Melatonin improves the composition of Gut microbiota in C57BL/6J mice fed with high fat-high fructose diet and/or subjected to chronodisruption.

Introduction

NAFLD/NASH is a complex disease that may result from interactions between genetic and environmental factors, eating habits, and alterations in gut microbiota (Amarapurkar et al., 2007; Buzzetti et al., 2016; Q. Liu et al., 2019; Younossi et al., 2019). The role of gut microbiota in the development of obesity, diabetes, atherosclerosis and in the progression of NAFLD to NASH has received increased attention since these diseases share a common mechanism of the immune system activation (P D Cani et al., 2008; Q. Liu et al., 2019; Schachter et al., 2018; Shah, 2019; Shen et al., 2017). Intestinal microbiota plays an important role in the development of inflammation, especially in the progression of NAFLD to NASH. Metabolites originating from gut microbiota are lipopolysaccharide (LPS), lipoteichoic acid, peptidoglycan, flagellin and bacterial DNA that have been attributed to immune system activation wherein; LPS is a major inducer of the inflammatory response in NAFLD/NASH (Abu-Shanab & Quigley, 2010; Federico et al., 2016). The altered intestinal microbiota is considered to be an indicator for the manifestation of NASH (Abu-Shanab & Quigley, 2010; Brandl & Schnabl, 2017; Ji et al., 2019).

The human and rodent gut microbiota is mainly composed of two dominant bacterial phyla, Firmicutes and Bacteroidetes that represent more than 90 percent of the total community and other subdominant phyla including Proteobacteria, Actinobacteria, TM-7, Deferribacteres, Tenericutes and Verrucomicrobia (Magne et al., 2020; Mahowald et al., 2009; Qin et al., 2010). The Firmicutes/Bacteroidetes (F/B) ratio is extensively recognized as an important index of gut microbiota health and an indicator of intestinal homeostasis (Li & Ma, 2020). The F/B ratio has been associated with different pathological states of diseases such as aging (Ma et al., 2020; Spychala et al., 2018), NAFLD/NASH (Fadieienko et al., 2021; Monga Kravetz et al., 2020; G. Wang et al., 2020), atherosclerosis (Xiong et al., 2019), diabetes (Ahmed et al., 2019; Salah et al., 2019), obesity (Koliada et al., 2017; Stojanov et al., 2020), inflammatory bowel diseases (Stojanov et al., 2020; Yañez et al., 2021) and many other neurological diseases such as such as stroke (Spychala et al., 2018), Dementia (Hoffman et al., 2017; Saji et al., 2019,

2022), Parkinson's (Bicknell et al., 2022; Yan et al., 2021), and Alzheimer's disease (Doifode et al., 2021). Diabetes induced in high-fat diet fed mice is characterized by reduced numbers of Bifidobacterium species in caecal content, higher plasma LPS levels along with low-grade inflammation (Patrice D Cani et al., 2007).

Stressor such as high calorie diet, altered sleep-wake cycle, and excessive light at night disturbs the host circadian system which influences the gut microbiome (Patrice D Cani et al., 2007; Z. Liu et al., 2020; Matenchuk et al., 2020; B. Wang et al., 2020; Wu et al., 2018). Gut bacteria possess their own daily rhythmicity in a light/dark cycle and melatonin has been implicated in reprogramming the gut microbiota (Yin et al., 2020). Disturbances or alterations in the microbiome rhythms may contribute to an increased risk of metabolic syndrome associated diseases including fatty liver disease (Ahmed et al., 2019; Fadieienko et al., 2021; Koliada et al., 2017; Monga Kravetz et al., 2020; Salah et al., 2019; Xiong et al., 2019). Excessive light interferes with the endocrine system and alterations in gut microbiota contributes to the increasing rates of lipid associated metabolic diseases (Hong et al., 2020). In C57BL/6J mice, disruption of circadian rhythm leads to the development of metabolic syndrome through alterations in gut microbiota (Cheng et al., 2021). Melatonin is known to improve lipid and glucose metabolism and also influence the gut microbiota communities in animal models and in humans (Hevia et al., 2015; Tiao et al., 2014; Yin et al., 2018). In C57BL/6J mice, constant nocturnal light (ZT12 to ZT24 i.e. 8 p.m. to 8 a.m.) oral administration of 0.4 mg/ml melatonin ameliorated gut microbiota dysbiosis, prevented body weight gain, decreased adipocyte size and improved overall lipid metabolism (Hong et al., 2020). In mice fed with high fat diet, oral melatonin supplementation alleviated lipid accumulation, reversed gut microbiota dysbiosis, improved Bacteroides and Alistipes-mediated acetic acid production and caused overall reprogramming of gut microbiota (Yin et al., 2018). In ICR mice, oral supplementation of 0.2 mg/ml melatonin for 2 weeks increased richness (as per Chao 1 and ACE indices) and metabolism of intestinal microbiota, intestinal morphology (villus : crypt ratio) with little effect on the apoptosis of intestinal cells (Ren et al., 2018). The above published literature establishes a link between melatonin and intestinal microbiota in pathophysiology of metabolic diseases wherein; high calorie diet and light rhythm disruption have also been studied in experimental models.

Our study investigates alterations in gut microbiota (faecal bacterial community abundance and diversity of the microbe composition) of high fat-high fructose diet fed C57BL/6J mice model of NAFLD/NASH and/or photoperiodic manipulations induced chronodisruption. The merits of daily evening intraperitoneal administration of melatonin (10 mg/kg B.W) in preventing the disease induced alterations in gut microbiota are also studied. Correlations of neurobehavioral perturbations and its implications in disease pathology have been studied in subsequent chapter.

Experimental groups:

1. Control
2. Chronodisruption (CD)
3. High fat-high fructose diet (H)
4. High fat-high fructose diet + Chronodisruption (HCD)
5. Chronodisruption + Melatonin (CDM)
6. High fat-high fructose diet + Melatonin (HM)
7. High fat-high fructose diet + Chronodisruption + Melatonin (HCDM)

Fresh fecal samples were collected from the control and experimental groups of mice and transferred to the lab for metagenomic studies. DNA were isolated separately using a commercially available kit according to the manufacturer's instructions (QIAGEN Stool kit; QIAGEN, CA) and metagenomic DNA was used as a template in PCR to amplify 16S r DNA.

Parameter tested:

1. Bacterial diversity and richness (Shannon, Chao and ACE) indices
2. Firmicutes/Bacteroidetes ratio to understand the extent of gut dysbiosis
3. Relative abundance of major and minor OTUs
4. F/B ratio correlations with NASH (morphometric and histopathological) characteristics

The experimental protocol for the present study is depicted in Fig. 3.1. Detailed methodology is described in materials and methods section.

