THE EFFECT OF PROBIOTICS ON METABIOME IN THE INTERLEUKIN-10 GENE DEFICIENT MICE USING CORRELATION NETWORKS

By

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ABSTRACT

THE EFFECT OF PROBIOTICS ON METABIOME IN THE INTERLEUKIN 10 GENE DEFICIENT MICE USING CORRELATON NETWORKS USING CORRELATION NETWORKS

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George Mason University, 2014

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Crohn's disease, an Inflammatory Bowel Disease, is a chronic inflammation of the gastrointestinal tract, attributed to many factors such as genetics, environmental factors, microbial infections, and the immune system. When mucosal irritants, such as luminal antigens and microbes disrupt the epithelial barrier, chronic inflammation occurs. Of the various ecological niches on the human body, the gut microbiome is the most complex. The interactions between the gut microbiome, its metabolome, and the host are in a dynamic state. Hence, a snapshot of the metabolome, and microbiome of the gut will elucidate some of the shifts that occur when the system is perturbed and these can be analyzed using Correlation Networks. We used an Interleukin-10 gene knock-out mouse model that spontaneously develops colitis, to study Crohn's disease. A probiotic preparation, VSL#3, attenuates inflammation, reduces mucosal levels of pro-inflammatory cytokines and restores gut barrier function. Correlations Networks analysis of the microbiome, metabolome, and immunome is a systems biology approach that is an

effective way to gain insight into the metabolome-microbiome-host, relationships and functions. Therefore, we modeled the metabolome, microbiome, and immunome to understand the effect of probiotics on IL10-gene deficient mice.

Aim: The goal of the study is to determine the effect of probiotics on (i).Metabolite-metabolite, (ii). Microbiome-microbiome, and (iii). Microbiome-cytokine correlations in IL10 gene deficient mice.

Methods: In this study, the wild-type and IL10 gene deficient mice ($IL10^{-/-}$) were treated with probiotic formulation VSL#3 or control diet for 14 days and sacrificed. Two experiments were performed. In the first experiment, the liver and caecal contents metabolites were analyzed by NMR spectroscopy. In the second experiment, in addition to analyzing cytokines in the colon and ileum using electro-chemiluminescent multiplex kit, microbiome abundances for Cecal Contents and Cecal Mucosa were determined using Multi-Tag Pyrosequencing method.

Results: There is a striking reversal of liver metabolite correlations from negative in control-$IL10^{-/-}$ mice (C_IL-10) to positive in probiotic- $IL10^{-/-}$ mice (P_IL10), compared to the subtle differences in cecum metabolite correlations. Pyruvate metabolism, Glycolysis/Gluconeogenesis pathway and Krebs-Cycle in the liver, and Butanoate, Methane, Tryptophan, Phenylalanine and Tyrosine, Glycine, Serine and Threonine metabolism, and Krebs cycle, are significantly different between C_IL10 and P_IL10 correlation networks.

Microbiome-microbiome correlations network analysis shows that Bacteroidetes and Firmicutes increase in Cecal Contents, whereas, Proteobacteria, Bacteroidetes, and

Firmicutes increases in Cecal Mucosa of P_IL10 mice. The interactions involving *Bacteroides, Paraprevotella, Roseburia*, and *Robinsoniella* are significant different in the Cecal Contents and Cecal Mucosa, correlations networks between C_IL10 and P_IL10.

Probiotics establish Actinobacteria, and Proteobacteria in Cecal Contents and Cecal Mucosa networks in C_IL10. Tissue differences between Cecal Contents and Cecal Mucosa microbiomes correlations networks of C_IL10 and P_IL10 indicates that Proteobacteria are absent in C_IL10 Cecal Contents, present in C_IL10 Cecal Mucosa, present in P_IL10 Cecal Contents, and absent in P_IL10 Cecal Mucosa. Significant differential correlations analysis between C_IL10 and P_IL10 indicates that *Barnesiella, Butyricimonas, Parabacteroides, Paludibacter, Syntrophococcus, and Roseburia* have significantly differential correlations in both Cecal Contents and Cecal Mucosa networks. Proteobacteria (*Parasutterella* and *Ralstonia*) are absent in Cecal Contents and present in Cecal Mucosa significant correlation differential networks.

