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Characterization Of Clostridium Cochlearium As A Potential Probiotic For Obesity Management

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CHARACTERIZATION OF *CLOSTRIDIUM COCHLEARIUM* AS A POTENTIAL PROBIOTIC FOR OBESITY MANAGEMENT

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Advisor Date

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DEDICATION

To big round eyes,

For the

worries, and missings, hidden in tiny tears

the dull, lonely hours, you hold so many years,

and yet

the brightest souls that I've ever seen

endless sweet songs whispering life's mean...

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CHAPTER-1

1. Introduction

1.1 Current global health threats

Worldwide, obesity has nearly tripled over the last four decades, in turn escalating numerous other metabolic ailments. In 2016, more than fifty percent of the world's adult population was overweight or obese [1]. In the United States, that is more than 71 percent [2]. The global prevalence of diabetes among adults has almost doubled since 1990 (National Center for Health Statistics 2017-2018). Moreover, obesity is associated with other serious health risks such as coronary heart disease, end-stage renal disease, certain types of cancer, and fatty liver disease. This epidemic of noncommunicable diseases was recognized as a new global health threat by the World Health Organization in 2018 [2, 3]. Overweight and obesity are linked with more deaths globally than malnutrition or underweight [1]. Obesity is preventable.

The fundamental approach to prevent obesity is to maintain the energy balance between calorie intake and calorie output. Avoiding calorie-dense food and increasing physical activity are the gold standard approach to maintain caloric balance. However, other factors such as genetics or psychological conditions should also be considered.

Despite much research and public health campaigns, obesity and associated metabolic complications keep rising worldwide. The advancement of next-generation sequencing technology and recent gnotobiotic mouse studies have revealed a strong connection between the gut microbiome and the host phenotype. It was found that germ-free mice are resistant to a high-fat diet, but once they were colonized with an obese microbiome, they developed obesity [4, 5]. This observation was quite analogous with fecal transplant studies in lean and obese human

volunteers[6]. The gut microbiome has attracted significant research interest recently regarding the etiology of obesity and associated comorbidities.

1.2 Gut microbiome

The human gut is a habitat for a very diverse range of symbiotic organisms and other microbes, collectively termed the gut microbiome. The microbiome and the host genome together produce a whole array of metabolites and signaling molecules such as secondary bile acids, short-chain fatty acids (SCFA), choline, Gamma-aminobutyric acid (GABA), serotonin, and more that can determine the metabolic phenotype and disease condition in the host. While many gnotobiotic mouse studies suggest that the microbiome is an influential environmental factor for host metabolism, mounting evidence of its contribution to human physiology emphasizes that the gut microbiome is an essential metabolic organ for human existence. It contains 3.8×10^{13} organisms, with thousands of different species [7]. Nearly half of all cells in the human body are microbial, and their collective genome includes one hundred and fifty times more genes than the entire human genome [8-10]. This ecosystem is an open and integrated unit composed of very diverse yet interactive indigenous microbes. These play a role in the community, contributing significantly to the whole microbiome ecosystem [11]. Functional communities exquisitely regulate biochemical pathways to determine the host metabolism. In the presence of non-native organisms, the inhabitants may amend their roles to keep the ecosystem balanced in the habitat. Consequently, this leads to the invention of probiotic-mediated gut microbiome alterations with the aim of positive changes in the host metabolic pathways [10, 12, 13].

1.3 Probiotics

Probiotics are "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host" [14]. Probiotic organisms are mainly sourced from the human gut and traditional fermented foods such as yogurt, cheese, pickles, or kefir grains. A narrow range of organisms, such as *Lactobacillus* and *Bifidobacterium*, mostly dominate probiotic research and the probiotic industry [15-18]. Other probiotics currently present in the field are *Saccharomyces, Streptococcus, Enterococcus, Escherichia,* and *Bacillus*. Though there is a vast diversity in the gut microbiome, novel probiotics are yet to be explored. Despite the unfavorable condition of the gastrointestinal tract (GIT), probiotics be live to perform their function(s). The major drawback of classical probiotics is their lack of persistence in the hostile environment of the GIT. In this context, the *bacillus* species, which are spore forming bacteria, have attracted more attention in the probiotic industry because of their ability to survive in unfavorable GIT conditions. There is evidence that vegetative cells, as well as spores, demonstrate probiotic activity. In spore formers, it was further found that some of the dynamic changing conditions such as acidity and bile concentrations immensely impact spore germination and outgrowth in the GI tract [19-21]. Though spore-forming probiotics are preferred, only several species exist from genus *bacillus* in the probiotic field [22].

With the development of metagenome sequencing and better culturing methods, probiotic research is stepping into a new era. So far, probiotics have only been in food supplements, yet soon, next-generation probiotics (NGPs) will likely come under a drug regulatory framework aimed at preventive and treatment measures. *Faecalibacterium,* or *Akkermansia,* are some of the uncommon genera classified for potential therapeutic species. Recently, non-canonical probiotic families abundant in the colon, such as *clostridiales* and *Bacteroidales,* are being explored as

potential NGPs. Several species of *Bacteroidetes* such as *Bacteroides fragilis* strain ZY-312, *Bacteroides xylanisolvens*, and *Bacteroides acidifacien* have already been considered as potential probiotics[23-26]. However, the other common genus, *Clostridium*, has not been explored to the same extent as *Bacteroides. Clostridium butyricum* is the only species in that genus found to be reviewed for over 50 years to treat *Clostridium difficile* infections, Helicobacter pylori infections, high cholesterol levels, and cancer [27].

1.4 Clostridium cochlearium

Nonpathogenic clostridium species, especially spore formers, could be potential therapeutic probiotics [28]. *Clostridium cochlearium* has shown various positive influences on gut physiology [28-30]. *C. cochlearium* is a gram-positive obligatory anaerobic spore-forming bacterium which usually presents in pairs and short chains [31]. *C. cochlearium* ferments glutamate via the methyl aspartate pathway to produce butyrate [28, 32]. Butyrate is frequently considered a health beneficial short-chain fatty acid. It is the primary energy source for the gut epithelial cells, and it involves regulating cellular metabolism and immune responses through gene expressions and cell signaling pathways [33-35]. Despite its close genetic relationship to the human pathogen *C. tetani*, *C. cochlearium* is a non-toxin-forming, nonpathogenic bacterium present in the human gut [36, 37]. It was identified to utilize glucose, galactose, glycerol, and ribose as a carbohydrate source [38], be able to ferment gelatin [39], convert tyrosine to phenol [40], and produce indole and H_2S [38] in previous studies. Further, it utilizes serine, methionine, threonine, leucine, alanine, histidine, aspartate, and tyrosine, and produces amino butyrate [40]. A recent study reported that *C. cochlearium* showed significantly higher electroactivity compared to other pre-selected mouse gut bacteria, revealing its capability of extracellular electron transference based on either redox-active molecules or planktonic cells [41].

However, there are few direct studies available on this species. The available literature only discusses the properties limited to its electrochemical activity [41] and amino acid metabolism [42-44]. Except for some genomic evaluations, *in vivo* study of its health effects on an animal model has never been performed. Specifically, information is lacking about its biological effects on the fundamental facts, such as growth conditions and viability in unfavorable gastrointestinal conditions, which are crucial for novel probiotic development.

Therefore, the goal of the present study was to determine the properties of *C. cochlearium* for growth and survival in *in vitro* conditions and investigate the effects and underlying mechanisms of administration of *C. cochlearium* on obesity and host metabolic processes using a high-fat diet-induced obese mouse model. We hypothesized that *C. cochlearium* is a potential probiotic to prevent obesity and related metabolic complications.

1.5 Specific aims

The existing literature does not provide essential preliminary data such as optimum growth conditions or *in vivo* viability potential in human gastrointestinal tract conditions and biological effects of *C. cochlearium*. Therefore, the specific aims were as follows.

Specific Aim 1

To determine the viability of *Clostridium cochlearium* in simulated gastrointestinal tract conditions and its optimal *in-vitro* growth conditions.

Specific Aim 2

To determine the potential anti-obesity effects of *Clostridium cochlearium* on a diet-induced obese mouse model.

Specific Aim 3

To determine the underlying mechanisms of the effects of *Clostridium cochlearium* on host metabolism.

CHAPTER-2- SPECIFIC AIM 1: TO DETERMINE THE VIABILITY OF CLOSTRIDIUM COCHLEARIUM IN SIMULATED GASTROINTESTINAL TRACT CONDITIONS AND ITS OPTIMAL IN-VITRO GROWTH CONDITIONS.

2.1 Introduction

2.1.1 Next-generation probiotics

The global probiotic market is in high demand, one of the most dominant source of nutrition supplement production. The probiotic organisms featured in these products are mainly based on human gut commensals or traditional fermented food. Probiotic research or commercial probiotic products are limited to two genera, which include *Lactobacillus* species and *Bifidobacterium* species [8]. Most of the probiotics in the market, including commonly exploited strains, are considered GRAS (generally regarded as safe) by food safety authorities.

With the development of better culturing techniques, affordable metagenome sequencing, and analytical tools, probiotics research has recently been exposed to a new era. The human gut microbiome is unique to each individual. Probiotic therapeutics can be one of the innovative approaches for initializing personalized medicine. Next-generation probiotics are a new and promising direction to explore the connection between the microbiome and disease management [27]. *Faecalibacterium, Akkermansia, Bacillus spp*. are some of the uncommon genera identified as novel biotherapeutics [27].

2.1.2 Spore forming bacteria

One of the primary challenges of probiotic supplements is ensuring their viability in the GIT. Though classical probiotics show exciting health effects, their survival rate in the GIT can be substantially low. Spore-forming bacteria attract greater attention due to their ability to produce spores in unfavorable GIT conditions. Sporulation is a survival strategy of bacteria to preserve the organism from hostile environments for vegetative cells [45]. *Bacillus* spp, have been thoroughly

investigated for both spores and vegetative cells and their cell cycles in the GIT [19, 22, 46]. It has been demonstrated that the bacilli spore can safely transit across the stomach, germinate, and grow in the upper intestines and sporulate in the lower intestines to be excreted as spores [46, 47]. The dynamic changing conditions of the upper GI tract, such as gastric acidity and bile concentration, are critical to trigger spore germination [47-49]. The most common spore-producing genus in the colon, *Clostridium*, has not been explored to the same extent as *Bacillus* spp. However, *Clostridium butyricam*, *Clostridium difficile*, and *Clostridium perfringens* spores have been studied previously [50].

2.1.3 Physiological conditions of the human gastrointestinal tract

To survive in GI transit, the probiotic must be resilient to the detrimental physiological conditions in the upper gastrointestinal track especially the stomach, duodenum, and proximal ileum. Hence, low acidity and bile salt tolerance are critical factors for bacterial survival and colonization in the human gut. The tolerance capacity of the GI tract tolerance is usually analyzed by either the bacterial growth rate or bacterial growth curve under the presence of experimental conditions[51] .

It is important to simulate the exact physiological condition for an *in-vitro* digestion model to understand the behavior of probiotics in *in-vivo* conditions. The pH, bile salts, and other digestive enzyme concentrations of the human GIT depend on the individual and the food ingested [52]. The physiological properties of the human GIT under normal condition are depicted in Figure 1.

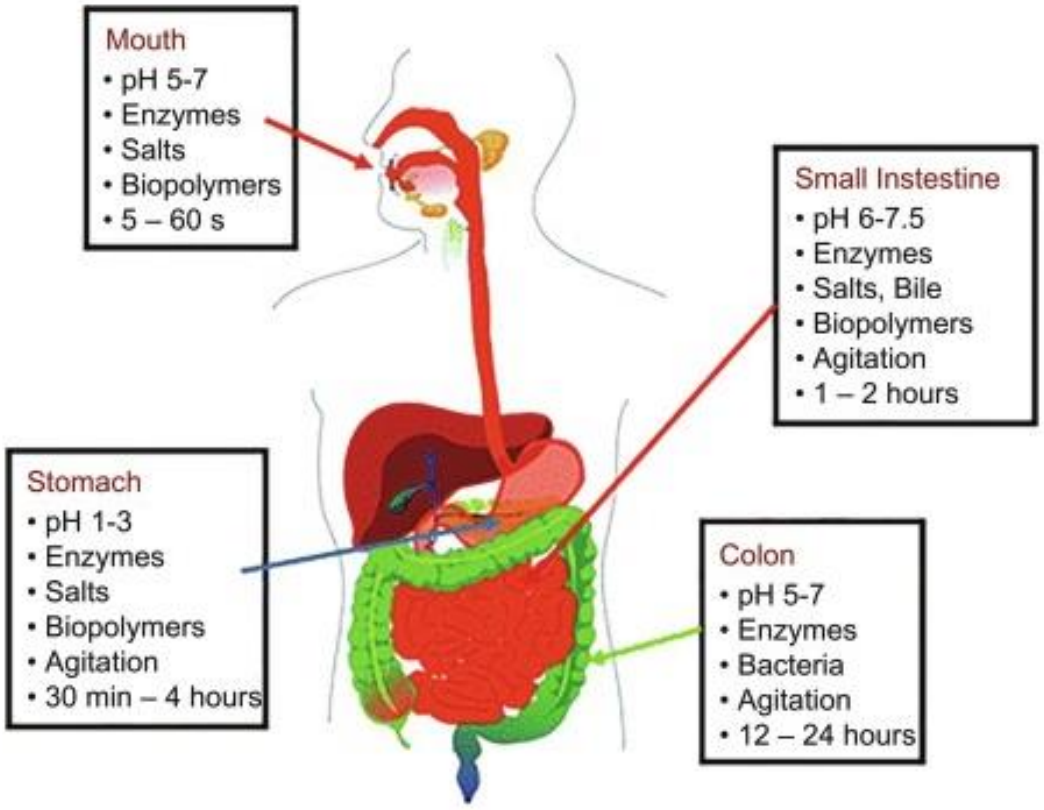

Figure1. The physiological conditions of the gastrointestinal tract [53].

The duration of the gastric phase is usually one to two hours. The intraluminal pH of the stomach in the fasting state is around 1-3, which rapidly increases to 5.5 - 6.5 in the small intestines. After entering the duodenum, chime mixed with bile salts, sodium bicarbonate, and enzymes causes the pH to increase as it reaches up to7.5 in the terminal ileum[54]. The pH drops to 5.7 in the cecum due to SCFA production from gut flora and gradually increases to 6.7 in the rectum. In the fed state, the bile salts in the duodenum are around 5-15 mM, while in the fasted state, it can be as low as 4.3-6.4 mM [53].

2.1.4 Influence of prebiotics and cross-feeding interactions on host metabolism

Prebiotics are non-digestible food ingredients that can benefit host health by promoting the growth and activity of microflora in the gut. A food ingredient can be considered as a prebiotic if it cannot be hydrolyzed or absorbed in the upper part of the GIT, an energy substrate for beneficial commensal bacteria in the gut, or able to provide beneficial effects for host health. The most common prebiotic category is the non-digestible carbohydrates[55]. Prebiotics, mainly resistant starch (RS) and non-starch polysaccharides, have been revealed to increase in SCFA production. Fermentation of some RS such as insulin, Galactooligosaccharides (GOS), and fructooligosaccharides (FOS), are favored by the butyrate-producing bacterial groups [56-58]. They have been reported to increase immunoregulatory interleukins (adiponectin, IL-6) and improve leaky gut, decrease high-fat diet-induced lipopolysaccharides (LPS), and pro-inflammatory cytokines (TNF-α, IL-1β) [59, 60]. Bacterial utilization of energy-rich substrates broken down by another species is called substrate cross-feeding. Similarly, utilization of the metabolic end products of one species by another species is called metabolic cross-feeding. Vuyst et al. (2015) proposed two distinct mechanisms of cross-feeding between the genus *Bifidobacteria* and butyrate producing colon bacteria. Butyrate-producing groups were observed to consume the end products (lactate and acetate) or partially broken down products released by the bifidobacterial metabolism [61] . Bacterial cross-feeding has a huge impact on the net SCFA production in the colon and thus determines the diet-induced alterations in the microbiota and the host pathophysiology. Ultimately, these alterations generate a basis for developing multiple metabolic modifications.