Experimental design

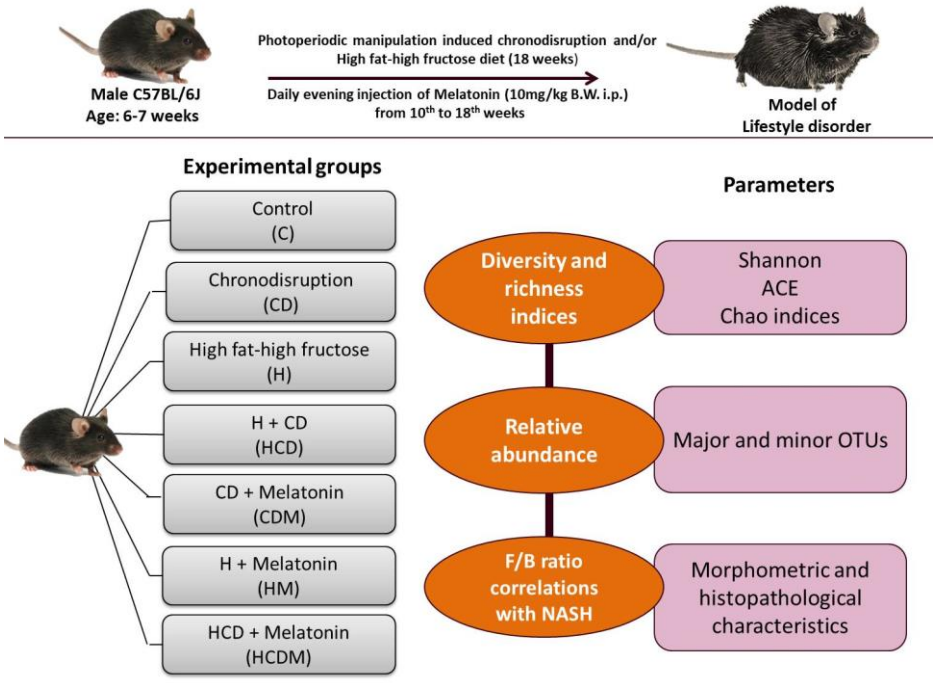


Figure 3.1: Experimental design for 16s rRNA metagenomic analysis of gut microbiota.

Results

Quantification of metagenomic DNA for NGS studies

Aim of this study was to compare the faecal bacterial community abundance and diversity of the microbe in various experimental groups envisaged herein. Quantification of faecal metagenomic DNA for NGS studies was performed using Qubit ® 3.0 Flurometer (Thermo Fisher Scientific, USA). The concentration of DNA in each sample was estimated to be 7.4 ng/µl (C), 20.7 ng/µl (CD), 5.02 ng/µl (H), 5.11 ng/µl (HCD), 2.33 ng/µl (CDM), 9.52 ng/µl (HM) and 8.13 ng/µl (HCDM). The obtained DNA was then used to prepare the amplicon library.

Bacterial diversity and richness indices of faecal microbiota of C57BL/6J mice subjected to high fat-high fructose diet and/or chronodisruption

The number of operational taxonomic units (OTUs) was lower in H, HCD and HM than CDM and HCDM groups with the CD group recording the least index (Fig. 3.5). The number of OTUs increased with the number of sequences in all the experimental groups except H and HCD groups. The number of unique sequences were highest in melatonin treated (HM>HCDM>CDM) groups but, the control showed the highest number of OTUs (Fig. 3.5). Diversity indices showed a similar trend. The Shannon, ACE and Chao indices were decreased in CD, H and HCD groups but, melatonin treatment (CDM, HM and HCDM) had accounted for moderate/no improvement (Fig. 3.6).

Microbial composition of major phyla (Bacteroidetes & Firmicutes) in faecal microbiota of C57BL/6J mice subjected to high fat-high fructose diet and/or chronodisruption

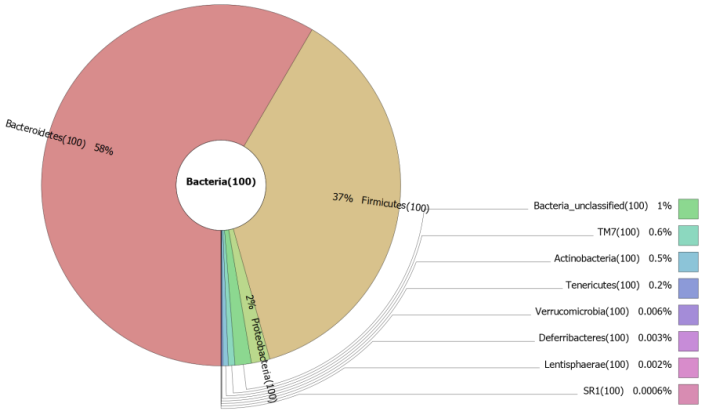
Among the bacterial phyla in the faecal sample of the experimental groups, the major percentage relative abundance was of Bacteroidetes, Firmicutes, and Proteobacteria, followed by Actinobacteria and TM-7 while the remaining (< 1% 16S rRNA reads) were minor phyla (Fig. 3.7 & 3.8). Bacteroidetes and Firmicutes contributed more than 90% of the relative abundance in the gut microbiota, while the remaining sub-dominant phyla were less than 10%. Bacteroidetes (58%) and Firmicutes (37%) were recorded as the dominant phyla in control group. But in disease control groups (CD, H and HCD) the

Firmicutes were clearly the most abundant with (CD-61%, H-69% and HCD-80%) as compared to control (i.e. 37%) whereas; melatonin treatment had lowered their levels (CDM-34%, HM-18% and HCDM-44%). Further, the Bacteroidetes phyla was found to be decreased (CD-33%, H-23% and HCD-15%) in the above mentioned groups as compared to control (58%), but the relative abundance was reversed in the melatonin treated (CDM-59%, HM-79% and HCDM-52%) groups (Fig. 3.2, 3.3 & 3.4).

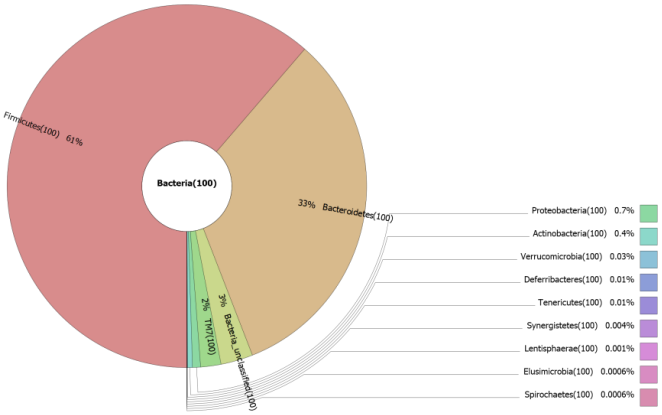
Microbial composition of minor order/genus in faecal microbiota of C57BL/6J mice subjected to high fat-high fructose diet and/or chronodisruption

An increase in relative abundance of the Group-Ruminococcaceae and Order-Desulfovibrionales were observed in the CD, H and HCD groups, but the same was reversed following melatonin treatment (CDM, HM and HCDM) (Fig. 3.10). Alistipes (an indole positive minor genus) were decreased in all the three disease control (CD, H and HCD) groups, but no change was observed in melatonin treated (CDM and HCDM) groups. The HM group recorded % relative abundancy comparable to that of the control group (Fig. 3.11). Further, Helicobacter abundance was found to be increased in H group whereas; no change was recorded in CD, HCD, CDM, HM and HCDM groups. Mucispirillum abundance was found to be increased in H, HM and HCDM groups whereas; the remaining groups showed no change (Fig. 3.11).