Conclusion: Our results show that probiotics affect the metabolome, and microbiome correlation networks in IL10-knock-out mice. Probiotics also affect tissue specific differential microbiomes in the gut.

1: INTRODUCTION AND LITERATURE REVIEW

The term 'biome' refers to a habitat and the organisms in it. The term 'microbiome' is therefore the collection of microorganisms inhabiting a biome. The genomes of all members of the microbial community encode a variety of enzymes that produce a range of metabolites or the metabolome (Turnbaugh *et al.*, 2008). We define the interactions and function of the microbiome and host as the Metabiome (Gillevet *et al.*, 2010).

The function of the microbiome thus depends on its member composition and the metabolites they produce in that particular habitat. Therefore, we must analyze both the metagenome and metabolome to understand a role of microbiome in a habitat.

1.1. Microbiome Analysis

Microbial community profiling involves two main methods that do not require cultivation of organisms. The first method uses 16S rRNA (for archae and bacteria) or 18S rRNA (eukaryotes) gene sequences as phylogenetic markers to determine the diversity of the organisms in the sample (Microbiome). The second uses shotgun sequencing on community DNA to elucidate all genes (Metagenome) from all organisms in the sample (Hamady *et al.*, 2009).

1.2. The Human Intestinal Microbiome

The human microbiota consists of approximately 10^4 bacterial taxa that exist in various ecological niches on the human body. The most complex of all is the gut microbiome that contains some 10^{12} bacteria/gm of feces in the average human (Savage, 1977). Several studies have investigated the human or mouse intestinal microbiomes, using metagenomics. Gill *et.al.* compared ~78 Mb of unique metagenomic DNA sequence from gut of two healthy humans. They identified large number of bacterial genes and found that the human microbiome is significantly enriched for metabolism of glycans, aminoacids, xenobiotics, and vitamin biosynthesis. Eckburg *et.al.* carried out 16S rRNA analysis of 3 human gut microbiomes. They found 395 phylotypes at the strain level. Major species found were *Bacteroides, Clostridium, Eubacterium*, and *Ruminococcus*. Of the 395 phylotypes, 80% of species were species yet to be cultured (Eckburg *et al.*, 2005). In another study, analysis of 16S rRNA sequences from distal intestinal microbiota of obese mice, lean mice and wild type mice revealed that the obese mice had 50% reduction in abundance of Bacteroidetes and an equal proportion increase in abundance of Firmicutes. This study indicated an association of intestinal microbiome composition with obesity (Ley *et al.*, (2005).

1.3. Inflammatory Bowel Diseases

These are diseases caused by the inflammation of the gastrointestinal tract. Crohn's disease and ulcerative colitis are two main types of inflammatory bowel diseases. Their etiology is poorly understood, and many factors such as genetics, environmental factors, microbial infections and the immune system are attributed to inflammation of the intestinal mucosal surface (Baumgart *et al.*, 2007a).

1.3.1. Crohn's Disease (CD)

Crohn's disease is a chronic inflammation of the gastrointestinal mucosa. It can affect the gastrointestinal tract at any location, although the prominent sites of initial diagnosis are the terminal ileum, and colon. The symptoms can include diarrhea, abdominal pain, fever, bowel obstruction and even passage of mucus and blood. Further progression of the disease results in complications such as strictures, abscesses, or fistulas (Baumgart *et al.*, 2007b).

There is no direct laboratory diagnostic test for CD. Hence, the patient's history, physical exam, and other clinical tests including endoscopic and radiological tests are considered at the time of diagnosis. Furthermore, there is no cure and management of the disease involves inducing remission with drugs and surgery (Baumgart *et al.*, 2007b).

