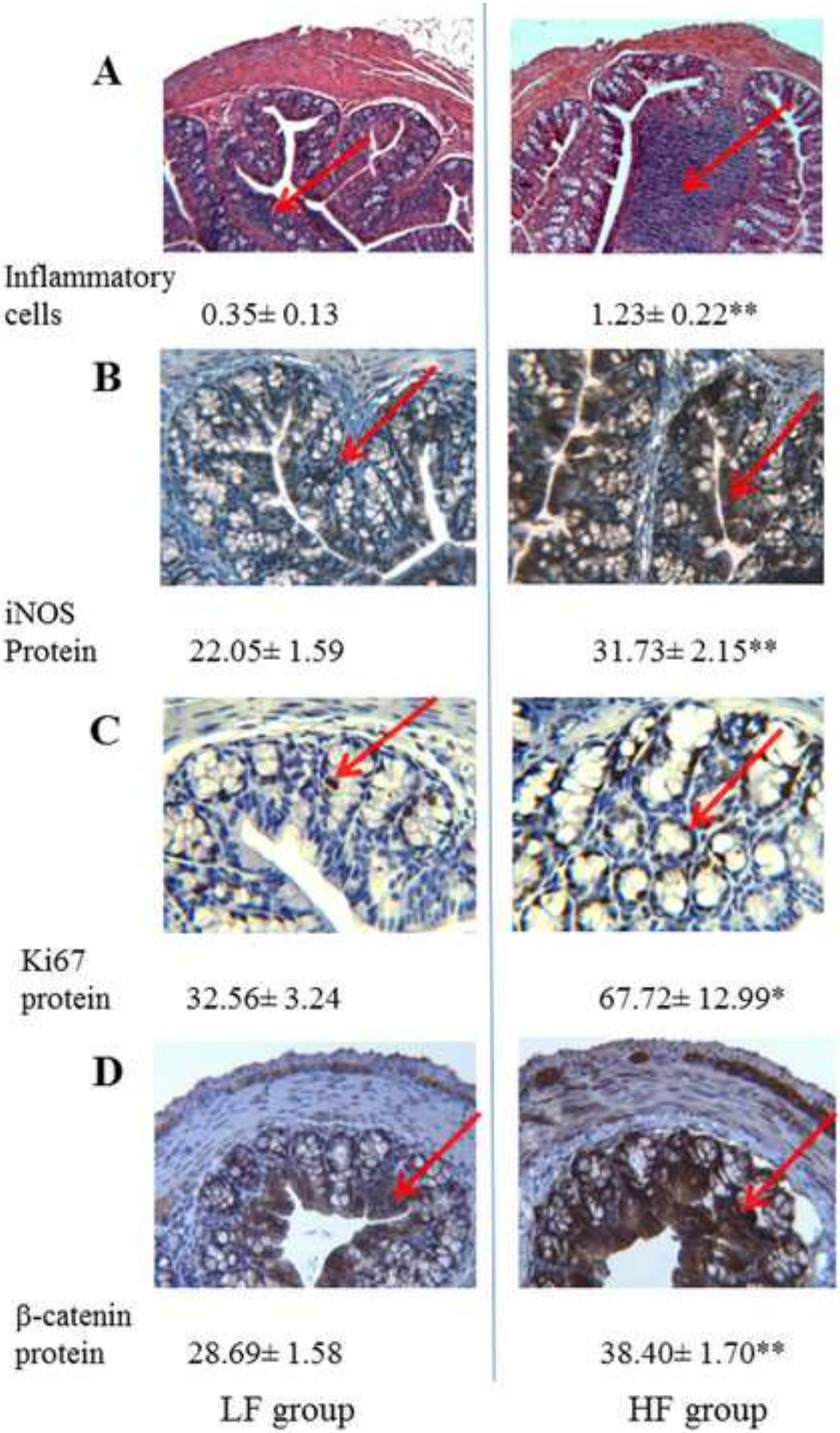

A
Inflammatory cells
0.35± 0.13
1.23± 0.22**
B
iNOS Protein
22.05± 1.59
31.73± 2.15**
C
Ki67 protein
32.56± 3.24
67.72± 12.99*
D
β-catenin protein
28.69± 1.58
38.40± 1.70**
LF group
HF group