(A) Control



(B) CD



(C) CDM

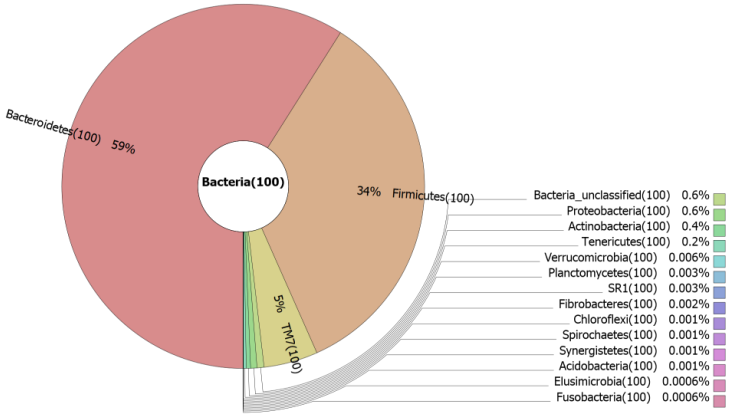
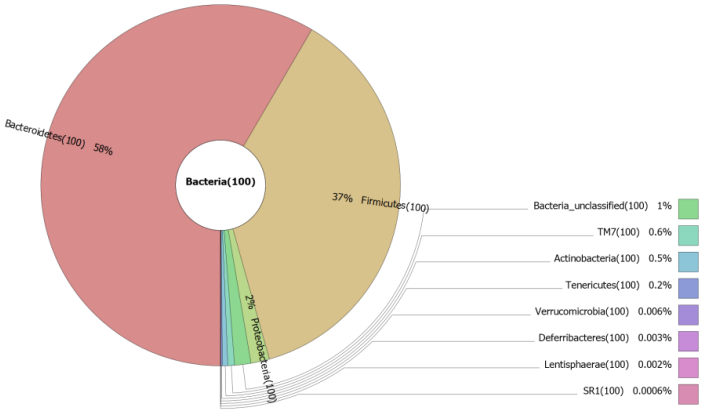
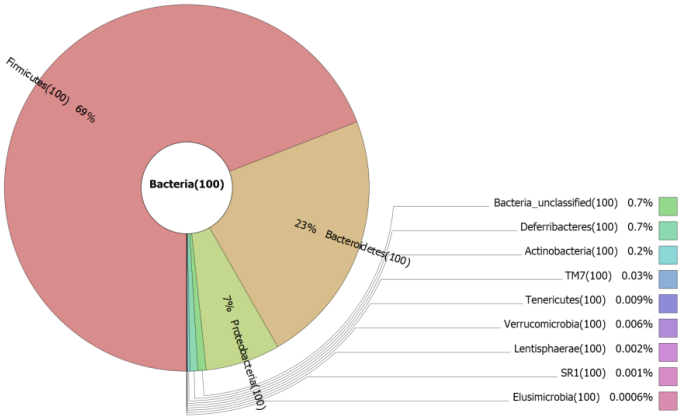


Figure 3.2: The percentage relative abundance of identified gut microbiota in mice subjected to chronodisruption (CD) and exogenous melatonin administration (CDM).

(A) Control



(B) H



(C) HM

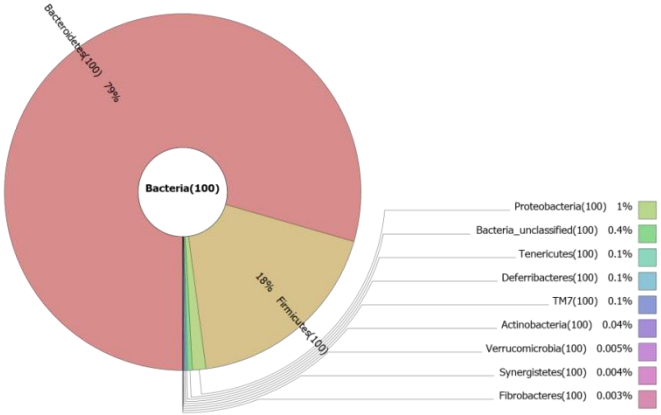
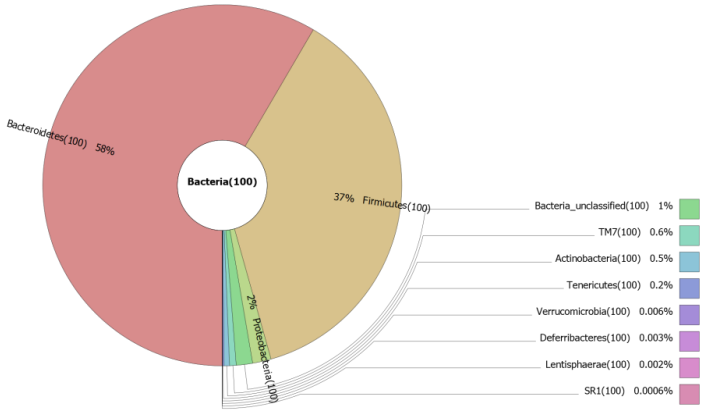
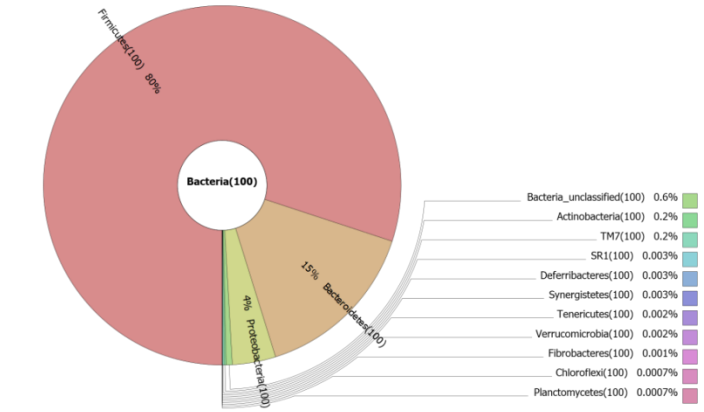


Figure 3.3: The percentage relative abundance of identified gut microbiota in mice subjected to high fat-high fructose diet (H) and exogenous melatonin administration (HM).

(A) Control



(B) HCD



(C) HCDM

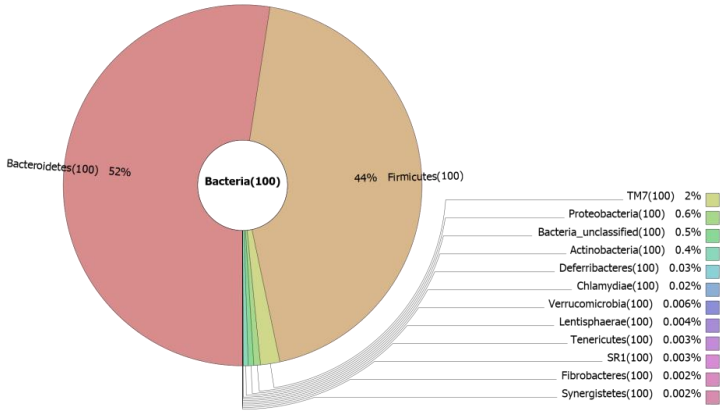


Figure 3.4: The percentage relative abundance of identified gut microbiota in mice subjected to the combination of both high fat-high fructose diet and chronodisruption (HCD) and exogenous melatonin administration (HCDM).