2.1.5 Bacterial growth conditions

The first step in probiotic development is to optimize *in-vitro* growth conditions. Commercial bacterial culture media are generally optimal, but modification of large-scale cell cultures is necessary to ensure cost-effectiveness. In addition, media pH, temperature, and other cross-feeding interactions should be considered to enhance the bacterial growth rate.

In the current study we first determined the viability of *C. cochlearium* in simulated gastrointestinal tract conditions and its optimal *in-vitro* growth conditions.

2.2 Methodology

2.2.1 Bacterial culture

C. cochlearium strain was purchased from ATCC (Manassas, VA). They were enumerated and cultured using anaerobic media and conditions according to the recommended method [62].

2.2.2 Culture media preparation

Peptone Yeast Glucose (PYG) broth media was prepared according to the recipe, cooled under nitrogen gas, and glucose, tween 80 and cysteine were added before dispensing into Hungate tubes under the same gas-phase then autoclaved at 121 ℃. 0.1% w/v. Resazurin solution (Thermofisher Acros Organics, Morris Plains, NJ) was used to identify the presence of anaerobic conditions.

2.2.3 Determine optimal pH

The pH of the media was adjusted from pH 2 - 11 with 1M Na_2CO_3 and 1M HCl using a pH meter before preparing Hungate media tubes. Then media tubes were inoculated with 10^9 CFU *C. cochlearium* 100 μL, followed by transferring 500 μL of cultured media in duplicates into a 48-well plate anaerobically, and optical density (OD) was recorded using HTS 7000 Biotek epoch

plate reader (PerkinElmer, Waltham, MA) every 30 minutes for twelve hours. All the readings were taken at 37 ℃ under anaerobic conditions.

2.2.4 Determine bile salt tolerance

Media tubes were prepared with corresponding amounts of bile salts to achieve the final concentration range 0.01% w/v, 0.05% w/v, 0.1% w/v, 0.2% w/v and 0.3% w/v desired for the experiment. Each tube (10 mL) was inoculated with 10^9 CFU *C. cochlearium,* and *500* μL of cultured media was transferred anaerobically into a 48-well plate, and optical density was recorded at 595 nm in duplicates every 30 minutes for fifteen hours using HTS 7000 Biotek epoch plate reader (PerkinElmer, Waltham, MA). All the readings were taken at 37 ℃ under anaerobic conditions.

2.2.5 Determine optimal temperature

Newly prepared PYG media tubes were inoculated with 10^9 CFU of *C. cochlearium* and incubated under different temperatures for 12 hours. Five hundred μL of culture was transferred from each Hungate tube into a 48-well microplate using an anoxic syringe, and optical density was recorded at 595 nm in duplicates in hourly intervals using HTS 7000 Biotek epoch plate reader (PerkinElmer, Waltham, MA).

2.2.6 Prebiotic supplements

The PYG media tubes were prepared without glucose, and sterile prebiotic solutions with final concentration 0.2% w/v were added to each Hungate tube before autoclaving. Each tube was inoculated with 10^9 CFU of *C. cochlearium, and* 500 μL of cultured media was transferred anaerobically into a 48-well plate, and optical density was recorded at 595 nm in duplicates every 30 minutes for fifteen hours using HTS 7000 Biotek epoch plate reader (PerkinElmer, Waltham, MA). All the readings were taken at 37℃ under anaerobic conditions.

2.2.7 Viability in simulated gastrointestinal digestion model.

Clostridium cochlearium was grown overnight to obtain 10^{10} CFU/mL, subsequently centrifuged to obtain the cell pellet, which was then washedwith PBS buffer, transferred to pH 2 media tubes and incubated for two hours at 37℃. Then, the acidic media was removed, and enteric digestion fluid (EDF), which was formulated to simulate the digestion process of the intestinal phase, was added into the tubes. EDF was prepared with the following formula: 0.5% w/v pancreatin (Sigma, St Louis, MO), 1.5% w/v bile salt (LP0055 OXOID, Ontario, Canada), 0.5% w/v amylase (Sigma, St Louis, MO), 0.1% w/v trypsin (Sigma, St Louis, MO), and 0.5% w/v lipase (Sigma, St Louis, MO) [63]. Samples were incubated for another two hours at 37℃ under anaerobic conditions.

After the four-hour digestion process, the bacterial pellets were transferred into regular PYG media tubes and incubated at 37℃, and optical density was recorded in duplicates every 6 hours. After each digestion in pH-2 and EDF, the tubes were vortexed well, and 1 mL of sample was obtained to harvest bacteria cells. The bacteria cells were pelleted, washed with sterile PBS, and stained with malachite green for microscopic analysis.

2.2.8 Clostridium cochlearium spore staining.

A drop (10^7 CFU/ml -10uL) of bacterial suspension was mounted on a glass slide, allowed to air dry and heat fixed. The smear was covered with a piece of paper towel, flooded with a 5% Malachite green stain, and steamed for 5 minutes. Then the slides were rinsed with water, and the cells were counterstained with safranin-O for 30 seconds. Finally, the smear was rinsed with water, dried, and observed under the light microscope and the electron microscope.

2.3 Results and discussion

2.3.1 Optimal growth conditions

The aim of the experiment was to determine the optimum temperature for *C. cochlearium* growth. The general optimum growth temperature for gut bacteria is the human body temperature, which is 37 ℃, but it can vary in *in-vitro* conditions. *C. cochlearium* grew in the range of temperature from 35 ℃ – 46 ℃ tested, as shown in Figure 2. The optimal temperature displayed was 43 ℃, and the best temperature range was 37℃ - 43℃. The bacterial growth rate decreased outside of that temperature range.

No significant difference was observed in the growth rate of *C. cochlearium* under different prebiotic supplements (Figure 3), but the final yield was significantly different. Two percent glucose was the carbon source available in regular PYG media. Galacto-oligosaccharide (GOS) significantly improved the bacteria yield than glucose. Prebiotics are mainly dietary carbohydrates, and galacto-oligosaccharides and fructans are the most recognized categories within that [60]. Fructans contain several prebiotics such as inulin and fructo-oligosaccharide (FOS) [60]; for this study, only FOS was included. Both GOS and FOS are oligosaccharides found in natural foods and have been shown to promote health-beneficial bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. in the human gut [64]. There was no difference in the effect between glucose and FOS. The PYG media without adding any carbon substrate resulted in low bacterial yield yet gave a similar effect as IMO supplementation.

C. cochlearium showed growth in pH 5 - 7 range, but pH 6 - 7 was the optimal range (Figure 4). *C. cochlearium* did not show substantial enumeration above pH 7 or below pH 4. While some bacteria such as *lactobacillus* spp., *streptococcus* spp., *Helicobactor pylori* are acidophiles several clostridiales such as *C. dificille* and *C. sordellii* have been identified as lacking several

proteins involved in acidic adaptation and broad range pH survival, which prevents them from growing under acidic environment. *C. cochlearium* displayed to be functional only in very narrow range of pH range indicating the incompetence of its acidic adaptability in the human GIT.

The aim of bile salt tolerance test was to determine the bile tolerance capacity of *C. cochlearium* in human small intestines. The bile salt available in the growth media significantly impacted the *C. cochlearium* growth (Figure 5). Bile salt concentration above 0.05% w/v showed inhibitory effects on *C. cochlearium* growth. The growth rate as well as the yield of *C. cochlearium* were considerably higher in the media without or with very little of bile salts (0.01 % v/w). The concentration of bile salts in small intestines ranges from 0.2% to 2% w/v [65]. Bile salts are antibacterial compounds that disrupt cell membrane, denature proteins, cause oxidative DNA damage, and stimulate host immune responses. Some bacterial species have adapted to the mammalian gut and developed resistance to bile salts through the remodeling of cellular responses [66]. Instead, it showed that *C. cochlearium* could not replicate under regular physiological bile salt range indicating potential bile salt intolerance.

3.3.2 Viability in simulated gastrointestinal digestion model.

As spore-forming bacteria, clostridiales can produce endospores under unfavorable conditions. Temperature and pH are critical factors that trigger spore formation of clostridiales [67, 68]. Heat shock is a common method to trigger bacterial sporulation [46, 54, 69]. In our results (Figure 6), *C. cochlearium* growth was not significantly impacted by the heat treatment. Both the high and low heat generated a similar response. The heat treatment reduced the growth rate and yield, and caused a longer lag phase compared to no treatment sample. This indicates possible sporulation during the heat shock. Spores take longer duration and sometime require external stimuli to germinate and outgrow compared to vegetative cells [20].

After determining the behavior of *C. cochlearium* in various pH, temperature, and bile salt concentrations, the responses were further analyzed under simulated GIT conditions. The incubation time and digestion media were selected according to the fasted condition of in-vitro digestion models mentioned in the literature [53, 54]. The gastric phase was tested with pH 2, and the intestinal phase was tested for addition of bile salts, pH 6.3, and digestive enzyme mixture. Live *C. cochlearium* bacteria and heat-treated bacteria were subjected to gastric digestion. The heat-treated sample was used under the assumption of that heat shock triggers sporulation, to distinguish the viability of spores and vegetative cells in the experimental conditions. There was no significant difference between heat-treated and none heat treated samples (Figure 7). The digestion treatment significantly lowered the yield of *C. cochlearium* compare to no treatment samples. However, *C. cochlearium* presented replication after 4-hour treatment of simulated GIT digestion. Analysis of spore formation under different pH ranges has revealed strain-dependent sporulation efficiency of genus clostridium previously. *C. dificille* and *Bacillus* spp. have depicted a low sporulation rate in an acidic environment. *C. perfringens* produces spores within a narrow range of pH 5.9-6.6, and *C. cellulolyticum* sporulated at pH 6.4 [21]. Moreover, *C. botulinum* spores shows an extended lag phase and decreased toxin production in the decreased temperature and pH [68]. We assumed that the high acidic and high bile salt concentration in the experiential setting triggered sporulation of *C. cochlearium* despite heat shock effect.

Spores can interact with bile acids along the GI tract. The spore germination is triggered by the assembly of small molecules called germinants with the membrane receptors of the spores. The germinants can be varied, including ions, amino acids, sugars, nucleotides, surfactants, which trigger irreversible germination to release Ca^{2+}- dipicolinic acid of the cell wall, increase water uptake, degrade the cortex, and outgrow the vegetative cell [70]. The host bile acid concentration

and composition, especially cholate derivatives, including taurocholate, glycocholate, cholate, and deoxycholate, can determine the germination of *C dificille* spores. In contrast, the deoxycholic acid has been shown to inhibit the *C. dificille* vegetative growth [21]. Further, chenodeoxycholate and lithocholate have been shown to inhibit taurocholate-mediated germination [71]. Hence, previous findings implied the importance of exploring the interactions of spore forming probiotics with host's bile acid metabolism.

Upon sensing unfavorable environmental conditions, the vegetative cell of *C. cochlearium* begins process of cell differentiation and sporulation. It is revealed that the clostridial cells form cigar shaped long sporangium with accumulated granulose at the initiation of sporulation. Then asymmetric division forms to give rise to pre-spore. The spore cortex and walls are formed subsequently before releasing the mature spore. Figure 8 shows the morphological changes *C. cochlearium* cells underwent to form spores in unfavorable in-vitro digestion conditions. The environment mimicking the pH of the stomach shows initiation of sporangium formation with endospores and free spores collectively counted for 68 present spores formed while 50% of them were free spores. Successive exposure to the enteric digestion fluid increased the free spore by 25% by increasing the number of total spores to 92%, suggesting the higher sustainability of *C. cochlearium* in the human gastrointestinal tract.

2.4 Conclusion

C. cochlearium cultures were tested in a variety of environmental settings to determine the optimum i*n-vitro* growth conditions. *C. cochlearium* shows optimum growth at temperature range 37 ℃ - 43 ℃, media pH range at pH 6 -7 and the presence of galactooligosaccharides in the media as the carbohydrate substrate. *C. cochlearium* do not replicate in extremely low pH (<pH 4) or high bile salt condition (> 0.1% w/v), indicating its incompetence to grow in the presence of

unfavorable conditions analogous to the upper part of the human small intestines. However, those unfavorable conditions trigger *C. cochlearium* endospore formation; thus, bacteria can survive in the GIT tract. Terminal ileum and colon is higher in pH and lower in bile concentration; thus, *C. cochlearium* might possibly colonize in the colon if it could survive throughout the stomach and the small intestines. The common challenge in probiotic development is to ensure the viability of probiotic in the GIT; hence, higher dosage or encapsulation techniques should be performed to confirm the probiotic travel to the colon safely to colonize in the gut. Current findings indicate *C. cochlearium* does not require extra preventive techniques to reinforce its viability in the GIT; thus, it is an ideal species for novel probiotic development.

CHAPTER-3- SPECIFIC AIM 2: TO DETERMINE POTENTIAL ANTI-OBESITY EFFECTS OF *CLOSTRIDIUM COCHLEARIUM* ON A DIET-INDUCED OBESE MOUSE MODEL.

3.1 Introduction

Multiple endogenous or environmental factors could contribute to obesity. There are complex deteriorations in metabolism over a period of time before presenting obesity and other noticeable health complications. It is important to select a surrogate animal model to mimic the molecular aspects of disease development when studying the pathophysiological effects of obesity. The C57BL/6 mouse is a particularly good model mimicking human metabolic imbalances presented in obesity because they develop insulin resistance, hyperglycemia and obesity when fed with a high-fat diet ad libitum. Germ-free (GF) mice studies and fecal transplant studies have repeatedly shown that the gut microbiome has an influence on obesity-related phenotype [4, 5, 72] , implying the potential of probiotic intervention to manage host health disparities [73-75]. Bile acid (BA) metabolism appears to interplay a key role in microbiome host synergism [65, 76, 77]. Bile acids are a vital metabolic component in dietary fat digestion and cell signaling. After being released into the intestinal lumen, 95% of bile acids are reabsorbed into the enterohepatic circulation, resulting in around 5% of bile acids being eliminated with feces. That is replenished by de novo synthesis of bile acids, which is around 200-600 mg daily in the liver [78]. However, a small fraction of bile acids (1%) escapes hepatic uptake, enters the systemic circulation, and reaches the peripheral tissues where they exert peripheral effects [15]. The bile acid pool is defined as the bile acids in the liver, gall bladder, intestines, and enterohepatic circulation. Before being secreted into the intestines, primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA) are conjugated with glycine or taurine to enhance their utilization for dietary lipid digestion. Conjugation increases bile acid solubility, thus better emulsifying activity as well as the

reabsorption efficiency. Gut microbes convert primary bile acids to secondary bile acids through biotransformation that increases the bile acids excretion with feces [79]. In mice most bile acids are taurine conjugated, while in humans they are glycine conjugated. Specifically, in mice a significant amount of CDCA is converted to α-muricholate (αMCA) and β- muricholate (βMCA) [80]. Primary bile acids that reach the large intestine (~5%) are bio-transformed by specific members of the gut microbiota via enzymatic reactions, deconjugation, dihydroxylation, and epimerization into secondary bile acids. Secondary bile acids increase the hydrophobicity of the bile acid pool, which is associated with greater toxicity. If being absorbed, secondary bile acids should be rapidly conjugated or sulfated to limit the damage to hepatocytes. Hence, only small amounts are absorbed, and the majority are excreted with feces. A recent study showed bile acid composition notably affected the spore germination of spore forming *clostridiales* [50]. The interchange of host bile acid composition and the bioactivity of the several *Clostridium* species have been presented in recent literature [21, 81]. In particular, TCA triggers the germination and outgrowth of *C. difficile*. Gut microbiome derived secondary bile acids decreased TCA mediated spore germination and outgrowth, growth kinetics, and toxin activity [82]. Even though this gut microbiome derived colonization resistance could be species or strain dependent, the outcome can impact the whole microbiome.