Figure 4

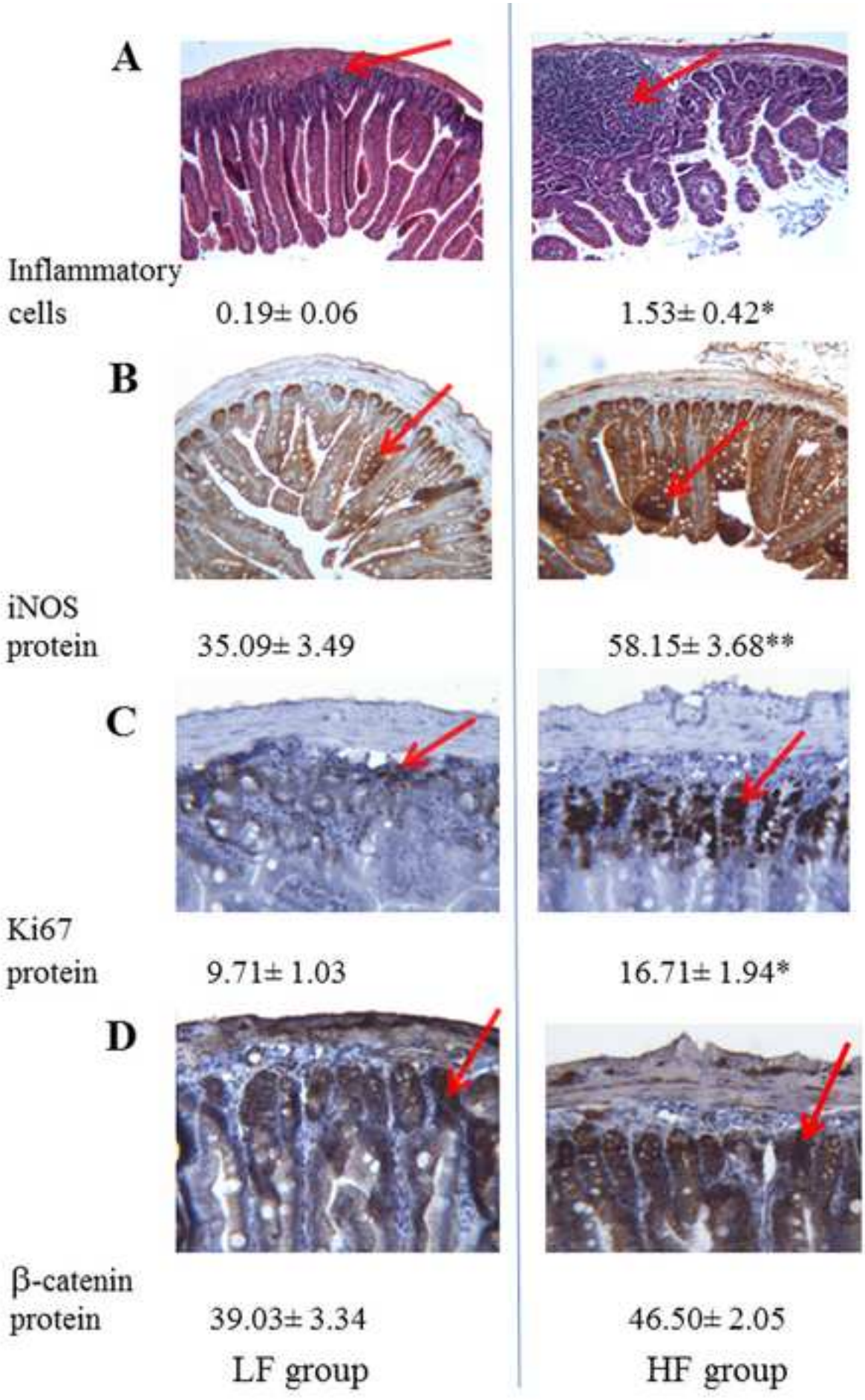

A
Inflammatory cells
0.19± 0.06
1.53± 0.42*
B
iNOS protein
35.09± 3.49
58.15± 3.68**
C
Ki67 protein
9.71± 1.03
16.71± 1.94*
D
β-catenin protein
39.03± 3.34
46.50± 2.05
LF group
HF group