1.3.2. Ulcerative Colitis (UC)

Ulcerative colitis is restricted to the colonic mucosa. The common symptoms include hematochezia (bloody stools) and passage of mucus. The diagnosis is done using endoscopic and histological analysis. Just as in CD, the management of the disease includes induction and maintenance of remission with drugs and surgery in life threatening situations (Baumgart *et al.*, 2007b).

1.4. Mouse Models of Crohn's Disease

Different kinds of mouse models are used for studying IBD, and each of them has specific advantages depending on the biological mechanism under study. There are chemically induced models, genetic models, and immunological models (Pizarro *et al.*, 2003).

Chemically induced models, require administration of a chemical agent such as TNBS (trinitrobenzene sulfonic acid) or DSS (dextran sodium sulfate) for induction of colitis. They are useful for studying inflammatory pathways and antigen-specific studies.

The immunological models or transfer cell models involve transferring T cells or bone marrow precursors into immunodeficient mice. Example is the $CD4^{+}CD45RB^{hi}$ T-cell adoptive transfer model of colitis. Genetic models include knock-out mouse models (where the gene is disrupted resulting in loss of activity) and a few transgenic models (where new genetic material is inserted into the germ-line). IL-10 knock-out mice spontaneously develop colitis (Kuhn *et al.*, 1993), while IL-2 gene knock-out mice develop a UC like disease (Sadlack *et al.*, 1993).

1.4.1. IL-10 Deficient Mouse

Interleukin-10 is an anti-inflammatory cytokine that is released by the T-cells and antigen presenting cells. IL-10 inhibits the release of other pro-inflammatory cytokines by cells such as monocytes, dendritic cells and macrophages. Thus, IL-10 acts as a suppressor of inflammation (Groux *et al.*, 2003).

Interleukin-10 gene knockout mice are a model of chronic inflammation (Rennick *et al.*, 1995). Interleukin-(IL)-10 deficient mice are widely used as the mouse model of colitis. The $IL10^{-/-}$ mice lose weight with aging, become anemic and exhibit chronic enterocolitis (4). IL-10 suppresses the immune inflammatory response to normal intestinal antigens. When the IL10 knockout mice are kept under specific pathogen free conditions, colitis is ameliorated. The enterocolitis in $IL10^{-/-}$ is due to dysregulated production of Th-1 type pro-inflammatory cytokines similar to that of Crohn's disease

(Kuhn *et al.*, 1993; Davidson *et al.*, 2000). When the IL10$^{-/-}$ mice were treated with IL-10 for 3 months, they gained weight, and administering IL-10 to weanlings for 3 months, completely prevented IBD. There is evidence that feeding probiotics to IL10$^{-/-}$ mice reduces inflammation (O'Mahony *et al.*, 2001).

1.5. Probiotics and Gut Health

The word "probiotics" was first introduced by Lilly and Stillwell in 1965. They originally defined it as a substance produced by one organism that contributes to the growth of another organism (Lilly et.al., 1965). According to the World Health Organization, probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit to the host. There is evidence that feeding probiotics reduces inflammation. *Lactobacillus sp.* was effective in preventing the development of spontaneous colitis in IL-10 deficient mice (Madsen *et al.*, 1999). Continuously feeding *Lactobacillus plantarun* attenuates established colitis in IL-10 deficient mice (Shultz *et al.*, 1998).

The etiology of IBD is not well known; no pathogen associated with IBD has been determined. Due to its beneficial effects, probiotics are used for the management of diarrheal diseases. Understanding the metabolic pathways involved can help in better management of these Inflammatory Bowel Diseases.