3.2 Materials and methods

3.2.1 *C. cochlearium* culture preparation

C. cochlearium strain (ATCC 17787) was purchased from ATCC (Manassas, USA) and cultured according to the manufacturers instructions. Cultures were grown to the exponential phase, harvested using anaerobic centrifugation, and resuspended in 25% glycerol, and then flash frozen to obtain samples and stored in -20˚C for use within one week. Prior to preparation of oral gavage samples, frozen bacteria were thawed and washed with phosphate-buffered saline and resuspended with sterile distilled water until the final concentration of 10^{10} colony-forming units per milliliter (CFU/mL) was reached.

3.2.2 Animals and Diet

This animal study was conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) of Wayne State University. Six to eight-week-old male C57BL/6 DIO mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed six animals per cage under constant temperature (24°C ± 1°C) and moisture of 40% ± 10% with 12-hour light-dark cycles and fed *ad libitum* with either a high-fat diet (D12492M - 60% kcal from fat) or a low-fat diet (D12450J - 10% kcal from fat) purchased from Research Diets Inc. (New Brunswick, NJ). Details are shown in Table 1.

3.2.3 Study Design

After seven days of acclimatization, all mice were randomly assigned into three experimental groups (n=12). Food and water were given *ad libitum*. High-fat diet (HF) control and low-fat diet (LF) control groups were gavaged with the vehicle of 200 µL of sterile water, and the treatment group (CC) group were orally gavaged with 200 µL of *C. cochlearium* 10^{10} CFU/mL and fed with high fat diet for 13 weeks. The basal parameters were recorded (body weight and

food intake) weekly while blood analysis (by tail vein puncturing) was done at 4th 8th and 12th week. Upon termination, mice were fasted for 8 hours and euthanatized by exposure to CO_2 and subsequent cervical dislocation. Blood samples were centrifuged to extract serum and flash frozen in liquid nitrogen, then intestinal content, and tissues (liver, intestines, cecum content, and fat) were obtained, rapidly snap-frozen in liquid nitrogen, and stored at -80°C for further analysis.

3.2.4 Blood collection and serum sample preparation

After each mouse was euthanized, and approximately 1 to 1.5 mL of blood was collected into a 1.5 mL microtube using heart puncture method. The blood sample was left undisturbed at room temperature for approximately 30 min and then centrifuged at 10 000 g for 10 minutes at 4 °C; the supernatant was aliquoted into microtubes for long term storage. All microtubes were stored in a - 80 °C freezer for future testing.

3.2.5 Fasting glucose and fasting insulin analysis

Fasting blood glucose (FBS) analysis was done each month of the study period. After eight hours of food deprivation, FBS was measured in each mouse by tail vein piercing (Accu-check glucometer - Roche, Indianapolis, IN). Fasting insulin was analyzed according to the manufacturer's instructions using Ultra-sensitive mouse insulin ELISA kit (#90080 Crystal Chem, Doners Grove, IL).

3.2.6 Oral glucose tolerance test (OGTT)

After eight hours of food deprivation, mice were given (oral gavage) 1 mg/kg body weight 10% w/v sterile glucose solution, then blood samples were obtained by tail vein piercing, and blood glucose level was measured using Accu-check glucometer (Roche, Indianapolis, IN) at 0, 15, 30, 60, and 120 minutes after glucose administration.

3.2.7 Serum cholesterol and triglycerides

Serum cholesterol and triglycerides were assessed using serum cholesterol and triglyceride liquid reagent kits (Pointe Scientific, Canton, MI).

3.2.8 Fecal calorie analysis

Twenty-four-hour feces were collected, air-dried and fecal energy (Pooled feces) of each group was measured by combusting duplicate fecal samples using Bomb Calorimeter (Parr, Moline, IL).

3.2.9 Body composition analysis

In the last week of the treatment, body compositions of each mouse were measured using EchoMRI-100 analyzer (EchoMRI, Houston, TX).

3.2.10 Statistical analysis

Results are presented as means ± SD. GraphPad Prism (Prism 9; Graph Pad Software Inc.) was used to perform statistical analysis. Student's t-test, one way or two-way ANOVA was used based on the number of independent variables involved, while repeated measure ANOVA was used to analyze means across one or more variables that are based on repeated measurements. For multiple comparisons, Tukey's post hoc test or Kruskal-Wallis test was performed according to data distribution. The significant difference is considered as $P < 0.05$.

3.3 Results and discussion

3.3.1 Effect on body weight gain & calorie intake

C. cochlearium administration significantly reduced high-fat diet-induced body weight gain on DIO mice (Figure 9). After 13 weeks of oral administration, the *C. cochlearium* treated group (71.69±14.94 %) showed 18% lower body weight gain compared to the HF (90.14±9.35%) group. The significant reduction of body weight gain by CC administration was first observed as

early as the 2nd week of treatment. Previously administration of various *lactobacilli* strains [83, 84] and *Bifidobacterium* strains [85, 86] have shown reduced body weight gain and adiposity in response to HFD on DIO mice. However, this is the first time that a potential probiotic showed such a weight-preventing activity after merely a week of dietary treatment. There was no significant difference in cumulative calorie intake between HF and CC groups (Figure 10), suggesting that the body weight reduction of CC supplementation could be due to its effect on energy expenditure. The high fat treated groups (CC and HF) showed significantly higher weight gain versus the control diet group confirming the fattening effect of a high-fat diet on DIO mice.

3.3.2 Effect on body composition and liver weight

Body composition analysis showed a significant reduction in fat mass (Figure 11-A) of the CC group compared to that of the HF group, while there was no significant difference in lean mass (Figure 11-B) between the two groups. The High-fat diet-fed mice (HF, CC) showed significantly higher fat mass than the low-fat diet-fed mice group, demonstrating the diet's effect on adipogenesis. No significant difference among the lean mass among the groups indicates the fat mass is the main contributor to the weight difference. There was no significant change in the liver mass (Figure 12-A) of the CC groups from the LF, but that was 35% lower than the HF group liver mass. Consistently the signs of lipid infiltration in CC group liver tissues (Figure 12-B) were less than that of the HF group, suggesting that high-fat diet-induced liver steatosis had been reversed by *C. cochlearium* intervention.

3.3.3 Effect on fecal calorie output

To assess the *C. cochlearium* mediated effects on host energy metabolism, we collected 24-hour feces, and fecal calorie output was determined. As expected, the LF (Figure 13) group fecal calorie output was significantly lower than the high fat diet fed groups. There was no significant

difference between the HF and CC groups. Weight gain reduction can be illustrated via the fundamentals of energy balance, such as low energy consumption, high-energy excretion, or high-energy expenditure. As the calorie intake (Figure 10) and fecal calorie output (Figure 13) did not significantly differ between the HF and CC groups, it is important to analyze the energy expenditure between CC and HF groups.

3.3.4 Effect on glucose metabolism

Probiotic studies repeatedly have shown favorable metabolic outcomes on the host [87-90]. The current study demonstrates the beneficial effects of *C. cochlearium* administration on lipid and glucose metabolism on high-fat diet-treated DIO mice.

Previous probiotic studies showed bodyweight reduction was often associated with positive metabolic outcomes, including improvement in insulin resistance and glycemic control [18, 91, 92]. The abundance of butyrate-producing bacteria in the gut has positively impacted insulin tolerance and type II diabetes mellitus [74, 93]. Correspondingly, our study shows that administration of butyrate-producing *C. cochlearium* significantly improved glucose homeostasis and insulin sensitivity in DIO mice (Figure 14). Fasting blood glucose was significantly lower in the CC group than that of the HF group from the 2nd month of the treatment. There was no significant difference in fasting blood glucose between CC and LF groups indicating that the high-fat diet-induced deterioration in glycemic control was improved in the CC group. Fasting serum insulin concentration values (Figure 15) further confirmed this observation. The CC group had a significantly lower plasma insulin concentration than that of the HF group but not significantly different from the LF group. Considering that the DIO mice model is severely insulin resistant, the data depicts insulin sensitivity and glucose tolerance improvement by C. *cochlearium* administration.

3.3.5 Effect on serum triglyceride and cholesterol

High-fat diet feeding was reported to increase weight gain, fat mass, hypercholesteremia, and glucose intolerance in DIO mice models. In addition, rapid lipid accumulation is connected with the predominant fatty liver condition [94]. As expected, mice in the high-fat diet fed groups presented elevated serum cholesterol (HF: - 158 ± 26.23 mg/dl, CC: - 112.7±20.12 mg/dl) compared to the low-fat fed group (LF: - 88.05±19.70) (Fig 17). Interestingly, *C. cochlearium* treatment decreased serum cholesterol by 29% compared to HF group while no significant difference with LF group was observed. No change in serum triglycerides levels was observed among groups (Figure 18). A recent probiotic study also noted that the short-term supplementation of probiotic consortium significantly reduced plasma cholesterol without changing the serum TG levels [95]. It was not clear why the high fat diet group serum triglycerides were not significantly different from those of the low-fat group. Probiotics have been identified to show hypocholesterolemic effects by altering cholesterol metabolism and transport [96-98]. Assimilation of cholesterol conversion of cholesterol to coprostanol or increase production of bile acids are identified mechanisms by which probiotics involve in impacting plasma cholesterol levels [98-100].

3.3.6 Effect on bile acid metabolism

All the serum bile acids species, including total bile acids, were higher in the HF group than the CC group. Tauro-conjugated primary bile acids were significantly decreased in the CC mice, resulting in a significantly lower total conjugated bile acid pool. Both primary and secondary bile acids groups were also significantly lower in the CC group. The bile acid pool is defined as the bile acids in the liver, gall bladder, intestine, and enterohepatic circulation. In the normal physiological condition, around 1% of bile acids in the enterohepatic circulation is spillover into

the systemic circulation [78]. Though the serum bile acids are not counted in the bile acid pool, serum bile acids composition can represent the composition of enterohepatic circulation [101, 102]. The significant reduction of serum bile acids level in the CC group could be due to low bile acid reabsorption through the intestinal wall. The hydrophobicity of bile acids as well as bile acid transporter expression levels could potentially affect the efficacy of bile acid reabsorption[103, 104]. Conjugation increases the bile acids solubility, thus better emulsifying activity as well as the reabsorption efficiency. Higher fecal excretion potentially lowered the bile acids in the entero-hepatic circulation, thus decreasing total bile acid pool and resulting in higher demand for *de novo* synthesis of bile acids in hepatocytes, which subsequently leads to the reduction of serum cholesterol [105]. High fat feeding is known to increases taurine conjugated bile acids [101]. Conjugated bile acids are metabolized by the microbes inhabited in the upper small intestines. Hence, altered microbiome in the upper small intestine can significantly affect bile acid composition [106]. Non-alcoholic fatty liver disease and insulin resistance have been associated with increased taurine conjugated bile acids and secondary bile acids [107]. Serum taurine conjugated bile acid level positively correlated with elevated plasma glucose and insulin resistance [106]. Manipulation of bile acid metabolism has been applied to control systemic lipid concentrations for decades. Diversity of intestinal bile acids composition has revealed to interfere with the enterohepatic circulation of bile acids, lowering plasma LDL cholesterol while raising HDL cholesterol, and apoA1 [108].

Administration of *C. cochlearium* significantly increased fecal bile acid excretion (Figure 21). Low level of tauro-conjugated bile acids indicates the bile acid deconjugation process is higher in the CC group. The total deconjugated bile acids are 10% higher in the CC group than the HF group. In the distal ilium, conjugated primary bile acids are deconjugated or dehydroxylated by

commensal gut bacteria with BSH activities, resulting in increased secondary bile acids, deoxycholic acid (DCA), and lithocholic acid (LCA) [109]. Recent mice studies have suggested taurine conjugated bile acids, especially TCDCA associated with induction of FXR signaling, microbiome alteration, and bile acid deconjugation [106, 110-112]. Both deconjugated bile acids and secondary bile acids are higher in hydrophobicity than their conjugated counterparts leading to a higher fecal excretion of bile acids [113]. Significantly high free CA and DCA can contribute to increased hydrophobicity of CC bile acids leading to higher fecal excretion. This explains the low serum bile acids in the CC group, possibly caused by a reduction in intestinal bile acids uptake. The significant increase in secondary bile acid and unconjugated bile acids level demonstrated high microbial biotransformation of bile acid in the CC microbiome. Additionally, unconjugated bile acids are less efficient at FXR activation [114]. We then analyzed the gene expressions related to bile acid metabolism.

3.4 Conclusion

In conclusion, we saw that daily treatment of *C. cochlearium* for 13 weeks exerts anti-obesity effects via reducing body fat mass. Obesity related other metabolic deteriorations, including hyperglycemia, impaired glucose tolerance, hypercholesteremia, and fatty liver condition, also improved, indicating the potency of *C. cochlearium* as a novel probiotic for treating obesity and associated metabolic complications. *C. cochlearium* treatment significantly alters bile acid composition, hence bile acid metabolism may play a role in C. *cochlearium* influence on host metabolism. The beneficial effects of *C. cochlearium* did not appear to have occurred through calorie (food) intake or fecal calorie output; thus, it is important to observe the energy expenditure of these mice to understand the fundamentals of the weight reduction ability of *C. cochlearium.*

CHAPTER 4 - SPECIFIC AIM 3; TO DETERMINE THE UNDERLYING MECHANISMS OF THE EFFECTS OF *CLOSTRIDIUM COCHLEARIUM* ON HOST METABOLISM

4.1 Introduction

4.1.1 Energy balance

Obesity is defined as a body mass index over thirty kg/m^2 or excessive fat accumulation that causes health risks. Obesity is often considered as being caused by excessive food intake and lack of physical activity. To be more precise, this concept should be viewed by using the terminology of energy balance. When the energy intake exceeds the energy expenditure, the positive energy balance results in energy storage increasing body mass, of which typically 60 to 80 percent is body fat [115]. Body weight gain is the result of positive energy balance over a given period. Energy intake involves energy consumption and energy harvest through GIT, while energy expenditure counts through resting metabolic energy and the thermic effect of food (TEF), which is the energy cost of absorbing and metabolizing food consumed as well as the energy expended through physical activity. Any other factors such as genetic or environmental that impact body weight must occur through one or more these components of energy balance.