Figure 5

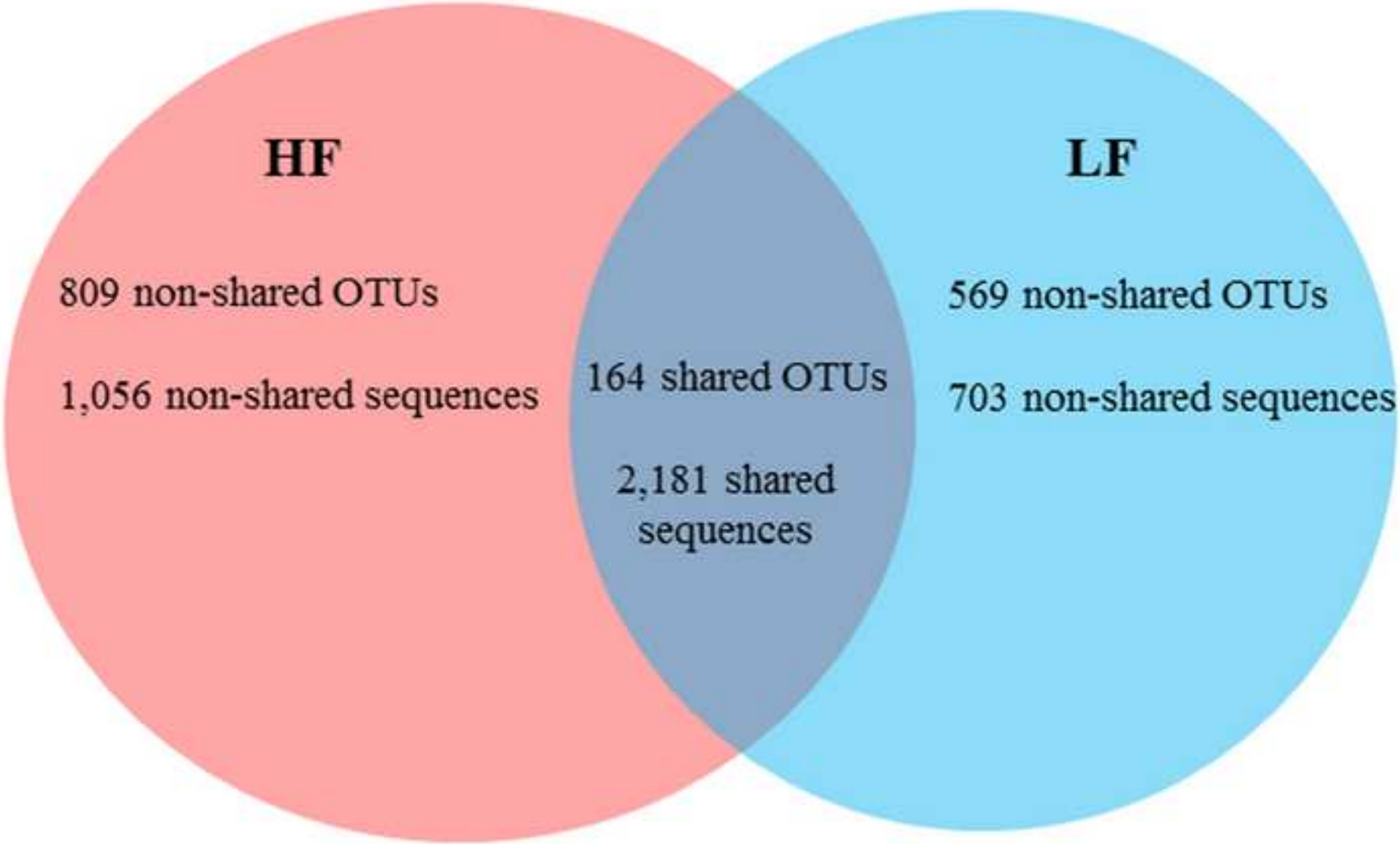
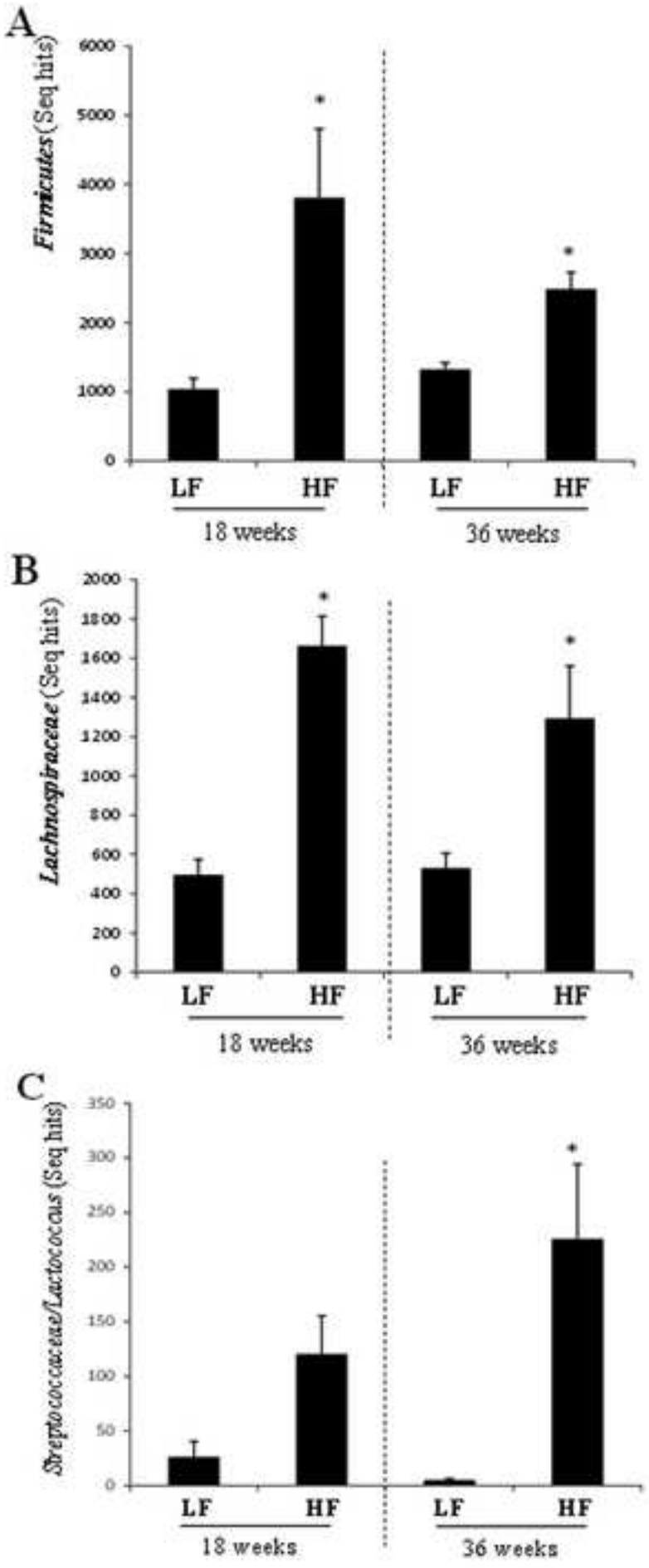

HF
809 non-shared OTUs
1,056 non-shared sequences
164 shared OTUs
2,181 shared sequences
LF
569 non-shared OTUs
703 non-shared sequences

Figure 6


A
Firmicutes (Seq hits)
6000
5000
4000
3000
2000
1000
0
LF
HF
LF
HF
18 weeks
36 weeks
B
Lachnospiraceae (Seq hits)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
LF
HF
LF
HF
18 weeks
36 weeks
C
Streptococcaceae/Lactococcus (Seq hits)
350
300
250
200
150
100
50
0
LF
HF
LF
HF
18 weeks
36 weeks