1.5.1. Vsl#3 Probiotic Diet

Assessment of the effectiveness of probiotics involves testing mixtures of different bacterial strains to attenuate inflammation in different animal models. One such widely used mixture is an oral preparation VSL#3. It is a mixture of eight live bacterial

species, (*Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium breve, Lactobacillus acidophilus, Lactobacillus paracasei, Lactobacillus bulgaricus, Lactobacillus plantarum, and Streptococcus salivarus subsp. Thermophilus*) and contains $3x10^{11}$ colony forming units (CFU/gm). VSL#3 preparation shows significant attenuation of inflammation in chemically induced colitis in rats (Shibolet *et al.*, 1999). Administering VSL#3 to IL-10 deficient mice reduces mucosal levels of pro-inflammatory cytokines and restores the gut barrier function (Madsen *et al.*, 2001).

1.6. Cytokines

Cytokines are small peptides produced by immune cells. They are key signaling molecules in the intestinal immune system, capable of inducing, prolonging and even terminating inflammation. (Rogler and Andus, 1998). The two forms of IBD are thought to be due to immune imbalance, loss of immune tolerance, and increased levels of inflammatory cytokines. In IBD, there is an imbalance between pro-inflammatory and anti-inflammatory cytokines. Examples of the pro-inflammatory cytokines include tumor necrosis factor alpha (TNF-α), Interleukins 1Beta (IL-1β), IL-6, IL-8, IL12, and Interferon-Gamma (IFN-γ), whereas, examples of anti-inflammatory cytokines are IL-4, IL-5, IL-10, and Tumor Growth Factor –Beta (TGF-β).

1.6.1. Pro-inflammatory Cytokines

IL-1β is produced by macrophages in the inflamed mucosa. The effect of IL-1β is controlled by the IL-1 receptor antagonist (IL-1ra) produced by epithelial cells. A decreasing ratio of IL-1ra/IL-1β is correlated to higher IBD activity (Sanchez-Munoz et

al., 2008). TNF-α is produced by innate immune cells, macrophages and differentiated T-cells. Increased levels of TNF-α are observed in IBD patients (Reimund *et al.*, 1996). IL-2, is produced by T-helper 1 (Th1) cells. Increased levels of IL-2 are observed in patients with Crohn's disease whereas the levels are decreased in Ulcerative colitis (Van Kemseke *et al.*, 2000)). IL-6 is produced by macrophages in the gut. Increased levels of IL-6 are observed in the IBD patients (Louis *et al.*, 1997). IL-8 is produced by macrophages, epithelia cells and fibroblasts (Rogler and Andus, 1998). Increased levels of IL-8 are observed in diseased patients. IL-8 is regulated by IL-1 and TNF-α. IL-12 is produced by activated macrophages. It induces Th1 cell differentiation. It causes mucosal inflammation in animal models of CD (Rogler and Andus, 1998). Interferon- γ (INF-γ) is produced by the Th1 cells and Natural Killer (NK) cells. Increased levels of INF-γ are detected only in the mucosa (Rogler and Andus, 1998).

1.6.2. Anti-inflammatory Cytokines

IL-4 is anti-inflammatory cytokine. It inhibits TNF- and IL-1, but induces IL-1ra (IL-1 receptor antagonist). IL-4 levels are decreased in CD than UC. IL-5 is produced by T- helper 2 cells, a subset of T-helper cells. Increased levels of IL-5 are observed in UC patients (Rogler and Andus, 1998). IL-10 is produced by T-cells, and B-cells. It inhibits pro-inflammatory cytokines, and hence attenuates inflammation. In the absence of IL-10, increased levels of IL-12 and INF-γ are detected. (Kuhn *et al.,* 1993)

1.7. Role of Cytokines and Micro-biomes in Mucosal Immunity

The pathogenesis of IBD involves an imbalance of the immune system towards luminal antigens such as dietary factors and microorganisms. The mucosal surfaces in the gastrointestinal tract are in continuous contact with the luminal antigens, and are covered by epithelial cells which secrete mucins. Mucins form a barrier that prevents large particles including bacteria from contacting the epithelial layer. The epithelial layer functions like a leaky barrier, supporting fluid exchanges and other tissue specific functions. When mucosal irritants disrupt the epithelial barrier, chronic inflammation occurs. In a healthy gastrointestinal tract, an intact epithelial cell layer is maintained by apical junctional complex, composed of tight junction and adherens junction, both of which regulate barrier function. However, it is the tight junctions that are the principal determinants of mucosal permeability, and susceptible to modulation by the cytokines (Turner, 2009).