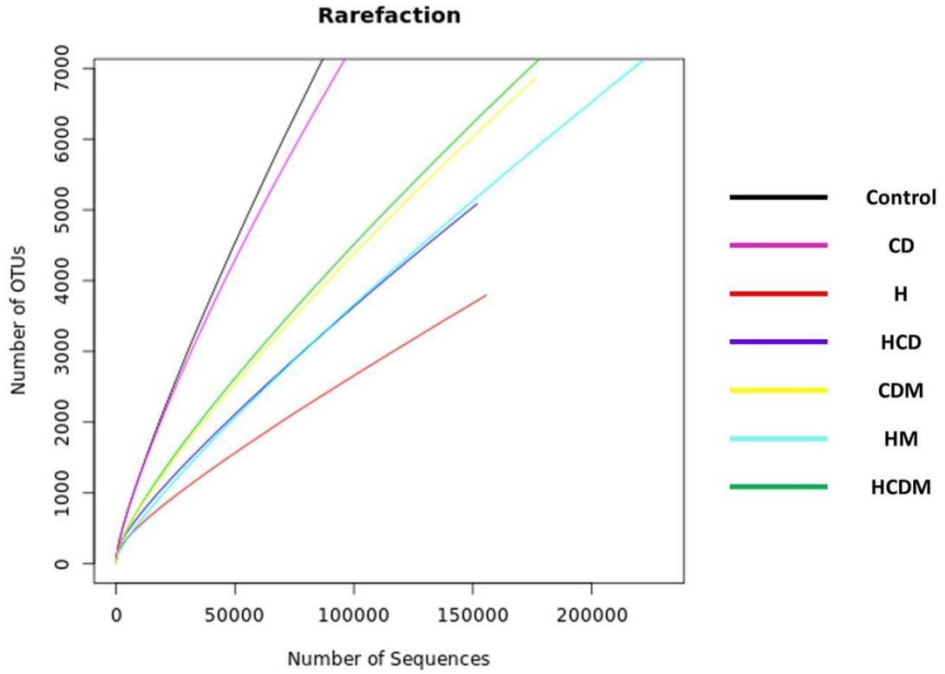


Figure 3.5: Rarefaction curves of microbial clusters in fecal samples of C57BL/6J mice subjected to high fat-high fructose diet and/or chronodisruption (CD, H and HCD) groups. Exogenous melatonin administration increased the species richness in (CDM, HM and HCDM) groups. The Y-axis indicates the number of operational taxonomic unites (OTUs) and each line for samples contains 97% confidence intervals.

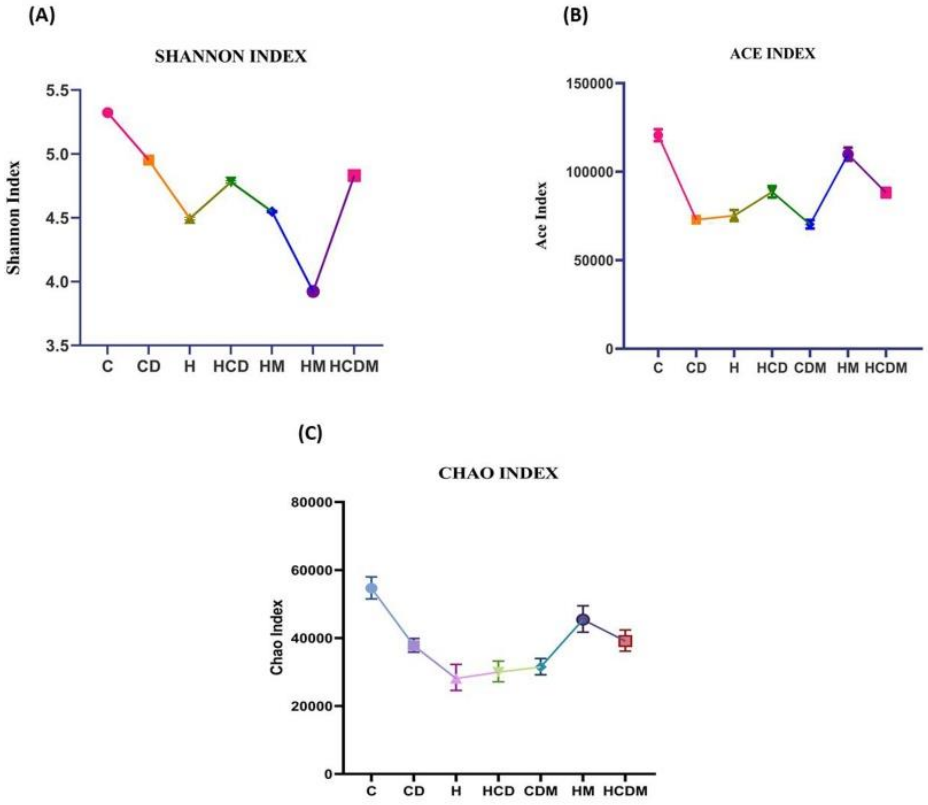


Figure 3.6: (A) Shannon (B) ACE and (C) Chao indices to access the species diversity and richness of the gut microbiome in the faecal sample of C57BL/6J mice subjected to high fat-high fructose diet and/or chronodisruption.