Indirect calorimetry measures the energy produced by a living organism by placing the subject in a calorimeter and recording its oxygen consumption, carbon dioxide production, and preferably nitrogen excretion. This produces a more reliable estimate of energy expenditure (EE) in mice, calculated by a formula made from the Weir equation [116].

EE = ((3941(VO2) + 1106 (VCO2)) *1.44

VO2 = Volume of O2 consumption (ml/h/kg)

VO2 = Volume of CO2 production (ml/h/kg)

4.1.2 Regulation of bile acid synthesis

Bile acid synthesis involves dozens of enzymes through two major pathways, the classic pathway, which is the predominant pathway, and the alternative pathway. The major difference between these pathways is the chemical modification of steroid rings undergone in the production of bile acids from cholesterol. The rate limiting enzyme of the classic pathway is CYP-7-α1hydroxylase (CYP7A1). Bile acids are primarily ligands for both the nuclear farnesoid X receptor (FXR) and the membrane bound Takeda G protein-coupled receptor 5 (TGR5). Bile acid activated TGR5 signaling associated with cAMP induced thyroxin (T_4 to T_3) and uncoupling protein-1 (UCP-1) activation thus increase energy expenditure in brown adipose tissues, as well as glucagon-like peptide (GLP-1) to improve glucose tolerance[117]. Murine studies have shown that bile acids induce TGR5 signaling in the order of TLCA > LCA > DCA > CDCA > CA to increase energy expenditure in brown adipose tissue [108]. On the other hand, FXR, which is highly expressed in the liver and intestines, is activated by free and conjugated-bile acids, with CDCA being the most effective bile acid ligand of FXR, followed by LCA, DCA, and CA. Hydrophilic bile acids ursodeoxycholic acid (UDCA) and αMCA and βMCA are FXR antagonists.

FXR is the key regulator of bile acid synthesis, biliary secretion, intestinal bile acids reabsorption, fecal excretion, and hepatic bile acid uptake from the portal vein, which consequently has an impact on cholesterol, lipids and glucose metabolism. Additionally, low FXR expression or absence of intestinal FXR shows increased level of serum and hepatic bile acids, cholesterol, triglycerides, and pro-atherogenic serum lipoproteins [118].Hepatic FXR activates small heterodimer partner (SHP) and liver receptor homolog-1 (LRH- 1) and subsequently represses the activation of CYP7A1, the rate limiting enzyme of bile acid synthesis. It was found that the

intestinal, but not hepatic, FXR signaling pathway involved in the bile acids derived metabolic changes [110, 119].

4.1.3 FXR/FGDF15 Axis

Intestinal FXR mediated bile acid regulation is reported to be significantly associated with the gut microbiome. Intestinal bile acids trigger Intestinal FXR, which induces fibroblast growth factor 15 (FGF15) to activate FGF receptor 4 to inhibit CYP7A1 and ultimately to inhibit bile acid synthesis. FXR activation also induces bile acid transporter expression such as BSEP, OSTα/β and while inhibiting NTCP. The FXR-dependent FGF15/FGFR4 gut-liver signaling pathway maintains the bile acid synthesis and enterohepatic circulation, which consequently has an impact on cholesterol, lipid, and glucose metabolism. The reduction of intestinal FXR mediated bile acid uptake into portal blood circulation results in higher fecal excretion of bile acids. Low delivery of bile acids back to the liver lowers the intercellular bile acids, thus increasing liver bile acid synthesis [120, 121]. Studies conducted with various mouse models, such as gnotobiotic, anti-biotic treated, FXR/ FGF15 ablated, have explored how gut microbiome influences the bile acid composition mediated FXR- FGF15 pathway to induce adipogenesis. A human study has shown that antibiotic treatment impacted FXR mediated alterations of gut microbiome, bile acid metabolism and insulin sensitivity [12]. It was found that intestinal FXR knock out mice decreased *lactobacillus* spp., and BSH activity in the intestinal content significantly and became resistant to high fat diet induced obesity [112]. FXR deletion is associated with protection against weight gain, glucose, and insulin intolerance despite higher plasma triglyceride levels. However, the precise role of FXR is still debatable, as conflicting findings report FXR signaling promotes [112] or protects [110, 122] against obesity.

Taurine conjugated bile acids such as TCA, TCDCA [119] were found to be better FXR agonist whereas T-β-MCA,UDCA [123] and Gly-MCA are strong FXR inhibitors. Though TCDCA is a weak activator of FXR, in the presence of TCA, it also can be a FXR inhibitor [112]. The resistance to adipogenesis and the beneficial lipid profile observed in germ free mice relates to higher TCA mediated FXR activation and CYP7A1 inhibition. Conventionally raised (CONV-R) mice are dominated by T-β-MCA mediated FXR suppression, thus CYP7A1 activation. Additionally, FXR agonist TCA and TCDCA treatments activate intestinal FXR- FGF15 pathway [124].

4.1.4 Reverse Cholesterol Transport

As an end product of cholesterol catabolism, a bile acid pool plays a major role in cholesterol homeostasis. The daily production of bile acids, which is approximately 500 mg, accounts for 50% of cholesterol turnover. De-novo synthesis of cholesterol accounts for more than 50% of total cholesterol production by the liver, while others are derived from dietary sources. In addition to de-novo synthesis, the major source of cholesterol for cells is receptor mediated cholesterol uptake from lipoproteins. Excess CL that exceeds hepatic needs is usually packed into VLDL and then transported to peripheral tissues after conversion to LDL. Liver X receptors (LXRs – LXRα, LXRβ) are cholesterol sensing nuclear receptors activated by cholesterol derivatives. LXRα is expressed more in the liver, adipose tissue, and macrophages, while LXRβ is expressed ubiquitously. LXR promotes reverse cholesterol transport (RCT), the process of cholesterol delivery from peripheral tissues to liver for excretion. Elimination of excess cholesterol from the peripheral tissues and lipid laden macrophages occurs through the reverse cholesterol transport (RCT) pathway. RCT is an important preventive mechanism against atherosclerosis development [125]. LXR induces a cluster of lipo-proteins genes, including Apo A, and Apo E and number of

other genes involved in cellular CL efflux mechanism. Several studies have reported the different stages of reverse cholesterol transport, clarifying the formation of nascent HDL and their remodeling into mature HDL by receiving cholesterol esters through ABCA1 and SR-B1 [126] via LCAT before returning to the liver. SR-B1 and ABCA1 are key devices of cholesterol efflux from cholesterol laden arterial wall macrophages. After returning to the liver by HDL, cholesterol is further converted into bile acids. Mice lacking LXRα reported showing an accumulation of cholesterol in the liver when they were fed a high cholesterol diet. Moreover, LXR activation was identified to improve GLUT4 mediated insulin tolerance in both humans and mice [127, 128]. Overall, LXR is a key regulator of lipid and carbohydrate metabolism. In humans, mature HDL particles transfer CL to LDL-C catalyzed by the cholesteryl ester transfer protein (CETP) [125], which makes the human cholesterol metabolism differernt from rodents.

4.1.5 Sterol regulatory element–binding proteins (SREBPs)

SREBPs are key regulators of lipid homeostasis, which can activate the expression of more than 30 gene-related pathways. The transcription factors SREBP1 and SREBP2 are the predominant isoforms within that family [129]. At normal physiological conditions, SREBP-1c favors fatty acid biosynthesis, which involves acetyl-CoA carboxylase (ACC) and fatty acid synthase FASN, while SREBP-2 favors cholesterol synthesis, including the rate limiting enzyme of indigenous cholesterol synthesis HMG-CoA reductase. Patients undergoing bile acid sequestrant therapy or ileum resection show derepression of CYP7A1 due to low bile acid absorption, thus depleting hepatic cholesterol and leading to increased SREBP2 activity [105]. Both the SREBP and CYP7A1 are found to be regulated by the liver X receptor (LXR) family of proteins [130, 131].

4.1.6 Sphingolipid pathway

Furthermore, Intestinal FXR shows a correlation with serum and ileal ceramide levels. Endogenous FXR agonist also depicts the activation of ceramide and sphingolipids pathways [121]. Mice lacking certain species of ceramides have shown protection against diet induced obesity and insulin resistance. Conjugate bile acids also activate the sphingolipid pathway [132]. Activation of S1P2 by conjugated bile acids inhibit CYP7A1 and bile acid synthesis by activating SHP to inhibit gene transcription [132].

4.2 Materials and methods

4.2.1 Metabolic Chamber-metabolic activity analysis

In the last week of the study, mice were housed individually in the TSE PhenoMaster metabolic cage system (TSE systems, Chesterfield, MO), and oxygen consumption (VO_2), CO_2 production (VCO_2) and total energy expenditure were recorded every 39 minutes (time points) for five days to generate the respiratory quotient, and total energy expenditure. Temperature in the metabolic chamber was kept constant at 24 °C, and animals had free access to food and water.

4.2.2 Quantitative PCR

Total RNA was extracted from liver tissues using the RNeasy Mini Kit (Invitrogen) following the manufacturer's protocol. Complementary DNA was synthesized using iScript reverse transcription supermix, and real-time quantitative PCR was performed using SsoAdvanced universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Targeted Gene-specific primers were used, and all the results were normalized with the housekeeping gene β-actin, and GAP-DH and relative gene expressions were determined using the Livac method.

4.2.3 Statistical analysis

Results are presented as means ± SD. GraphPad Prism (Prism 9; Graph Pad Software Inc.) was used to perform statistical analysis. Student's t-test, one way or two-way ANOVA was used based on the number of independent variables involved, while repeated measure ANOVA was used to analyze means across one or more variables that are based on repeated measurements. For multiple comparisons, Tukey's post hoc test or Kruskal-Wallis test was performed according to data distribution. The significant difference is considered as $P < 0.05$.

4.3 Results and discussion

4.3.1 Effects on metabolic activity

A diurnal comparison (3-4 full light/ dark cycles) was conducted to evaluate the energy expenditure of the mice. The metabolic activity of the mice in the CC group and the HF group were assessed every thirty-nine minutes for five days, and the data of the middle three-day were used to determine their metabolic activity. We observed significantly higher energy expenditure in the CC group versus the HF group both in the light and the dark cycles of the day as shown in **Figure 25**. The O2 consumption (**Figure 23**) and CO2 production (**Figure 24)** were also significantly higher in the treatment group. Interestingly, another Butyrate-producing bacterium, *Eubacterium hallii,* has recently been reported to increase energy expenditure in db/db mice[87]. Butyrate supplementation was previously reported to improve insulin sensitivity and energy expenditure in DIO mice [133]. The observed improvement of insulin sensitivity, and bodyweight reduction, and the energy expenditure of DIO mice upon *C. cochlearium* treatment could be due to its ability of butyrate production [116]. In addition, the increased bile acid pool and individual bile acid species have shown a positive correlation with thermogenesis and protection against diet-induced obesity previously via the TGR5 signaling pathway and UCP1 activated non-shivering

thermogenesis in brown adipose tissues[134, 135]. We found several genes related to thermogenesis (**Figure 26-B & Figure 29-B**) overexpressed in the C. *cochlearium* treated group, possibly implying the upregulation of cellular thermogenesis mechanisms.

4.3.2 Effects on hepatic and intestinal gene expressions

Hepatic bile acid receptor gene expressions (**Figure 26**) showed a trend of upregulation, but the differences were not significant. The rate-limiting enzyme of the bile acid synthesis in the liver, which is CYP7A1 (**Figure 27-C**), is significantly upregulated in the CC group. It is important to consider that the upregulation of hepatic bile acid synthesis is triggered by low intracellular bile acid concentration. Liver FXR/SHP axis activation requires high intracellular bile acid concentration to induce the feedback inhibition of the CYP7A1 reaction[136]. It was found that the effective engagement for CDCA to give a half-maximal response (EC50) to trigger FXR is around 17 μM, and more than 100 μM for TCA under normal physiological conditions [136]. However, the intestinal FXR-FGF15 axis is identified as a more physiologically relevant pathway to regulate the bile acid feedback mechanism via enterohepatic circulation. Importantly, this pathway is independent of the hepatic FXR. Significantly low intestinal FXR and reduced FGF15 expression in the CC group indicate the absence of feedback inhibition of bile acid synthesis in the CC group. FGF15 is found in the ileum's absorptive cells ,which plays a major role in bile acid absorption[137]. Both the lowered intestinal FXR and FGF15 indicate bile acid absorption was reduced; thus, hepatic bile acid uptake through the enterohepatic circulation was low in the CC group. Previously, it has been reported that FXR-FGF15 axis is involved in modulate obesity [112]. Inhibition of intestinal FXR-FGF15 axis through probiotic treatment increased intraluminal retention of TCA, which was parallel to decreased absorption and low serum disposal [111]. Additionally, $FXR^{-/-}$and $FGF15^{-/-}$ animals showed CYP7A1 de-repression and higher bile acid

excretion with feces [111, 138]. A similar observation was made by the FXR antagonism [120, 123, 139]. Interestingly the phenotype was reversed with activation of the FXR transcriptional machinery by an FXR agonist [111]. This finding suggests that bile acid composition could play a critical role in FXR activation. In the CC group, we observed that CYP7A1 upregulation was driven by the inhibition of intestinal FXR mediated FGF15 signaling (**Figure 27 - A, B**) with no significant changes in SHP or other hepatic bile acids receptor expression (**Figure 26 - B, C**) levels. Different bile acids are endogenous FXR ligands with different affinities. Though both the conjugated and free bile acids can activate the intestinal FXR, there could be competitive inhibitory actions by the ligands with higher affinities. Thus, activation or repression of FXR has shown metabolic implications.

Higher bile acid excretion is a counterpart to an increase in fecal cholesterol and other lipid species. Low bile acids returning to the liver directly affects cholesterol metabolism. Notably, a recent study reported FXR activation significantly affects intestinal cholesterol absorption, bile acid composition, and reverse cholesterol transport to prevent atherosclerosis development [140]. To observe the impact of cholesterol metabolism, we analyzed gene expressions related to reverse cholesterol transport.

LXR, the critical regulator of RCT, induces a cluster of lipoproteins genes and several other genes involved in the cellular cholesterol efflux mechanism. *C. cochlearium* administration significantly upregulated the genes involved in RCT (**Figure 28**), including LXR, and target lipoproteins (Apo-A and Apo-E), and cholesterol transfer proteins ABCA1 and SR-B1. Hepatic LXR activation can be triggered by high cholesterol turnover to bile acids in the liver, stimulating other interconnected pathways to lipid and glucose metabolism. High cholesterol diet-fed mice, lacking LXRα, were reported to present reduced cellular cholesterol levels, demonstrating its

cholesterol clearance role. Overall, LXR is a crucial regulator of lipid and carbohydrate metabolism. Upregulation of reverse cholesterol clearance was an indication of improved serum lipid profile in the CC group, which could lead to lower lipid infiltration to hepatocytes. Lower serum cholesterol and lower hepatic steatosis in the CC group were compatible with the gene expression results involved in reverser cholesterol transport. Activation of LXR has been shown to increase β-oxidation and glucose oxidation in the white adipose tissues [141] as well as enhance energy expenditure in brown adipose tissues [142]. The lower fat mass and increased energy expenditure in the CC group might be triggered by LXR mediated metabolic modifications. Previous clinical studies supported the notion that regulation of SR-B1 and ABCA1 in humans is similar to that of rodents. Rodent models have shown that over expression of [125] SR-B1 decreased atherosclerosis. We suggest that the genes we focused on in this study related to reverse cholesterol clearance can be comparable to humans.