1.8. Probiotics, Micro-biome, and Mucosal Immunity

Many studies suggest an association between probiotics, gut microbiome and mucosal immunity. In one study, VSL#3 fed IL-10 deficient mice showed reduction in mucosal inflammation, improving the resistance to *Salmonella* infection (Madsen *et al.*, 2001). Another study suggests that feeding probiotic preparation VSL#3 to IL10-deficient mice reduces mucosal levels of cytokines, and restores gut barrier function (Madsen *et al.*, 2001). VSL#3 can modulate various signaling pathways that can affect mucosal immunity such as NF_KB (Nuclear Factor KappaB, Petrof *et al.,* 2004), PPARγ

(Peroxisome proliferator activated receptor gamma, Ewaschuk*, et al.,* 2006), and hsp (heat shock proteins, Petrof *et al.,* 2004) pathways.

Individual strain present in the VSL#3 cocktail, such as *Bifidobacterium breve* , targets the NF_KB pathway in the macrophages by decreasing lipopolysaccharide (LPS) binding to CD14 receptor (Menard *et al.*, 2004). *Bifidobacterium longum, Lactobacillus plantarum, Lactobacillus delbrueckii subsp. Lactobacillus bulgaricus Lactobacillus acidophilus,* and *Lactobacillus casei* also target the NF_KB pathway in the intestinal epithelial cells as well (Thomas and Versalovic, 2010).

1.9. Metabolomic Analysis

Metabolomics is the study of metabolism at the global level. It is defined as systematically identifying and characterizing all the metabolites (small molecules) produced in one or more organisms or a biological sample under a given environmental or physiological condition. It can be used to understand the role of microorganisms inhabiting the human body (Idle *et al.*, 2007).

A number of studies aimed at determining biomarkers for IBDs characterize the metabolite profiles of healthy and diseased patients or animal models. These studies involve the use analytical techniques such as Nuclear Magnetic Resonance Spectrometry (NMR) and Mass Spectroscopy (MS) to profile samples and multivariate analytical methods such as Principal Components Analysis (PCA), Partial Least Squares (PLS), and Discriminant Analysis (DA) to analyze the data. Some noteworthy studies that use these methods are as follows:

Williams *et al.* (2009), used NMR to profile urine samples from CD, UC, and healthy patients. They were able to cluster samples into groups using Principal Components Analysis (PCA), and identify cohorts using Partial Least Squares-Discriminant Analysis (PLS-DA). Murdoch *et al.* (2008), compared NMR generated urinary metabolite profiles from wild-type and IL-10 knockout mice, using 2-way Analysis of Variance (ANOVA), Principal Components Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA). The loadings plot of PLS-DA analysis, allowed them to distinguish the metabolites associated with the onset of the disease. Jansson *et al.* (2009), analyzed fecal samples using Ion Cyclotron Resonance Fourier Transform Mass Spectrometry. A combination of multivariate methods – PCA, Hierarchical Clustering Analysis (HCA), and PLS-DA were used to analyze the resulting data. Lin *et al.*, (2009) carried out non-targeted urinary metabolite profiling of IL-10 knock-out mice using GC-MS to identify potential markers of intestinal inflammation. They used multiple t-tests, and ANOVA to analyze their data. Martin *et al.* (2008), used the humanized genomic mice (germ -free mice colonized with human baby flora (HBF)) to assess the impact of probiotics (*Lactobacillus paracasei* or *L. rhamnosus*) on gut microbial functional ecosystem. They used NMR and Ultra Performance Liquid Chromatography Mass Spectrometry (UPLC-MS) analysis on multiple biofluids and tissues (plasma, urine, fecal extracts, liver tissues, and ileal flushes) to determine the host response to probiotic intervention. The NMR spectra were analyzed using Orthogonal-Partial Least Squares-Discriminant Analysis (O-PLS-DA). The multi-compartment metabolic data was integrated using Hierarchical Principal Components Analysis (H-

PCA). Martin *et al.* (2009), have used metabolite profiling of blood plasma to determine the metabolite changes during development (1, 8, 16, and 24 weeks) of colitis in IL-10 knockout mice. They analyzed the NMR data by PCA, Independent Component Analysis (ICA), PLS, and OPLS, to maximize discrimination between sample groups.