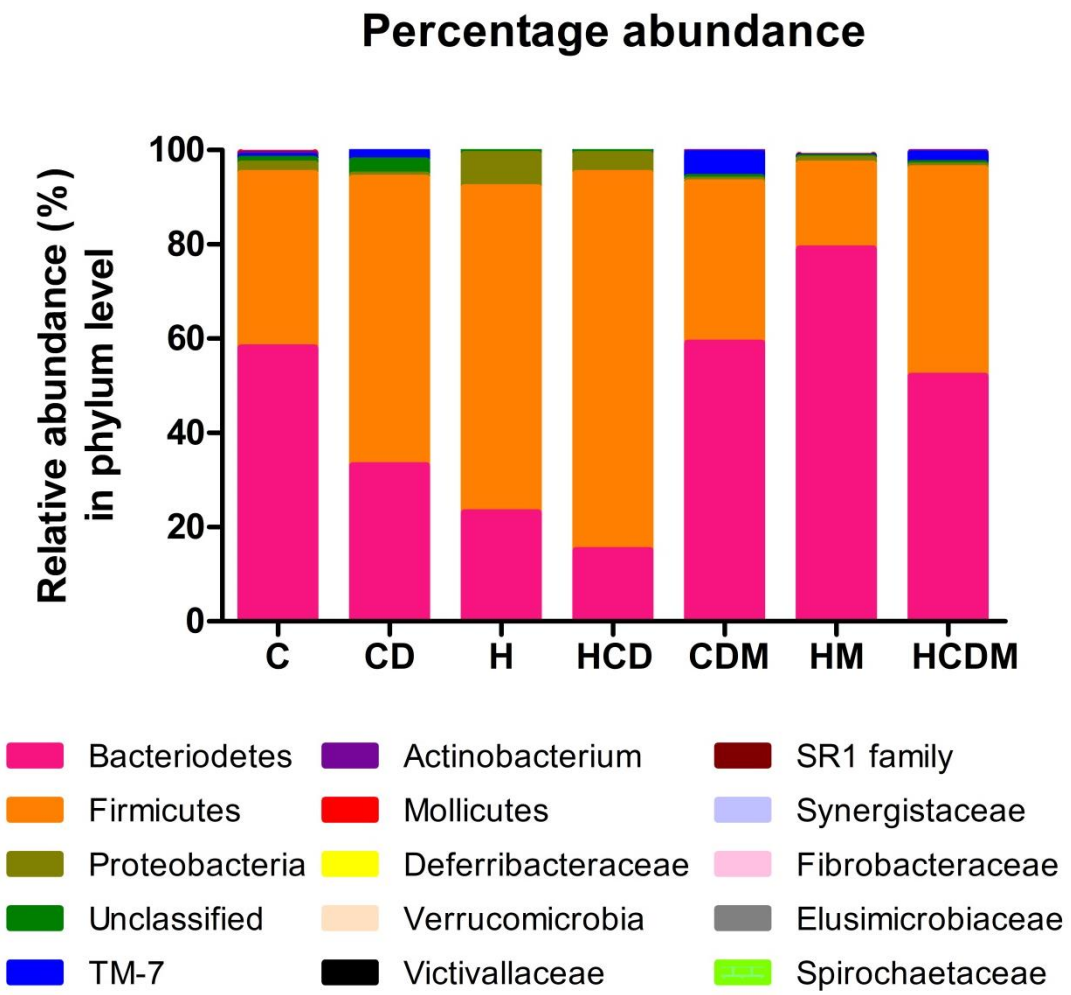


Figure 3.7: Stacked bar chart of relative abundance of the gut microbiota in faecal samples C57BL/6J mice subjected to high fat-high fructose diet and/or chronodisruption from pooled samples. Relative sequence abundance was calculated as the proportion of sequences belonging to a particular class of all 16S rRNA sequences recovered. The groups are C, (control); CD, (chronodisruption); H, (high fat-high fructose diet); HCD, combination of both (high fat-high fructose diet and chronodisruption); CDM, (chronodisruption and 10 mg/kg B.W i.p. melatonin); HM, (high fat-high fructose diet chronodisruption and 10 mg/kg B.W i.p. melatonin) and HCDM, (high fat-high fructose diet, chronodisruption and 10 mg/kg B.W i.p. melatonin).

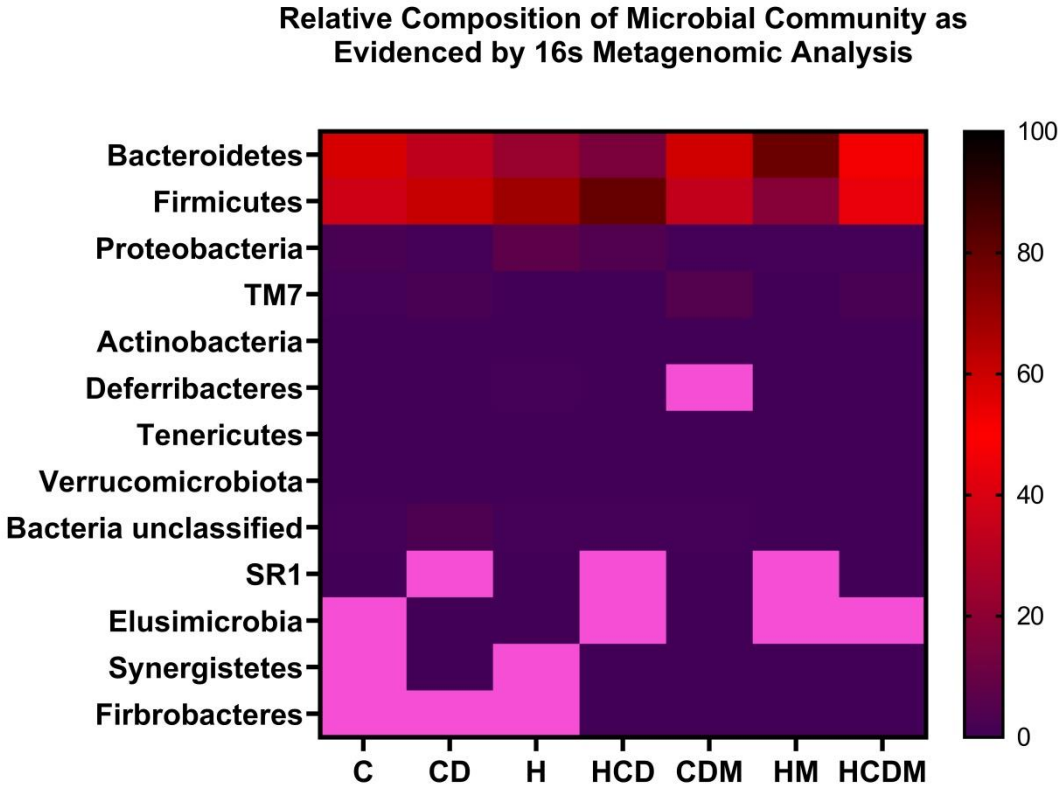


Figure 3.8: Heat map showing the relative abundances and distribution of representative 16S rRNA gene tag sequences classified at the genus level. The colour code indicates the percentage relative abundance ranging from purple (0 %) to orange (100%).

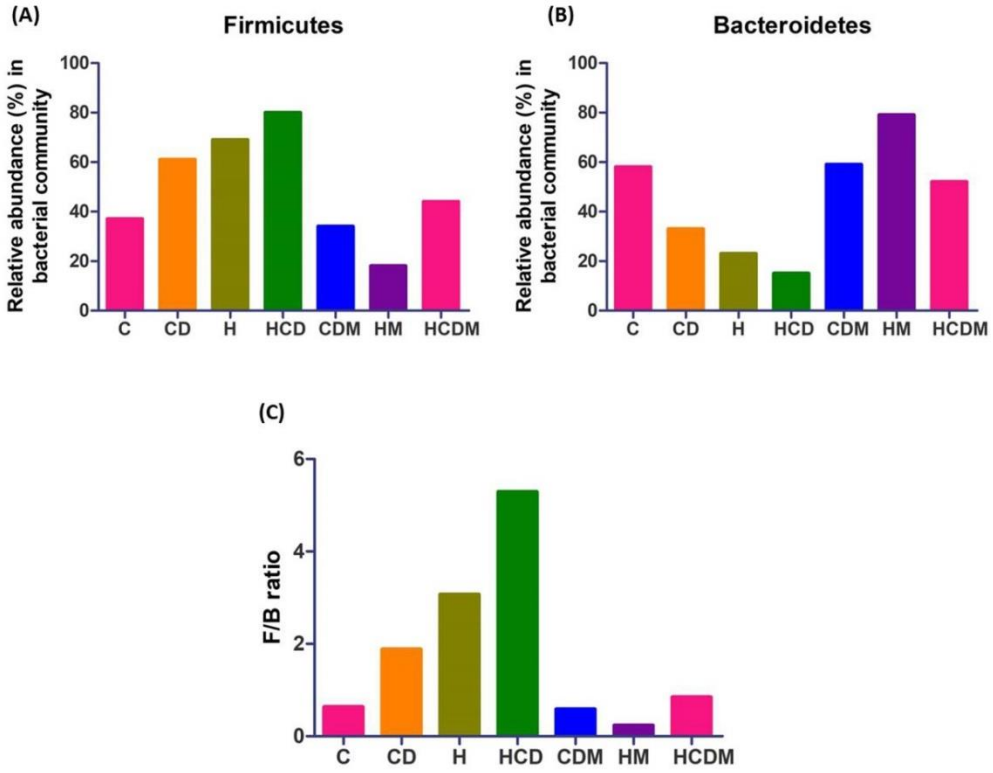


Figure 3.9: Increase in the abundance of Firmicutes and Bacteroidetes (F/B ratio) in faecal samples of C57BL/6J mice subjected to high fat-high fructose diet and/or chronodisruption for 18 weeks. An effect of timed administration of exogenous melatonin improves the F/B ratio as well as the abundance of Firmicutes and Bacteroidetes in gut microbiota.