Sterol regulatory element–binding proteins (SREBPs) are key regulators of lipid homeostasis, activating more than 30 gene-related pathways. The transcription factors, SREBP1 and SREBP2, are the predominant isoforms within that family [129]. At normal physiological conditions, SREBP-1 favors fatty acid biosynthesis while SREBP-2 favors cholesterol synthesis. We found that *C. cochlearium* administration significantly increased SREBP2 expression with no change in the SREBP1 (**Figure 29-A, B**). Conversion of cholesterol to bile acids reducing the intracellular sterol concentration can trigger SREBP2 activation. Patients undergoing bile acid sequestrant therapy or ileum resection have shown reduced intestinal bile acids absorption and de-repression of CYP7A1, thus depleting hepatic cholesterol, leading to increased SREBP2 activity [105]. The lower serum bile acid in the CC group may be an indication of a reduction of enterohepatic bile acid uptake; thus, C. *cochlearium* treatment showed a similar outcome in DIO

mice by increasing both the SREBP-2 (**Figure 29-B**) and CYP7A1 (**Figure 27**). The rate-limiting enzyme of endogenous cholesterol biosynthesis, HMG-CoA reductase, was not upregulated in the CC group (**Figure 29-C**). Angiopoietin-like 4 (ANGPTL4) is a primary regulator of lipoprotein metabolism by inhibiting lipoprotein lipase (LPL) activity [143]. LPL is the rate-limiting enzyme of TG hydrolysis circulating lipoproteins in the blood such as VLDL and LDL. However, not the liver-derived but the adipose-derived ANGPTL4 has effects on triglyceride regulation, suggesting a tissue-specific functional role of ANGPTL4 [144]. Overexpression of ANGPTL4 was observed to decrease LPL depended plasma TG and cholesteryl ester clearance [145]. Conversely, hepatic ANGPTL4 deficiency and deletion has increased hepatic lipase action and significantly reduced circulating cholesterol and TG levels. Moreover, ANGPTL4 promotes hepatic FA and cholesteryl ester uptake and oxidation, suggesting that the inhibition of ANGPLT4 in the liver protects against diet-induced obesity, liver steatosis, atherosclerosis, and glucose intolerance [144-146]. The CC group showed considerably repressed ANGPTL4 gene expression (**Figure 29-F**). *C. cochlearium* treatment showed a compatible mechanism by decreasing ANGPLT4 expression, serum cholesterol, liver steatosis, but serum triglycerides clearance was not observed as expected. A study reported that germ-free mice are protected against diet-induced obesity through increased AMPK activity, which induces the downstream target of fatty acid oxidation and elevated ANGPTL4, which in turn induces PGC-1α [147]. We found markedly higher PPAR-γ and PGC-1α expressions (Figure 29-D, E) in the CC group. Activation of PPAR-γ is associated with improved reverse cholesterol clearance and insulin sensitivity [148, 149]. Even though low adiposity in the CC group does not explain the function of PPAR-γ upregulation, activation of LXR and reverse cholesterol transport has been found to be associated with PPAR-γ activation

[150, 151]. PGC1-α is coactivated with PPAR-γ as well as a broad range of nuclear receptors and transcription factors to regulate diverse metabolic pathways including cellular energy homeostasis [147], and oxidative stress [148].

4.4 Conclusion

Obesity is a leading risk factor for many adverse metabolic effects such as fatty liver, hypercholesteremia, and insulin resistance. Many studies have demonstrated that FXR activation is associated with anti-lipogenic anti-lipidemic effects [152-156]. However, the role of FXR is still controversial. Accumulating evidence suggests that FXR ablation or FXR antagonism lower the lipogenesis in rodent models [111, 139, 157, 158]. The intestinal FXR pathway is significantly involved in the activation of bile acid synthesis. We found that *C. cochlearium* administration did not affect hepatic FXR but instead downregulated intestinal FXR repressing the endocrine feedback regulatory action of intestinal FXR-FGF15 axis. Inhibition of intestinal FXR signaling has been demonstrated to reduce obesity and insulin resistance by modulating bile acid metabolism and ceramide synthesis [120, 121]. Additionally, FXR has been identified to counteract proatherogenic responses in cardiovascular diseases [159] by regulating the enterohepatic circulation of bile acids and cholesterol. The FXR-dependent FGF15/FGFR4 gut-liver signaling pathway maintains the bile acid synthesis and enterohepatic circulation, which consequently impacts cholesterol, lipid, and glucose metabolism. The reduction of intestinal FXR mediated bile acid uptake into portal blood circulation results in higher fecal excretion of bile acids. Low delivery of bile acids back to the liver lowers the intercellular bile acids, increasing liver bile acids synthesis [120, 121].

Our data concludes that *C. cochlearium* administration increased bile acid deconjugation and fecal bile acid excretion; thus, reduced intestinal bile acid absorption consequently de-

repressed FXR/FGF15 inhibition circuit of CYP7A1 increasing bile acid biosynthesis. Along with the modulation of bile acid metabolism, we observed parallel regulation of reverse cholesterol transport. As primary nuclear receptors of the RCT and BA synthesis pathway, LXR and FXR combine multiple pathways to regulate glucose and lipid metabolisms. LXR activation stimulates lipid efflux from peripheral cells back to the liver for excretion through the RCT pathway. FXR mediated CYP7A1 activation converts excess cholesterol to bile acids, thus promoting lipid excretion through feces. These two metabolic pathways are highly interdependent and integrated to give a complementary effect. We showed that *C. cochlearium* administration interactively de-repressed bile acid absorption and activated RCT and bile acid biosynthesis to enhance lipid clearance. Thus, *C. cochlearium* could be a potential therapeutic probiotic for treating obesity and diabetes as well as hypercholesteremia. It was found that there is a strong regulatory relationship between the gut microbiome and bile acid biotransformation, such as deconjugation, dihydroxylation, or epimerization. Gut microbiome composition can alter the composition of individual bile acid species. Similarly, change in bile acid species can affect the gut microbiome composition.

However, further studies are needed to understand the impact of *C. cochlearium* on gut microbiome and the association of *C. cochlearium* mediated microbiome with bile acid composition and FXR/fgf15 singling pathway.

4.5 Tables and figures

Table 1. Nutrient composition and caloric content of the diets used in the mouse study.

	LF (D12450J)		HF (D12492M)	
Ingredient	gm	kcal	gm	kcal
Casein, 30 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	506.2	2024.8	0	0
Maltodextrin 10	125	500	125	500
Sucrose	68.8	275.2	68.8	275
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	20	180	245	2205
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
Overall	gm%	kcal%	gm%	kcal%
Protein	19.2	20	26	20
Carbohydrate	67.3	70	26	20
Fat	4.3	10	35	60
Total		100		100
kcal/gm	3.85		5.24	

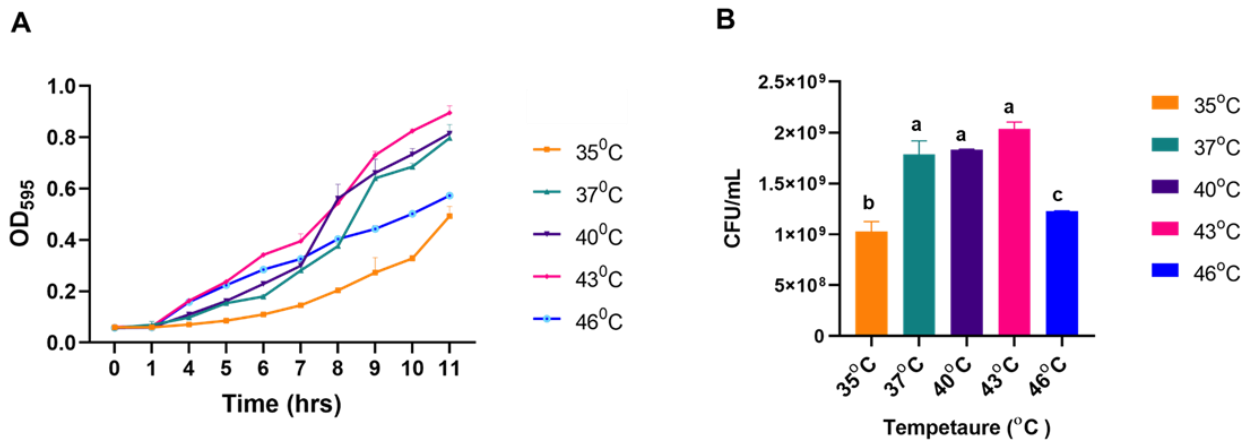

Figure 2. The optimum temperature of *Clostridium cochlearium* in-vitro growth.

C. cochlearium growth under 35 ℃, 37 ℃, 40 ℃, 43 ℃, and 46 ℃ temperature conditions were tested. The OD values were obtained every 30 minutes in duplicates for 11 hours. A) Growth curves of *C. cochlearium* B) Final colony forming units presented in experimented temperature samples. Results are expressed as the means ± SD.

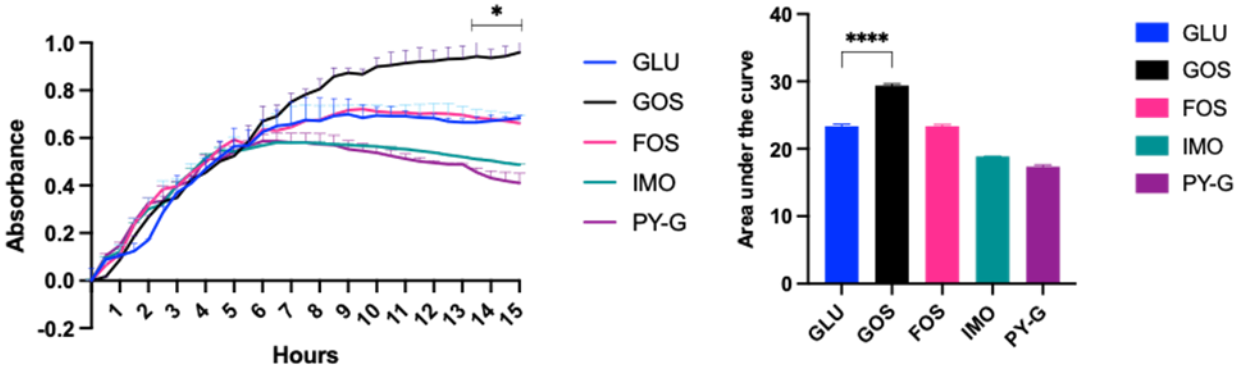

Figure 3. Optimum prebiotic supplementation for *Clostridium cochlearium* in-vitro growth.

C. cochlearium growth under different prebiotic supplementation was tested. Various media tubes were separately prepared containing 0.2% w/v prebiotic ingredients galactooligosaccharides (GOS), fructo-oligosaccharide (FOS), isomalto-oligosaccharide (IMO), glucose (GLU-positive control), and PYG media without glucose (PY-G negative control). These media tubes were inoculated with 10^9 CFU of *C. cochlearium* and incubated at 37℃. The absorbance values were recorded every 30 minutes in duplicates for 15 hours. A) growth curves of *C. cochlearium.* Statistical analysis was performed using repeated measure ANOVA. B) area under the curve of the *C. cochlearium* growth curves. Statistical analysis was performed using One way ANOVA. Results are expressed as the means ± SD, *P< 0.05, ****P< 0.0001.

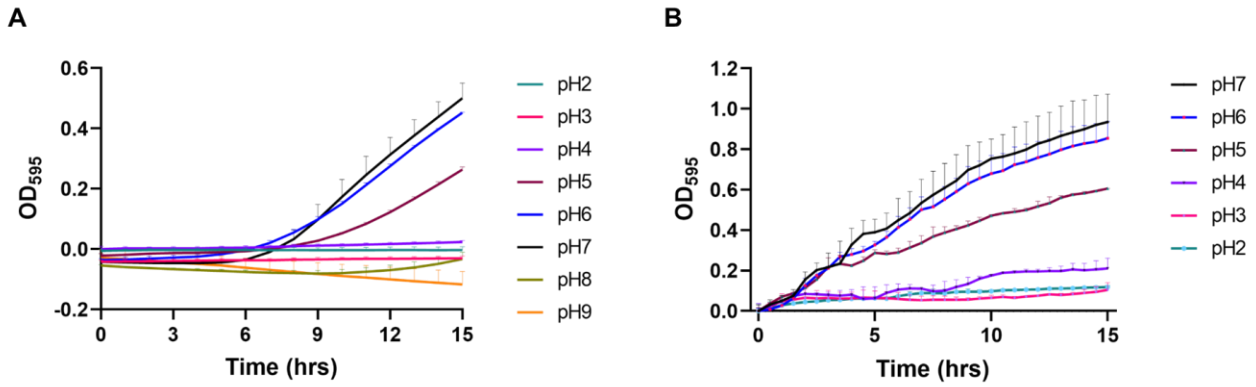

Figure 4. Optimum pH of *Clostridium cochlearium* in-vitro growth.

C. cochlearium growth curves in media with different pH levels was tested. The media tubes were adjusted to pH of 2, 3, 4, 5, 6, 7, 8, and 9, then inoculated with 10^9 CFU of *C. cochlearium* and incubated at 37℃. The OD values were obtained every 30 minutes in duplicates for 15 hours. A) *C. cochlearium* growth curves in pH 2 - 9. B) *C. cochlearium* growth curves in pH 2 - 7.

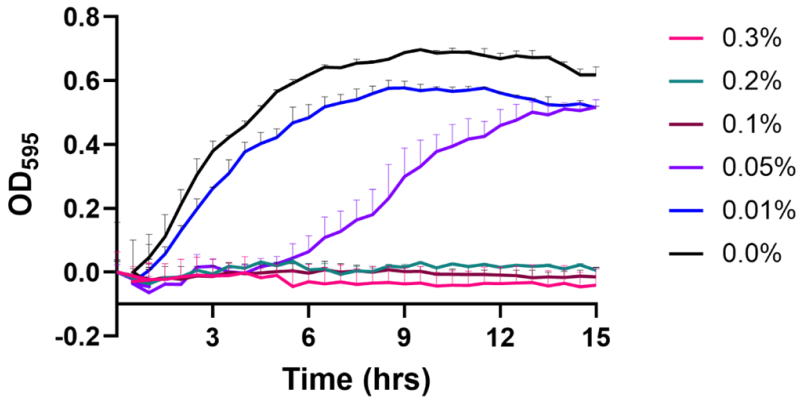

Figure 5. Bile salt tolerance of *Clostridium cochlearium.*

C. cochlearium growth under different bile salts concentrations was tested. The media tubes were prepared with 0.3%, 0.2%, 0.1% 0.05%, 0.01%, 0.0% w/v bile salts, inoculated with 10^9 CFU of *C. cochlearium* and incubated at 37℃. The OD values were obtained every 30 minutes in duplicates for 15 hours. The data are shown in means ± SD.