1.10. Metabolomic Analytical Approaches/Strategies

Different metabolomic analytical strategies answer different research questions, using different analytical techniques. Fiehn *et al.* (2002) described four approaches- target analysis, metabolite profiling, metabolomics, and metabolic fingerprinting.

1.10.1. Target analysis

Target analysis measures a few metabolites targeting specific substrates or products of an encoded protein; for example when studying the effect of a genetic alteration (Fiehn *et al.*, 2002). A major limitation is that the metabolites to be measured must be known, and should be available in purified form (Shulaev, 2006).

1.10.2. Metabolite profiling

Metabolite profiling is used when elucidation of the function of whole pathway or intersecting pathways is the goal. In this situation, it becomes necessary to identify and quantify the metabolites in those pathways (Fiehn *et al.*, 2002).

1.10.3. Metabolomics

In the metabolomics approach, all the measureable metabolites of the biological system are identified and quantified under a given set of conditions. When a single genetic change, affects the metabolite levels of unrelated biochemical pathways due to pleiotropic effects, the metabolomics approach is applied (Fiehn *et al.*, 2002).

1.10.4. Metabolic fingerprinting

Metabolic fingerprinting is a diagnostic tool to classify the samples rapidly according to their biological relevance or origin, such as to screen cell or tissue samples. It is not necessary to measure the levels of every metabolite, although the intracellular metabolome information is generated (Fiehn *et al.*, 2002).

1.11. Analytical Techniques for Detection of Analytes

The analytical techniques involve separation of the analytes followed by detection of the analytes. Separation of the analytes is usually by chromatographic methods such as Gas-chromatography (GC), High Performance Liquid chromatography (HPLC) and electrophoretic method such as capillary electrophoresis.

The commonly used analytical techniques for detection of the separated analytes in metabolomics are Nuclear Magnetic Resonance Spectrometry, (NMR), Gas-Chromatrography-Mass Spectrometry (GC-MS), Liquid Chromatrography Mass Spectrometry (LC-MS) and Capillary Electrophoresis Mass Spectrometry (CE-MS).

1.11.1. Nuclear Magnetic Resonance (NMR) Spectroscopy

Structural determinations of metabolites in pure and complex mixtures can be carried out with excellent quantitative precision. More than 100 samples/ day can be measured on a single spectrophotometer. Absolute concentrations are calculated when an internal standard of known concentration is added to the sample. The major disadvantage of NMR is it poor sensitivity and so is not useful for measuring low-abundance metabolites. It is non-destructive and no derivatization of samples is needed. NMR is used widely for metabolic fingerprinting, profiling and metabolic flux analyses (Weckwerth *et al.*, 2005, Shulaev, 2006).

1.11.2. Mass Spectroscopy

MS methods are used in metabolic fingerprinting, and metabolite identification. Due to high sensitivity, it can be used to characterize, identify and quantify a large number of compounds with a broad range of concentrations.

1.11.2.1. Gas Chromatography Mass Spectrometry

Gas Chromatography Mass Spectrometry (**GC-MS**) is most mature technology for rapid metabolite profiling. It can simultaneously profile and directly quantitate hundreds of chemically diverse compounds, volatile compounds. One limitation is its inability to study molecules that do not readily volatilize.