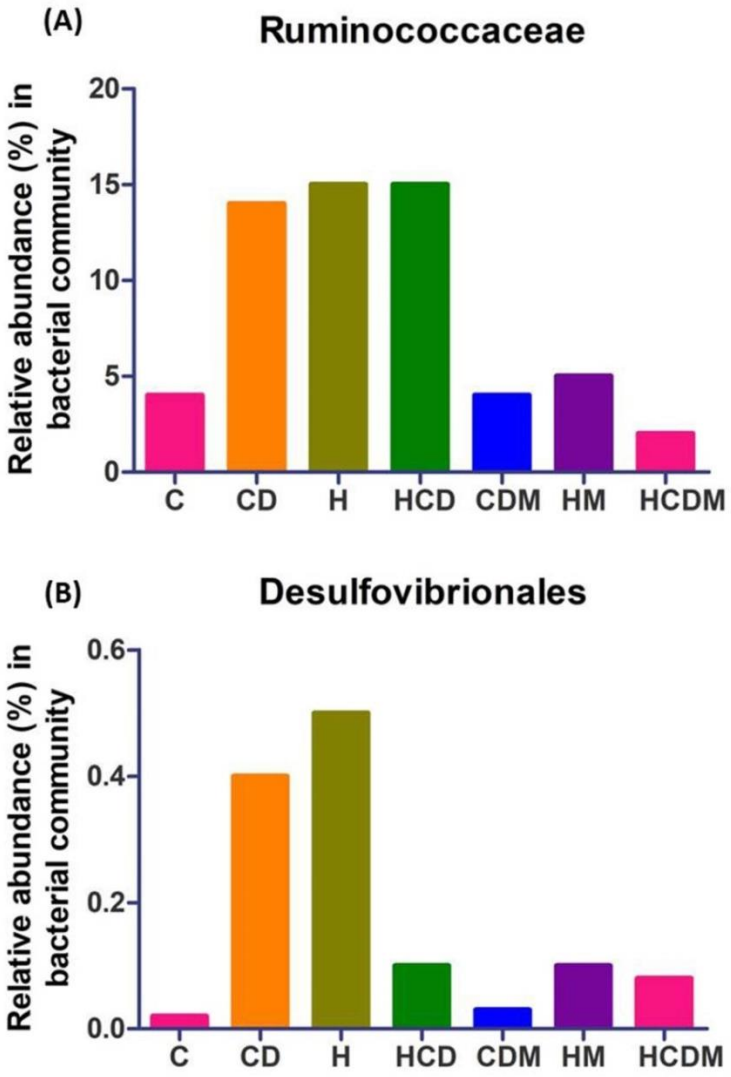


Figure 3.10: Alterations in the abundance of Ruminococcaceae and Desulfovibrionales in the C57BL/6J mice subjected to high fat-high fructose diet and/or chronodisruption for 18 weeks. Effects of timed administration of exogenous melatonin on the abundance of Ruminococcaceae and Desulfovibrionales in gut microbiota.

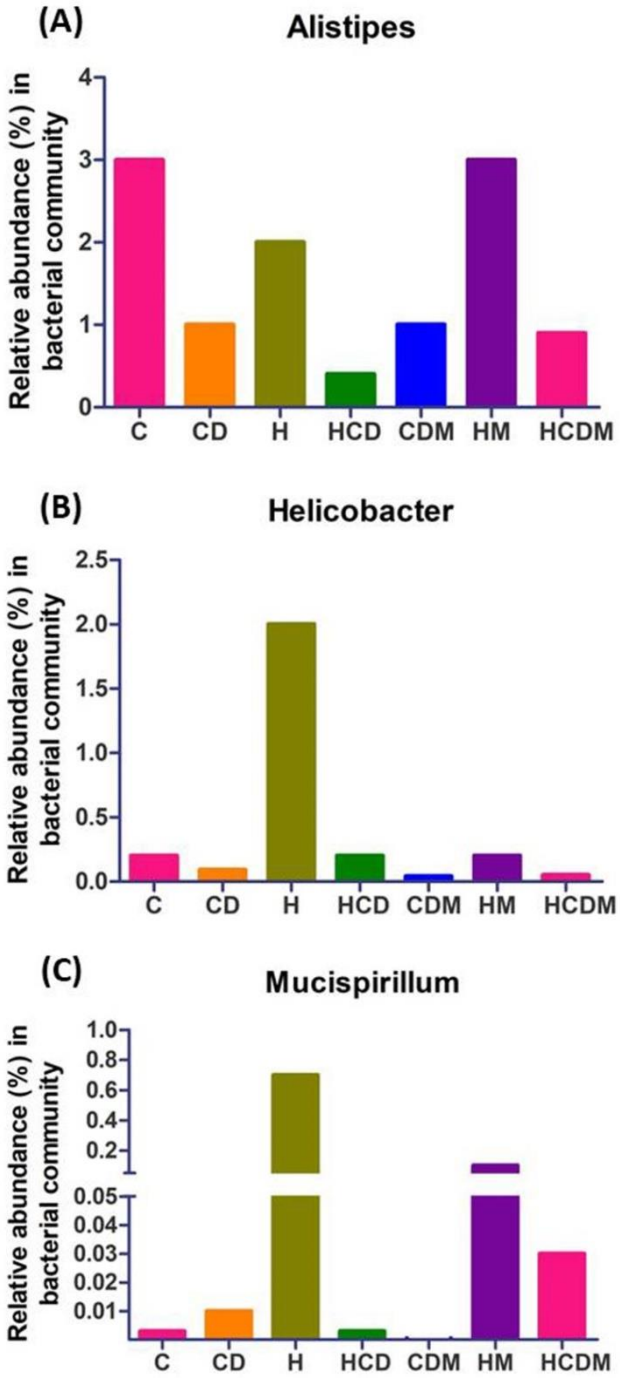


Figure 3.11: Alterations in the relative abundance of genus Alistipes, Helicobacter and Mucispirillum in faecal samples of C57BL/6J mice subjected to high fat-high fructose diet and/or chronodisruption for 18 weeks. Effects of timed administration of exogenous melatonin on the relative abundance of Alistipes, Helicobacter and Mucispirillum in gut microbiota.