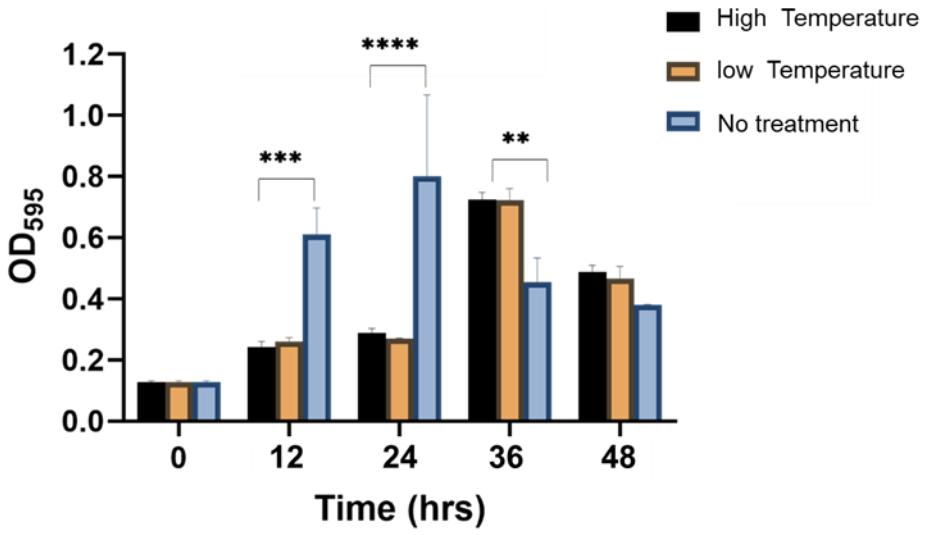

Figure 6. Effects of heat shock on *Clostridium cochlearium* growth.

Clostridium cochlearium overnight cultures were treated with high temperature (75℃), Low temperature (55℃), and no heat treatment (25℃) for 20 minutes, then transferred anaerobically into regular PYG culture media, and incubated at 37 ℃. The OD values were obtained in duplicates for 60 hours. Results are expressed as the means ± SD. Statistical analysis was performed using repeated measure ANOVA. *p< 0.05, **p<,0.01 and ***P< 0.001****P< 0.0001.

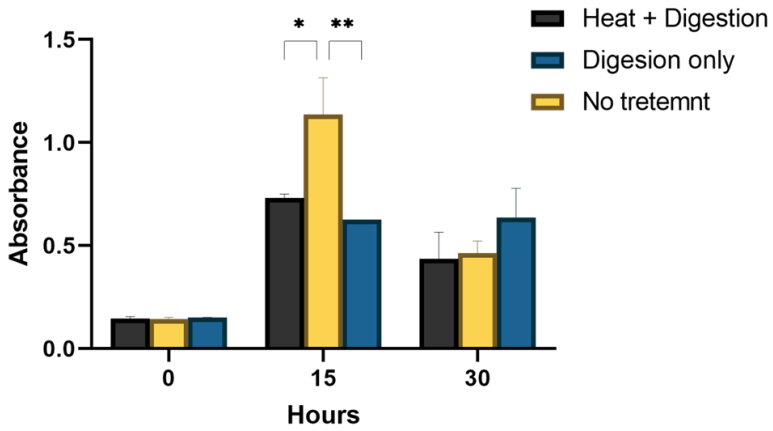

Figure 7. Effects of in-vitro digestion on *Clostridium cochlearium* growth.

Heat treated (75℃ for 20 minutes HD (Heat + Digestion)) and non-heat-treated (D (Digestion only)) *C. cochlearium* samples were incubated in simulated gastrointestinal media (2 Hours in pH-2 media and 2 Hours in enteric digestion fluid) for 4 hours at 37℃, then transferred anaerobically into regular culture media, and incubated at 37 ℃. No treatment was performed on the control (NT) sample. The absorbance was obtained in duplicates for 30 hours, and data is presented in 15-hour intervals. Results are expressed as the means ± SD. Statistical analysis was performed using repeated measure ANOVA. *$p < 0.05$, **$p<0.01$.

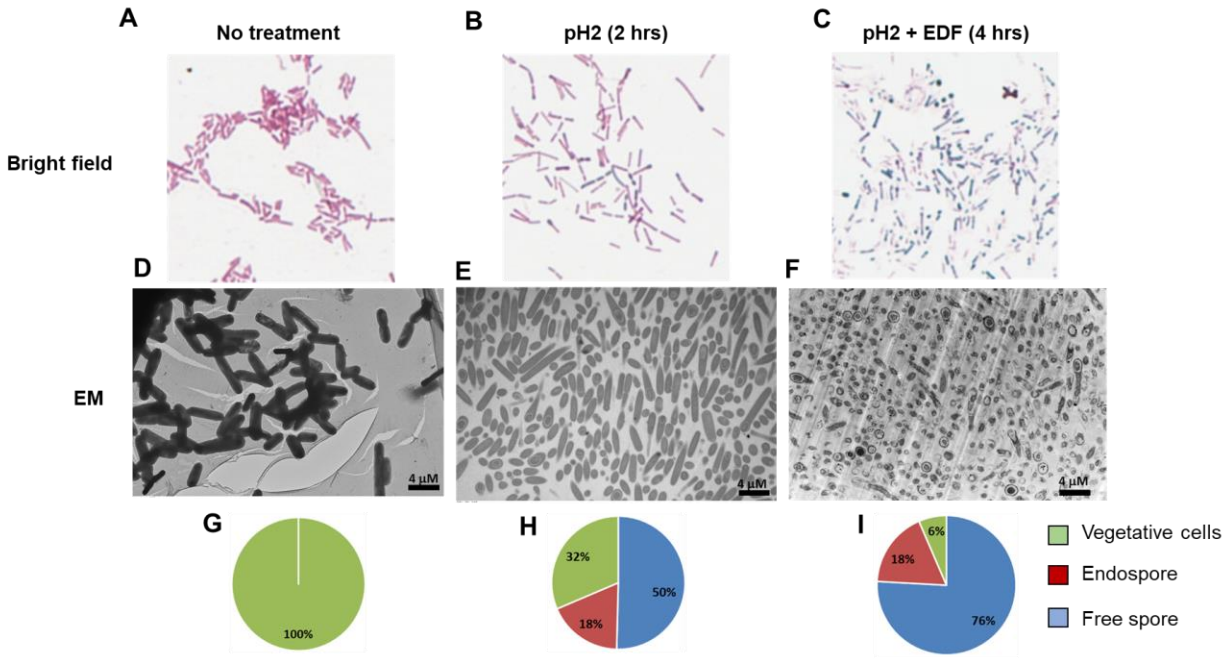

Figure 8. Spore formation of *Clostridium cochlearium* under simulated in-vitro digestion

Clostridium cochlearium cells were exposed to pH2 media treatment (B, E,) followed by enteric digestion fluid (C, F,) treatment. Images were taken under bright field (A, B, C), electron microscope (D, E, F). Cells of No treatment control (A, D). Percentage of *C. cochlearium* vegetative cells, endospore, free spore presented under no treatment (G) pH2 treatment (H) pH2 treatment + enteric digestion fluid treatment (F).

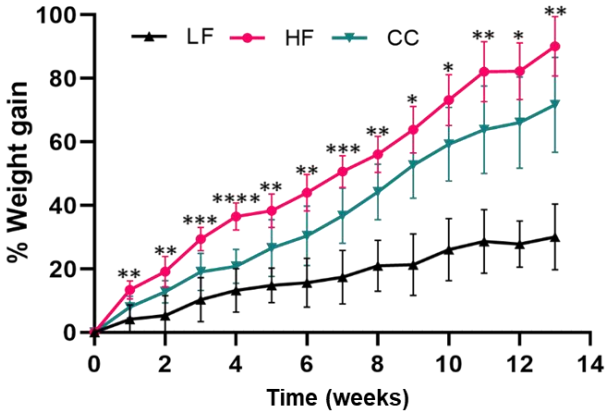

Figure 9. Effect of *C. cochlearium* administration on percent weight gain in DIO mice.

Male DIO mice (n=12 per group) were treated daily with vehicle or active *C. cochlearium* (CFU/mL=10^{10}) for 13 weeks. Results are expressed as the means ± SD. Statistical analysis was performed using repeated measure ANOVA. Significant differences are indicated as

* for P < 0.05, ** for P < 0.01, *** for P < 0.001 and **** for P < 0.0001.

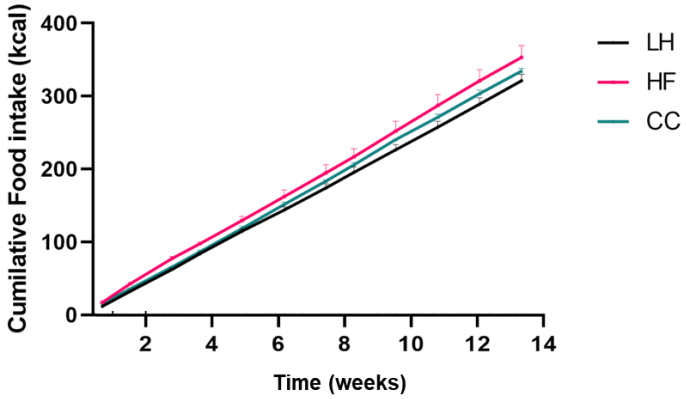

Figure 10. Effect of C. cochlearium administration on cumulative calorie intake in DIO mice. Male DIO mice (n=12 per group) were treated daily with vehicle or active *C. cochlearium* (CFU/mL=10^{10}) for 13 weeks. Results are expressed as the means ± SD. Statistical analysis was performed using repeated measure ANOVA.

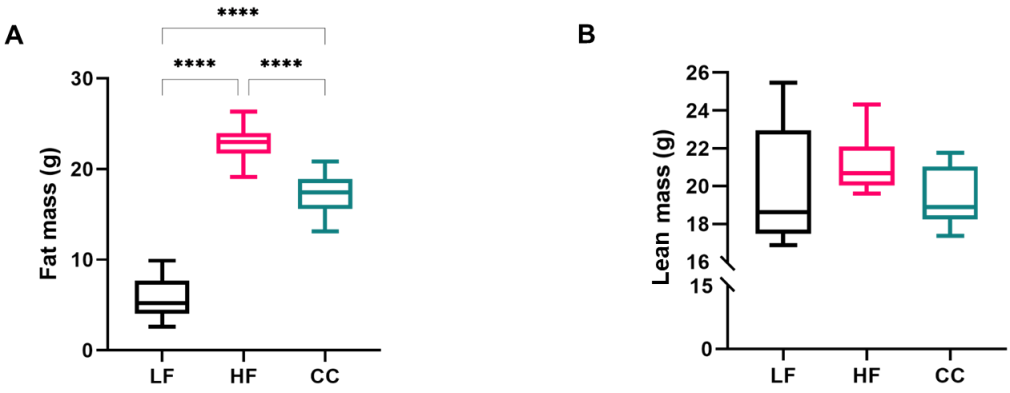

Figure 11. Effect of *C. cochlearium* on A) Fat mass B) Lean mass.

Male DIO mice (n=12 per group) were treated daily with vehicle or active *C. cochlearium* (CFU/mL=10^{10}) for 13 weeks. Results are expressed as the means ± SD. Statistical analysis was performed using one-way ANOVA. and **** represents P< 0.0001.

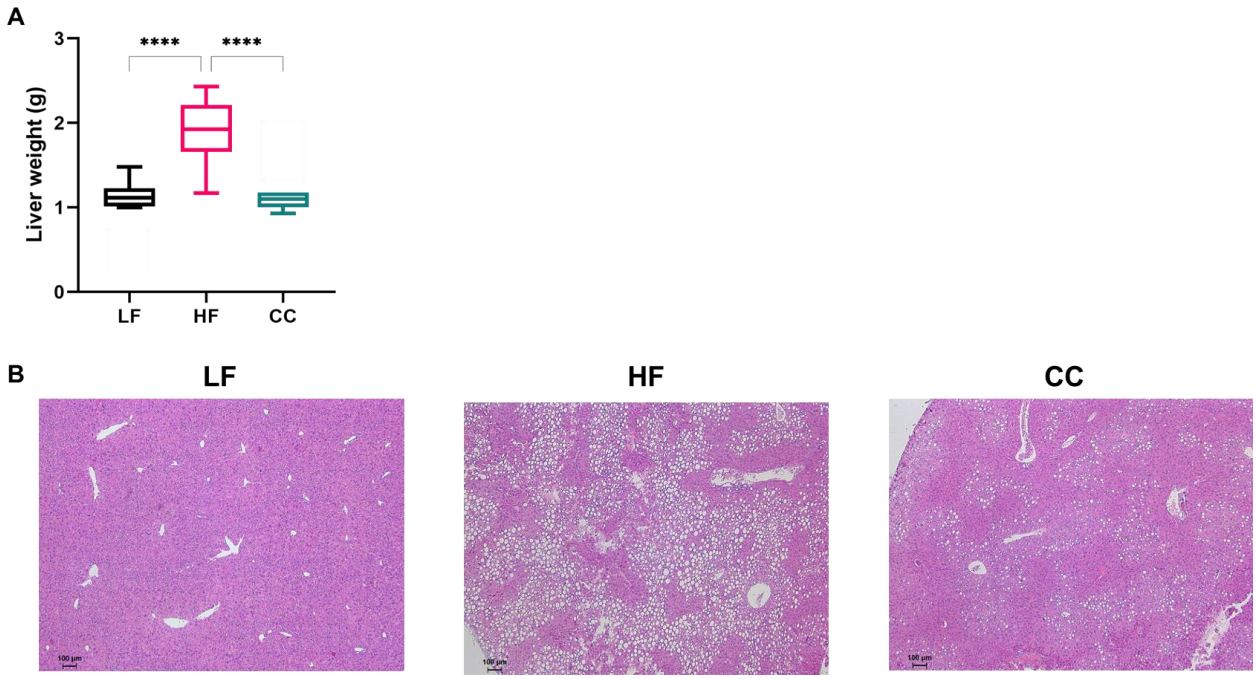

Figure 12. Effect of *C. cochlearium* on liver steatosis.

Male DIO mice (n=12 per group) were treated daily with vehicle or active *C. cochlearium* (CFU/mL=10^{10}) for 13 weeks. A) Liver mass B) Histology of Liver tissues showing fatty infiltration in the hepatocytes. Hematoxylin-eosin (HE) staining with 5X magnification. Results are expressed as the means ± SD. Statistical analysis was performed using one-way ANOVA. and **** represents P< 0.0001.

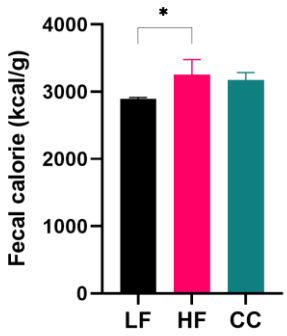

Figure 13. Effect of *C. cochlearium* on fecal calorie output.

Male DIO mice (n=12 per group) were treated daily with vehicle or active *C. cochlearium* (CFU/mL=10^{10}) for 13 weeks. Results are expressed as the means ± SD. Statistical analysis was performed using one-way ANOVA. and * represents $P< 0.05$.