1.11.2.2. Liquid Chromatography Mass Spectrometry (LC-MS)

Liquid Chromatography Mass Spectrometry has high sensitivity, and wider range of molecular mass detection. It is slow and can only do 20-100 samples/day. One limitation is in obtaining consistent quantitative precision. Other variations are High Performance Liquid Chromatography Mass Spectrometry (**HPLC-MS)** used for metabolomics of biofluids and Ultra Performance Liquid Chromatography Mass Spectrometry (UPLC-MS) that is 10-fold increase in speed and 3-5 fold increase in sensitivity.

1.11.2.3. Capillary Electrophoresis Mass Spectrometry (CE-MS)

Capillary Electrophoresis Mass Spectrometry first separates metabolites based on charge and size, followed by detection by MS. It has a high resolving power (10^5-10^6 plate number), very small sample requirements and a short analysis time. It is used for targeted and non-targeted metabolites analysis. Its significant advantage is its ability to separate cations, anions, and uncharged molecules in a single run. When the positive end

of a high voltage source is applied to the inlet electrode, and the negative end is applied to the outlet electrode, the uncharged molecules have the same velocity as the Electroosmotic flow (EOF), while cations migrate faster than the EOF, and anions migrate slower than the EOF. (Weckwerth *et al.*, 2005, Shulaev, 2006, Mehdi, 2002).

1.12. Analysis of Metabolomic Data Using Bioinformatics Tools

Depending on the analytical technique, different parameters are analyzed. When data is from GC-MS and HPLC-MS, the parameters analyzed are peak retention, intensity and mass/charge (m/z) ratio. For NMR, chemical shifts, bin integrations or metabolite concentrations are analyzed. Data analysis usually involves data normalization and multivariate statistical analyses. These analyses fall into three categories, unsupervised, supervised and nonparametric (see below).

Metabolomic data consists of a large number of variables (metabolites) and only a small number of samples. This high-dimensional data must be reduced to a fewer number of uncorrelated variables. This is done by using supervised or unsupervised methods. Most widely used data analysis methods for metabolomic data are the PCA, clustering, SOMs, PLS, DA and variations thereof. Some of these methods are described below.

1.12.1. Principal Components Analysis (PCA)

Principal Components Analysis is the most widely used first step in data analysis. PCA finds the linear combinations that maximize the variation in the data. The first component (PC1) yields the maximum variance, and the second component (PC2) explains the largest amount of the remaining variance. The PCs are orthogonal to each

other. The new variables are uncorrelated to each other. A plot of the PC1 versus PC2 aids in visualizing the separated classes (Steinfath *et al.*, 2008).

1.12.2. Hierarchical Clustering (HCA)

Hierarchical clustering is an unsupervised, pairwise dissimilarity clustering method, where the groups are linked in the order of closeness, to form a tree (dendrogram). In the agglomerative hierarchical method (most widely used), first all n observations are considered as groups. The groups are merged based on the criterion of inter group (cluster) dissimilarity matrix. The inter–cluster dissimilarity distance depends on the method of linkage distance calculation. The order in which the groups are linked is represented as a dendrogram. Groups that are very similar are linked at low heights, while groups with same inter-group dissimilarity between them are merged.

1.12.3. Self-Organizing Maps (SOM)

Self-Organizing Maps is an unsupervised learning technique that reduces high dimensional data into a map of one or two dimensions. It plots the similarities of the data by grouping similar data items together. The SOMs reduce dimensionality and display similarities (Kohonen *et al.*, 2007).

1.12.4. Partial Least Squares (PLS)

Partial Least Squares is an extension of the multiple linear regression method. It generates a weight matrix that maximizes the covariance between the responses and corresponding factors scores. Then least squares procedure is applied to determine the loadings (StatSoft, Inc., 2010).

1.12.5. Discriminant Analysis (DA)

Discriminant analysis is a mathematical maximization procedure. The goal is to determine uncorrelated linear combinations of the original variables. These linear combinations are called the discriminant functions. The discriminant functions are uncorrelated to each other. The DFs are determined until the maximum possible functions are determined. Samples are then classified into groups based on their DF score (Mertler *et al.*, 2002).