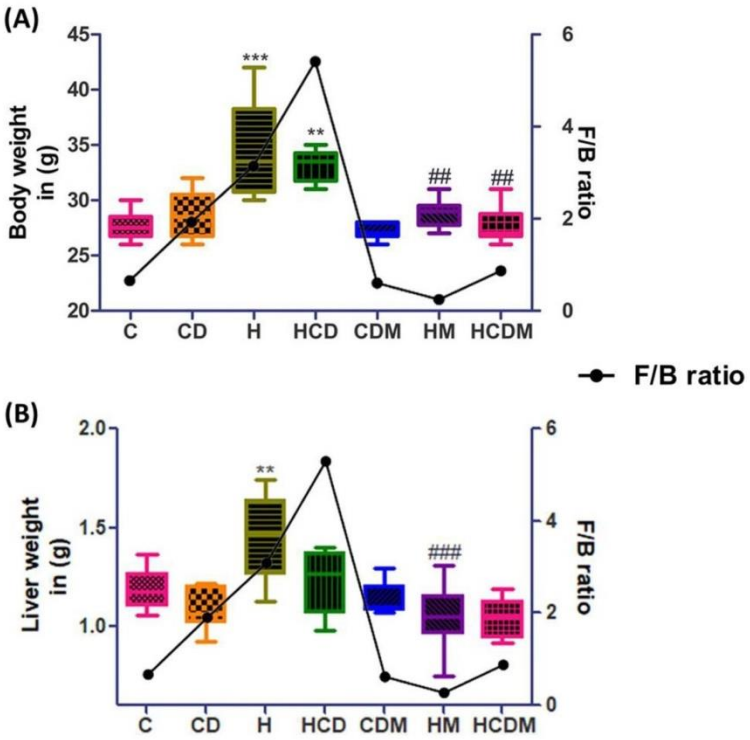


Figure 3.12: A positive correlation between the physical characters and F/B ratio in C57BL/6J mice subjected to high fat-high fructose diet and/or chronodisruption for 18 weeks. (A) Body weight and F/B ratio and (B) Liver weight and F/B ratio.

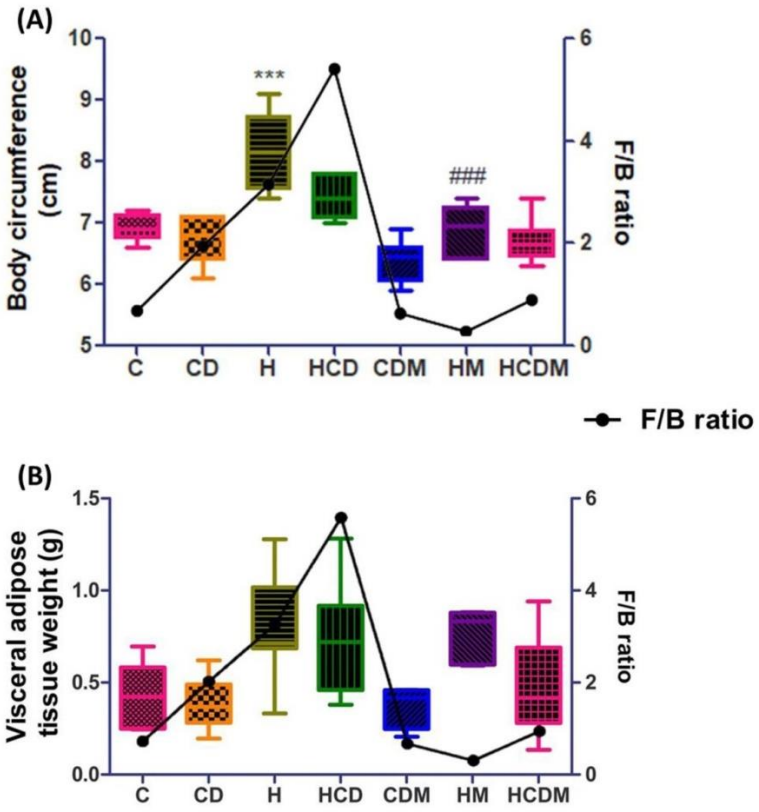


Figure 3.13: Correlation between the physical characters and F/B ratio in C57BL/6J mice subjected to high fat-high fructose diet and/or chronodisruption for 18 weeks. (A) Body circumference and F/B ratio and (B) Visceral adipose tissue weight and F/B ratio.

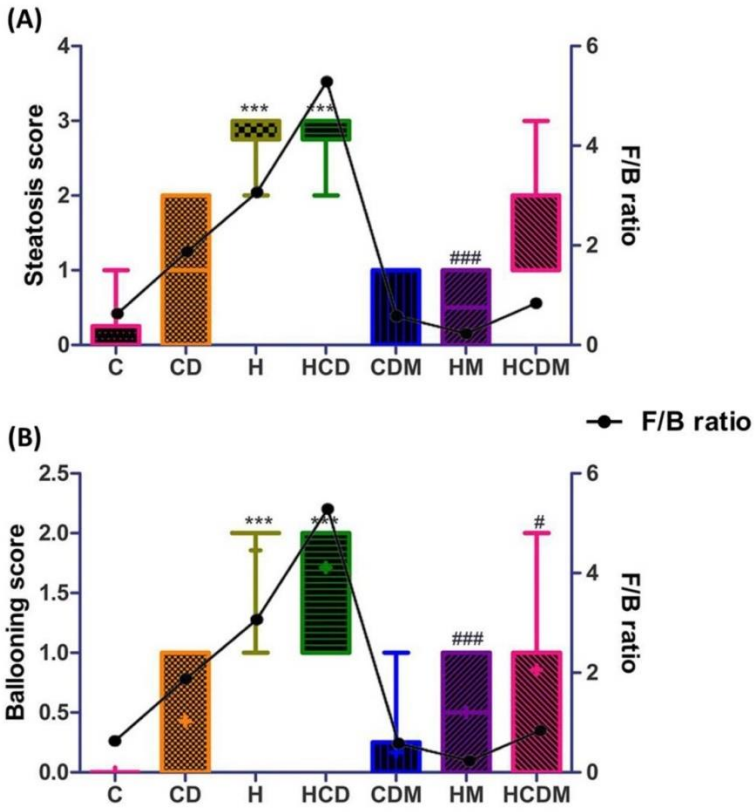


Figure 3.14: Correlation between the NASH histology and F/B ratio in C57BL/6J mice subjected to high fat-high fructose diet and/or chronodisruption for 18 weeks. (A) Steatosis score and F/B ratio and (B) Ballooning score and F/B ratio.