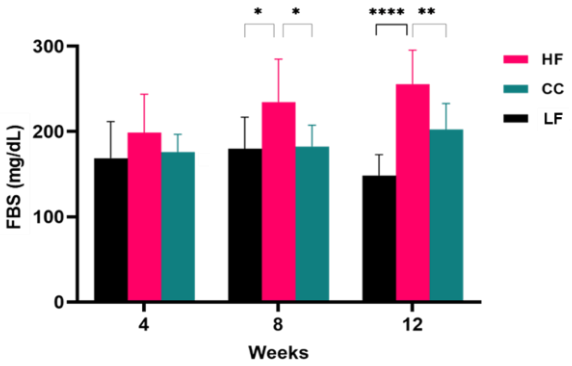

Figure 14. Effect of *C. cochlearium* on Fasting blood glucose

Blood glucose concentrations of DIO mice (n=12 per group), treated with *C. cochlearium* (CC) or the vehicle (HF, LF) after 8 hours fasting. Results are expressed as means ± SD. Statistical analysis was performed using one-way ANOVA. * for $P < 0.05$, ** for $P < 0.01$, and **** for $P < 0.0001$.

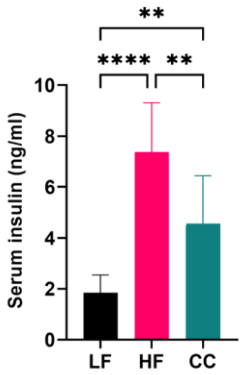

Figure 15. Effect of *C. cochlearium* on Fasting serum insulin.

Serum insulin concentration of DIO mice (n=12 per group), treated with *C. cochlearium* (CC) or the vehicle (HF, LF) after 8 hours fasting. Results are expressed as means ± SD. Statistical analysis was performed using one-way ANOVA. * for $P < 0.05$, ** for $P < 0.001$ and **** for $P < 0.0001$.

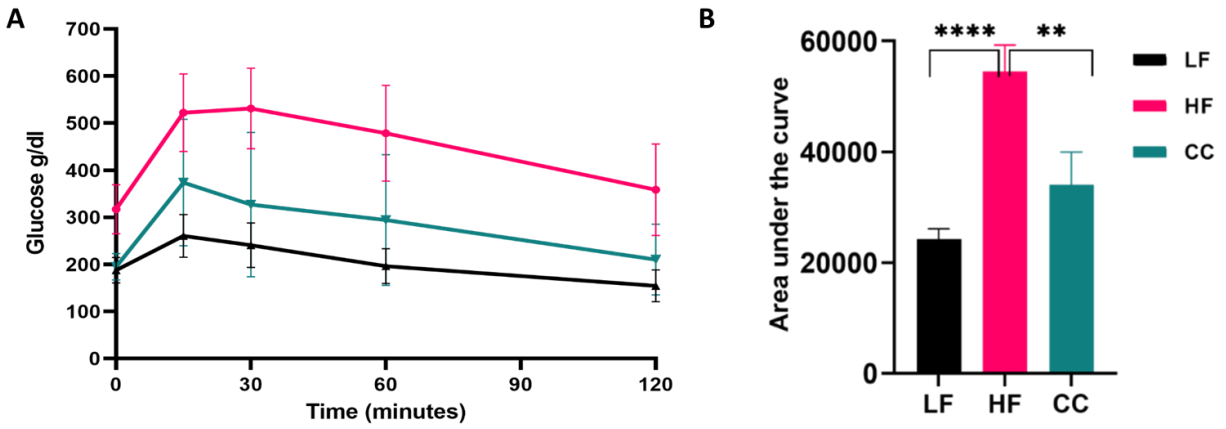

Figure 16. Effect of *C. cochlearium* on oral glucose tolerance test.

Oral glucose tolerance test in DIO mice (n=12 per group) treated *C. cochlearium* (CC group) or the vehicle (HF, LF groups) after 8 hours fasting. Blood glucose concentrations are taken at t = 0, 15, 30, 60 and 120-min after oral administration of (1 mg/kg BW) 10% w/v sterile glucose solution. B) The corresponding area under the curve (AUC) of oral glucose tolerance curve. Results were expressed as means ± SD.

Statistical analysis was performed using one-way ANOVA. ** for $P < 0.01$, and **** for $P < 0.0001$.

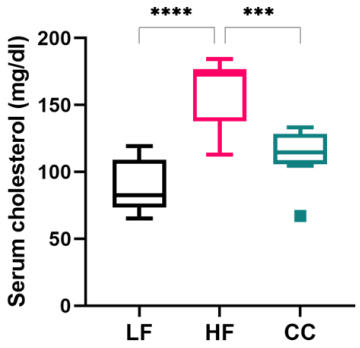

Figure 17. Effect of *C. cochlearium* on serum cholesterol

Serum cholesterol in LF, HF and CC groups after 8 hours fasting in DIO mice (n=12 per group) treated with *C. cochlearium* (CC group) or the vehicle (HF, LF groups). Results are expressed as the means ± SD. Statistical analysis was performed using one-way ANOVA. *** for $P < 0.001$ and **** for $P < 0.0001$.

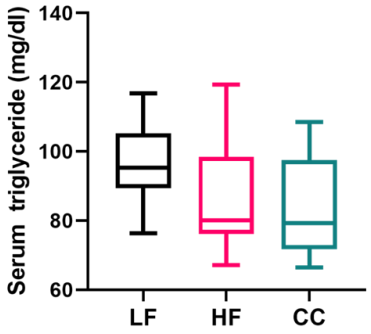

Figure 18. Effect of *C. cochlearium* on serum triglycerides

Serum triglycerides in LF, HF and CC groups after 8 hours fasting in DIO mice (n=12 per group) treated with *C. cochlearium* (CC group) or the vehicle (HF, LF groups). Results are expressed as the means ± SD. Statistical analysis was performed using one-way ANOVA.

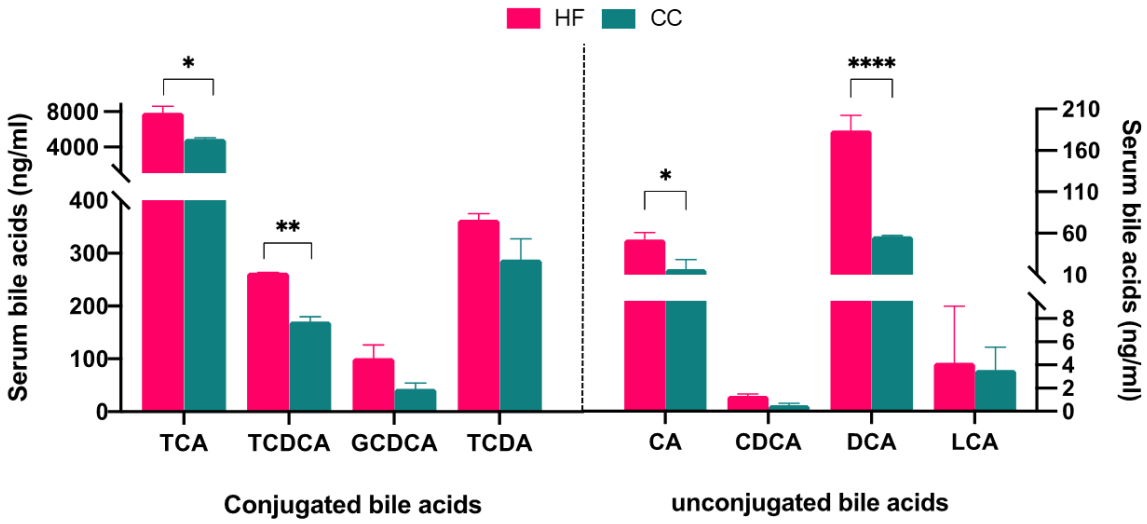

Figure 19. Effect of C. cochlearium on serum bile acids concentration (individual species)

Figure depicts serum bile acids composition; Results are expressed as means ± SD. Statistical analysis was performed using the Student's t-test. * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$ and **** for $P < 0.0001$.

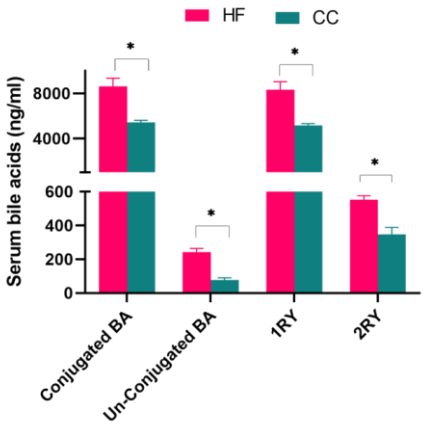

Figure 20. Effect of C. cochlearium on serum bile acids concentration (summery)

Figure depicts total conjugated, unconjugated, primary, and secondary bile acids in serum. Results are expressed as means ± SD. Statistical analysis was performed using the Student's t-test. * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$ and **** for $P < 0.0001$.

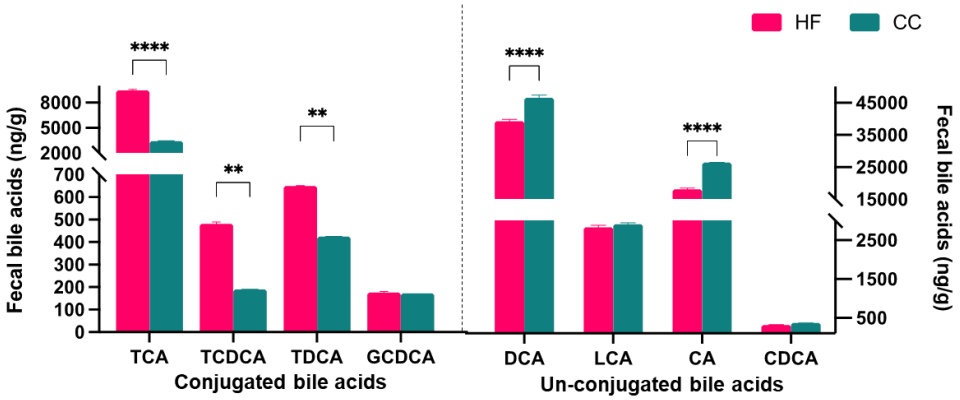

Figure 21. Effect of *C. cochlearium* on fecal bile acids concentration (individual species)

Figure depicts fecal bile acids composition. Results are expressed as means ± SD. Statistical analysis was performed using the Student's t-test. * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$ and **** for $P < 0.0001$.

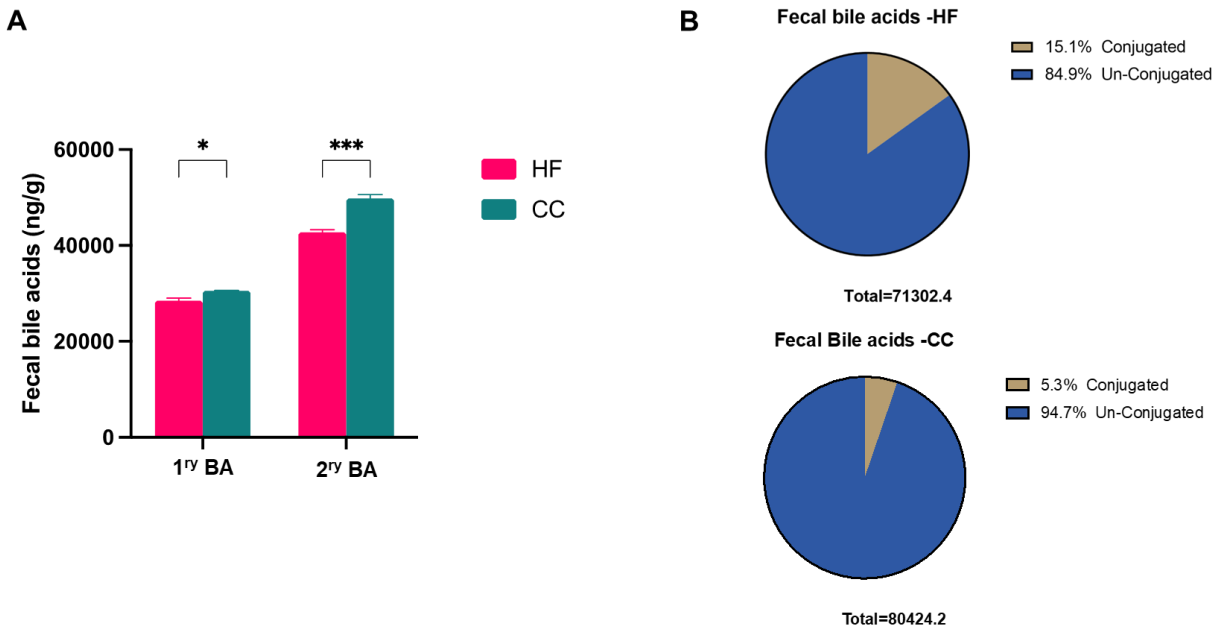

Figure 22. Effect of *C. cochlearium* on fecal bile acids concentration (summery)

Figure depicts A) total primary and secondary bile acids in feces. (B) percentage of conjugated and unconjugated bile acids in feces of HF and CC groups. Results are expressed as means ± SD. Statistical analysis was performed using the Student's t-test. * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$ and **** for $P < 0.0001$.

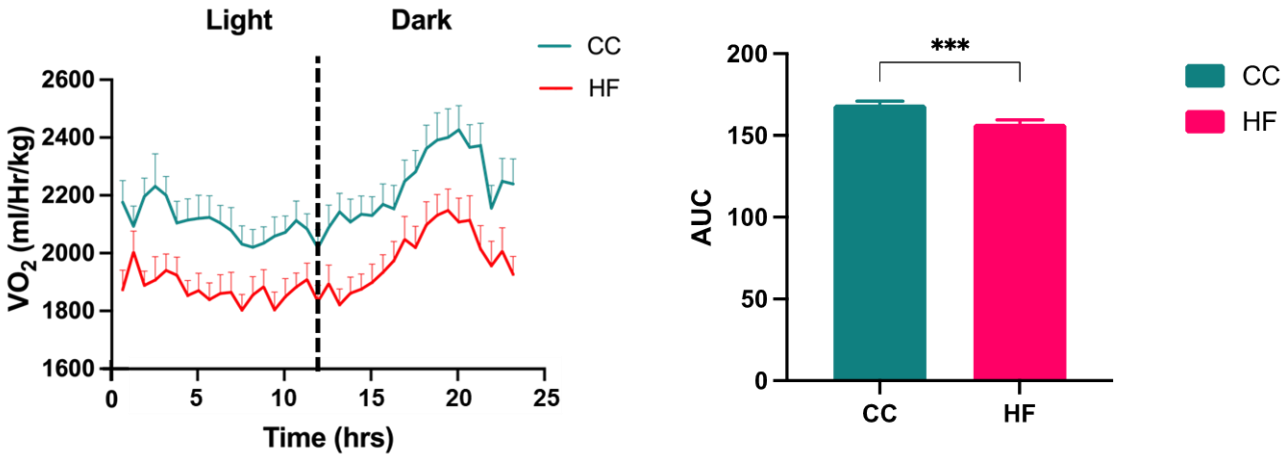

Figure 23. Effect of *C. cochlearium* on O_2 consumption.

energy expenditure DIO mice (n=12) were treated *C. cochlearium* (CC group) or the vehicle (HF group). Three-day data was averaged per time point per day; results are expressed as the means +SEM. Statistical analysis was performed using Student's t test. *p<0.05, **p<0.01 and ***p<.001.

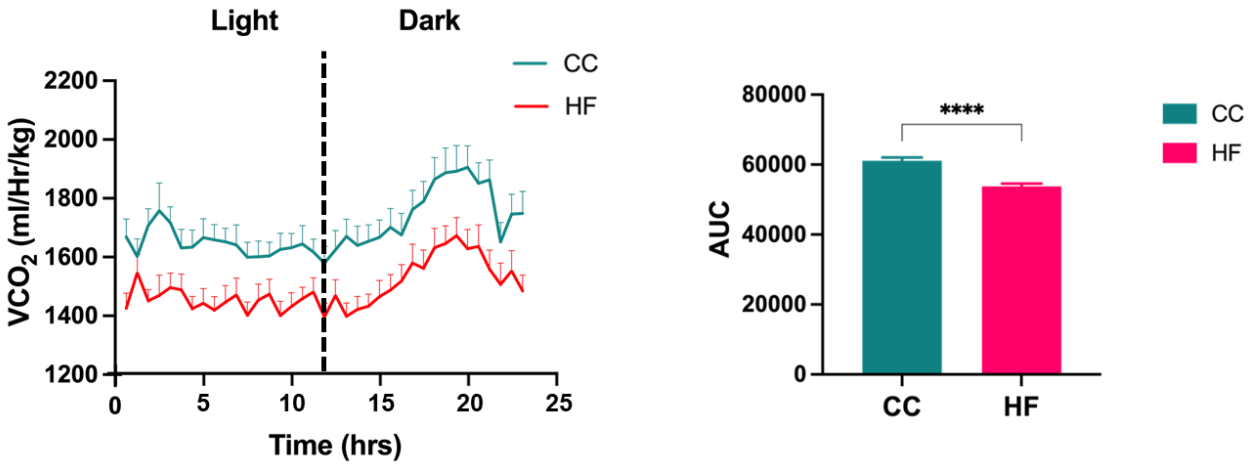

Figure 24. Effect of *C. cochlearium* on CO_2 production

DIO mice (n=12) were treated *C. cochlearium* (CC group) or the vehicle (HF group). Three-day data was averaged per time point per day; results are expressed as the means +SEM. Statistical analysis was performed using Student's t test. *p<0.05, **p<0.01 and ***P< 0.001.

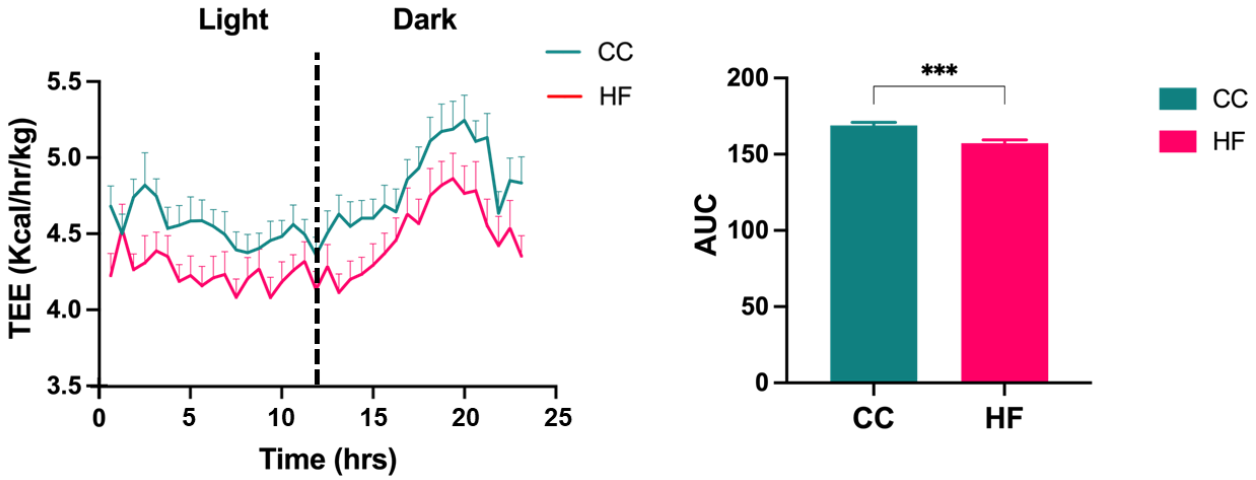

Figure 25. Effect of *C. cochlearium* on total energy expenditure

DIO mice (n=12) were treated *C. cochlearium* (CC group) or the vehicle (HF group). Three-day data was averaged per time point per day, and results are expressed as the means +SEM. Statistical analysis was performed using Student's T test. *p,0.05, **p,0.01 and ***P, 0.001.

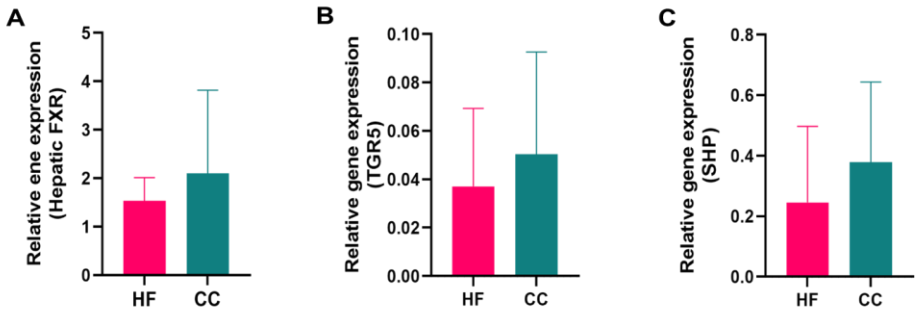

Figure. 26. Effect of *C. cochlearium* on hepatic bile acid receptor gene expressions.

Gene expression levels of bile acid receptors in liver tissues (A, B, C). Results are expressed as means ± SD. Statistical analysis was performed using the Student's t-test. * for $P < 0.05$.

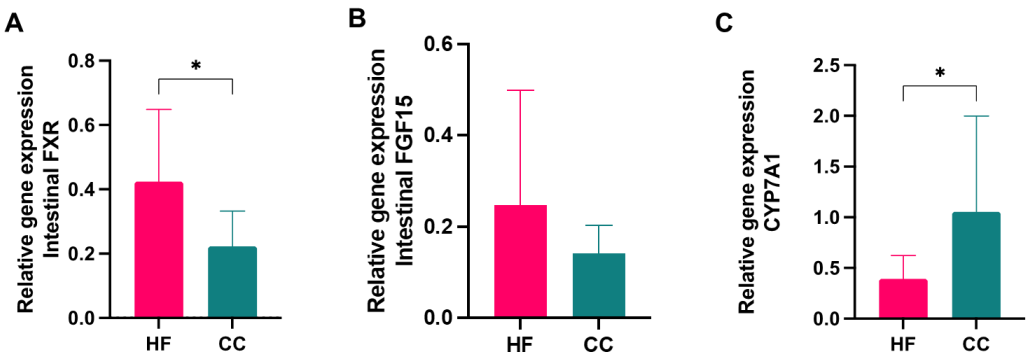

Figure. 27. Effect of C. cochlearium on intestinal bile acid receptor mediated feedback inhibition loop.

Gene expressions involve in bile acid biosynthesis (A) and Feedback inhibition of bile acid synthesis in intestinal tissues (B, C). Results are expressed as means ± SD. Statistical analysis was performed using the Student's t-test. * for $P < 0.05$.

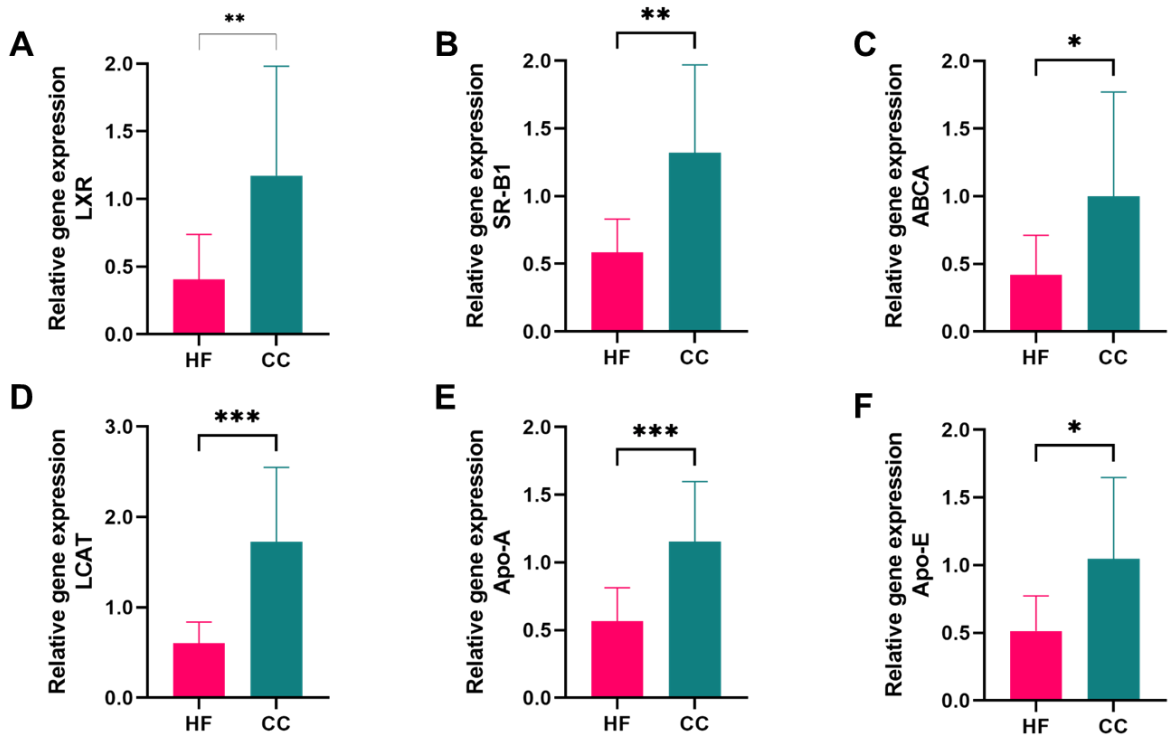

Figure. 28. Effect of C. cochlearium on gene expression levels involved in reverse cholesterol transport.

Figure depicts the gene expressions of lipoproteins (E, F), cholesterol transfer proteins (B, C) and catalyzers (A, D) involve in reverse cholesterol transport . Results are expressed as means ± SD. Statistical analysis was performed using the Student's t-test. * for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$ and **** for $P < 0.0001$.

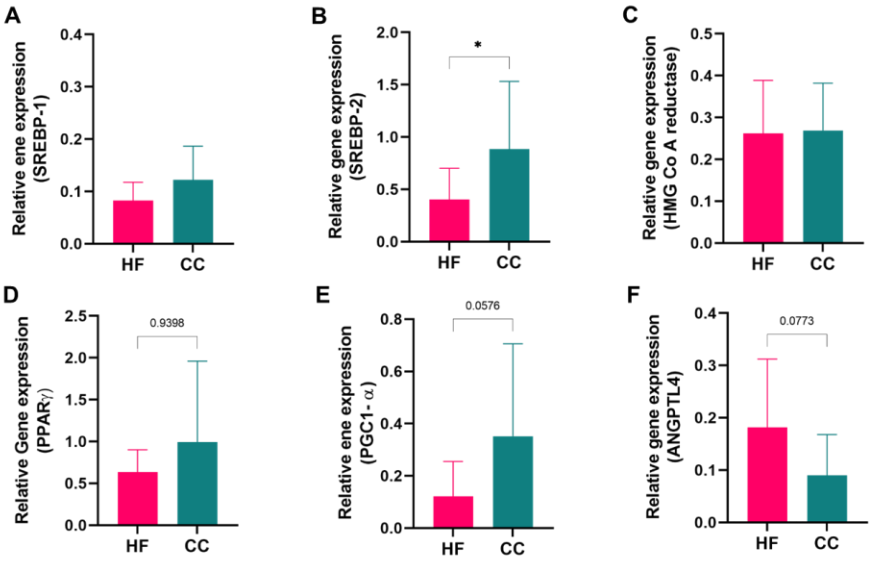

Figure 29. Effect of C. cochlearium on hepatic gene expressions involved in lipid metabolism. Results are expressed as the mean ± SD. Statistical analysis was performed using the Student's t-test. * for $P < 0.05$.

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ABSTRACT

CHARACTERIZATION OF *CLOSTRIDIUM COCHLEARIUM* AS A POTENTIAL PROBIOTIC FOR OBESITY MANAGEMENT

by

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August 2021

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Major: Nutrition and food science

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Emerging evidence indicates that manipulation of gut microflora is a potential therapeutic approach for managing obesity. Probiotic effects on host weight reduction have repeatedly been revealed through previous studies. Clostridium cochlearium is a butyrate-producing, spore-forming bacteria that have been reported to present in the mammalian gut. Our simulated Invitro digestion model revealed that *C. cochlearium* could survive in the unfavorable conditions of the human gastrointestinal tract, including low pH (pH2), high bile salts (1.5% w/v), and in the presence of enteric digestive enzymes. Daily Oral administration of *C. cochlearium* (10^9 CFU) for 14 weeks showed 18% bodyweight reduction in DIO (C57BL/6) mice primarily via fat mass reduction. Obesity-related other metabolic deteriorations, including hyperglycemia, serum glucose, and insulin intolerance, hypercholesteremia, liver steatosis, were also improved. A significant decrease in host energy expenditure and alteration of hot bile acid composition were observed in the *C. cochlearium* treated group. In addition, *C. cochlearium* treatment significantly upregulated gene expressions related to reverse cholesterol clearance and bile acids synthesis. *C. cochlearium* administration increased bile acid deconjugation and fecal bile acid excretion, thus

reducing intestinal bile acid absorption consequently derepressed FXR/FGF15 inhibition circuit of CYP7A1. Along with the modulation of bile acid metabolism, we observed parallel regulation of reverse cholesterol transport. These finding indicated that the anti-obesity activity of *C. cochlearium* occurred through altered bile acid composition, that increased energy expenditure and cholesterol turnover which ultimately improved host lipid and glucose metabolism. Thus, *C. cochlearium* could be a potential therapeutic probiotic for managing obesity, diabetes, and hypercholesteremia. Further studies are needed to understand the impact of *C. cochlearium* on the gut microbiome and the association of *C. cochlearium* mediated microbiome with bile acid composition and FXR/fgf15 singling pathway.

AUTOBIOGRAPHICAL STATEMENT

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EDUCATION

PhD in Nutrition and Food Science, Wayne State University (2016-2021)
MSc in Nutrition and Food Science, Wayne State University (2013-2015)
MSc in Chemistry, University of Sri Jayewardenepura. Sri Lanka, (2008-2010)
Bachelors of Ayurvedic Medicine and Surgery, University of Colombo, Sri Lanka (2001-2007)

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Graduate teaching assistance, Wayne State University. 2017-2021
Graduate research assistant, Wayne State University. 2016-2017
Ayurvedic Medical Officer, Sri Lanka, 2009-2012

LICENSES AND CERTIFICATIONS

HACCP, HACCP international Alliance (issued 2016)
ISAK, International society for the advancement of Kinanthropometry (issued 2017)
RCR, Responsible conduct of research, (2017)
Medical officer, Medical council in Ayurveda - Sri Lanka (2009)

PRESENTATIONS

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AWARDS & HONERS

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3. Patent pending; potential probiotic for prevention of diet induced obesity (2020)
4. 3rd place poster, Graduate research symposium, Wayne State University, (2018)
5. Parent Family Endowment Fellowship; Department of Nutrition and Food Science scholarship (2018)