Discussion

Gut microbiota is an essential mediator in health and disease because it interacts with various organs and systems in the body such as liver, lung, bone, brain, cardiovascular system thus indirectly regulates metabolism, immunity and pathogenesis of diseases (Amoroso et al., 2020; P D Cani et al., 2008; Michielan & D'Incà, 2015; Schachter et al., 2018). The microbiota plays an important role in food digestion and metabolism due to specific genes that encode for enzymes implicated in human digestion. Microbiota-derived metabolites such as bile acids, short-chain fatty acids (SCFA), branched-chain amino acids, trimethylamine N-oxide, tryptophan, etc. play an important role in the pathogenesis of metabolic disorders (Agus et al., 2021; Jiao et al., 2018; Swann et al., 2011). Thus, gut-derived metabolites act as a link between gut microbiota and metabolic physiology of humans. Gut microbiota has been implicated for their multiple roles in metabolic-related diseases such as NAFLD and/or NASH through alterations in glucose and fat metabolism, obesity induction, endogenous ethanol production, endotoxin production, triggering inflammatory responses (Brandl & Schnabl, 2017; Ji et al., 2019) (Aron-Wisnewsky et al., 2013; Shen et al., 2017). The main cause of progression of NAFLD is change in dietary habits i.e. shifts towards high calorie or high fat-high sugar diet, altered light/dark cycle, consequently causing dysbiosis of bacterial composition in the gut along with inflammation and activation of host immune system (Serino et al., 2012; Vitaglione et al., 2019; Wei et al., 2020). Constant light exposure coupled with HFD alters composition of gut microbiota and impairs gut barrier function resulting in dysbiosis and NAFLD/NASH in SD male rats (Wei et al., 2020).

Metagenomic studies confirm that various metabolic disorders such as obesity, type 2 diabetes, atherosclerosis, NAFLD or NASH, IBD etc. are linked with imperative alterations in the composition of gut microbiota (Koliada et al., 2017; Monga Kravetz et al., 2020; Salah et al., 2019; Stojanov et al., 2020; Xiong et al., 2019; Yañez et al., 2021). In a healthy gut, the major bacterial phyla constitutes of gram-negative Bacteroidetes, gram-positive Firmicutes and Actinomycetes (Dudek-Wicher et al., 2018). The most common are the gram-positive Firmicutes and the gram-negative Bacteroidetes, with several other sub-dominant phyla including Actinobacteria, Verrucomicrobia, and Deferribacterota (Eckburg et al., 2005). In our study, there was a significant change in

the gut microbiota composition in all the disease control (CD, H and HCD) groups with the higher indices of Bacteroidetes and lowered Firmicutes. These results are in agreement with studies on male SD rats subjected to continuous lighting regimen for 4 weeks wherein; higher Firmicutes and fewer Bacteroidetes were reported (Chu et al., 2020; Turnbaugh & Gordon, 2009). Also, a patient study conducted on Ukraine obese adult population had reported significantly higher relative abundance of Firmicutes and lower Bacteroidetes as compared to lean the subjects (Koliada et al., 2017). The Shannon index is a qualitative indicator of the number of various bacteria present in the stool sample (Kim et al., 2017). In high fat diet fed male SD rats, constant light exposure for 16 weeks altered the gut microbiota in the colon and promoted NAFLD/NASH progression with a decrement in the Shannon and Chao1 indices (Wei et al., 2020). Also, the Shannon and Chao indices were found to be decreased in high fat-diet fed Wild type (WT) and homozygous Nod1$^{-/-}$ (NOD1 KO) C57BL/6J mice (González-Ramos et al., 2020). In our study, microbiota diversity was quantified using Shannon, ACE and Chao indices and the same were found to be decreased in CD, H and HCD groups as compared to control. These results were in agreement with the published reports on SD rats fed with high fat diet coupled with constant light exposure and in patients with obesity (González-Ramos et al., 2020; Wei et al., 2020).

Altered gut microbiota and low-grade inflammation can be restored or can undergo a partial or total reversal by using probiotics, prebiotics, antibiotics or hormone supplement (Dudek-Wicher et al., 2018; Ianiro et al., 2016; Tuohy et al., 2003; Yildirim et al., 2019; Yin et al., 2020). Melatonin (N-acetyl-5-methoxytryptamine) is a circadian hormone with 400 times higher concentration in the gastrointestinal tract (Bubenik, 2002) and; has been recognized for the modulation of gut microbiota (Xu et al., 2017; Yin et al., 2018). Further, melatonin can modulate gut microbiota, relative abundances and motility of human intestinal bacteria (Enterobacter aerogenes) (Paulose et al., 2016). Oral melatonin (50 mg/kg body weight; BW) administration to HFD-fed mice was reported to favourably alter Firmicutes: Bacteroidetes ratio, diversity, richness and content of Akkermansia in small intestine with concomitant improvement in liver steatosis and systemic inflammation body (Xu et al., 2017). Published reports from our lab had reported that intraperitoneal melatonin (i.p. 10 mg/kg body weight) improves high fat-high fructose diet and/or chronodisruption induced liver steatosis, lipid metabolic

disorder and hepatic antioxidant status by resynchronizing the hepatic core clock genes in C57BL/6J mice (Joshi et al., 2021). Hence, it was considered pertinent to assess the efficacy of melatonin in inducing possible improvement in gut microbiota and Firmicutes: Bacteroidetes (F/B ratio) in high fat-high fructose diet and/or chronodisruption treated model of NAFLD/NASH. In the present study, an improvement in the abundance of Firmicutes and Bacteroidetes and the F/B ratio were recorded in all the melatonin treated (CDM, HM and HCDM) groups (Figure. 1, 2, 3 and 7). Also, melatonin treatment reversed the Chao and ACE indices in HM and HCDM groups but no change was observed in the CDM group. These observations are in agreement with the published literature that establishes a strong link between melatonin and gut microbiota.

Metagenomic studies confirm that high fat-high fructose and/or chronodisruption induced NASH is associated with significant alterations in composition of gut microbiota. However, efficient studies on the alterations of gut microbiota in the condition of chronodisruption and/or high calorie diet induced nonalcoholic steatohepatitis are lacking. Herein, we had correlated the F/B ratio with morphometric and histopathological changes associated with NASH. The F/B ratio when overlapped with body weight gain (Fig. 3.12), liver weight (Fig. 3.12), visceral adipose tissue weight(Fig. 3.13), body circumference (Fig. 3.13), steatotic and ballooning scores (Fig. 3.14) had revealed significantly higher indices in H and HCD groups but, the same were reversed in melatonin treated (CDM, HM and HCDM) groups. A positive correlation is therefore seen between the F/B ratio and morphometric and histopathological changes in NASH. Several studies had shown that melatonin ameliorates NAFLD/NASH in a variety of animal models (Hatzis et al., 2013; Joshi et al., 2021; Pan et al., 2006; H. Sun et al., 2016) by reducing body weight, anti-oxidant, anti-inflammatory, reprogramming gut microbiota composition (Berger et al., 2019; He et al., 2010; Joshi et al., 2021; C.-K. Sun et al., 2017; Tan et al., 2015; Yin et al., 2018) and our findings are in agreement with these reports.

In summary, the correlation between F/B ratio vs. morphometric and histopathological changes in NAFLD/NASH highlights the efficacy of melatonin in gut microbiome reprogramming. The investigation of gut microbiota may be more appropriate in between

the duodenum and the ileum of the small intestine to check its efficacy (Ignacio et al., 2016; Kiela & Ghishan, 2016). Herein, it may be noted that, the said inferences have limitations because the faecal samples were not collected from the proximal/distal end of small intestine but the faecal samples (excreta) were collected and pooled (n=6) for analysis. Possibly, changes in the dynamics of bacterial minor phyla in the absorptive regions of small intestine went undetected and a study on the same would unravel their role in lifestyle disorders.