1.12.6. Multivariate Analysis Of Variance (MANOVA)

Multivariate analysis of variance tests the significance of group differences. It can include multiple dependent variables and is useful in obtaining a holistic view of the biological process under investigation. The null hypothesis (Ho) states that population mean vectors are equal. Many test statistics can be used to test the null hypothesis, but the most widely used is the Wilk's Lambda. Its value ranges from 0 to 1. Smaller values of Wilk's Lambda indicate more evidence of differences in groups (Mertler *et al.*, 2002).

1.13. Metabolic Networks and Correlations

Metabolite profiles are snapshots of steady state levels predefined metabolite targets. Metabolite profiles aim to determine the effect of development, diet, or environment on the physiological condition of an organism or tissue. Since, the metabolite levels are the result of a network of metabolic pathways, the correlations between metabolites are like metabolic fingerprints and can be an effective way of gaining insight into the metabolic pathways involved (Steur *et al.*, 2003). Any changes in the metabolite levels such as during disease states will result in different correlation

matrices. The key is to understand the relationship of metabolite profile to the underlying biochemical reactions and regulatory networks.

Metabolic correlations analysis, as a novel concept was put forth in a study that compared tubers and leaves of 30-40 SS2 antisense potato plants to wildtype (Weckwerth *et al.,* 2004). They observed metabolite correlation differences in potato tubers and leaves. They tested 656 metabolites from tubers, of which 34 were significant at $p < 0.05$, and 18 had $p < 0.01$. In the case of leaves, 1216 metabolites were tested, of which 262 had significance of $p < 0.05$, and 98 were had $p < 0.01$. Topology of the metabolite correlation networks showed that the carbohydrate metabolism was altered in the SS2 antisense plants (Weckwerth *et al.*, 2004).

In our analyses so far, we have used a method put forward by Morgenthal *et al.* (2006). Morgenthal *et al.*, have compared the correlation patterns between different plant tissue types, and found that differences in tissue types are reflected in the correlation networks. The metabolite-metabolite correlation is determined using the pairwise Pearson Correlation Coefficients between the metabolites. The significance of the correlations is determined using the Student's t-transformation, and the differences between the correlations are tested using the z-transformation.

2: RESEARCH GOALS AND HYPOTHESIS

The goal of this study is to determine the effect of probiotics on the metabolite-metabolite, microbiome-microbiome, and microbiome-cytokine correlations in wild-type control mice versus IL10 deficient mice. Using the IL-10 deficient mice as the colitis model the metabolomic data from different organs or tissues can provide an understanding of the responses of the physiological processes to factors such as dietary environment, developmental or genetic changes.

Many studies have shown that probiotics are beneficial and attenuate intestinal inflammation (Shultz *et al.*, 1998) reference. However, there are no studies that evaluate the effect of probiotics on the metabolite levels, and hence the metabolic pathways in the IL-10 knockout mouse. This study aims at elucidating the metabolic network/ pathway differences, because it can help in better management of the inflammatory Bowel Diseases.

2.1. The Null Hypothesis

Feeding a probiotic diet to the IL-10 deficient mice will affect the metabolite-metabolite correlations. Thus, Ho = Probiotics do not affect metabolite-metabolite correlations and H1 = Probiotics affect metabolite-metabolite correlations.

2.2. The Experiments

2.2.1. Experiment for chapter 3

In this study, wild-type and IL10 deficient mice were treated with probiotic formulation VSL#3 for 14 days and sacrificed; their liver and caecal contents were analyzed by NMR. Thus, there were 4 groups for each tissue in this study, probiotic fed wild type mice (P_WT), probiotic fed IL10 deficient mice (P_IL10), control wild type mice (C_WT), and control-IL10 deficient mice (C_IL10). VSL#3 is a probiotic formulation consisting of 8 live bacterial strains: 3 *Bifidobacterium* strains, 4 *Lactobacillus* strains and 1 *Streptococcus* strain. It contains $3x10^{11}$ colony forming units (CFU/gm) of viable lyophilized bacteria.

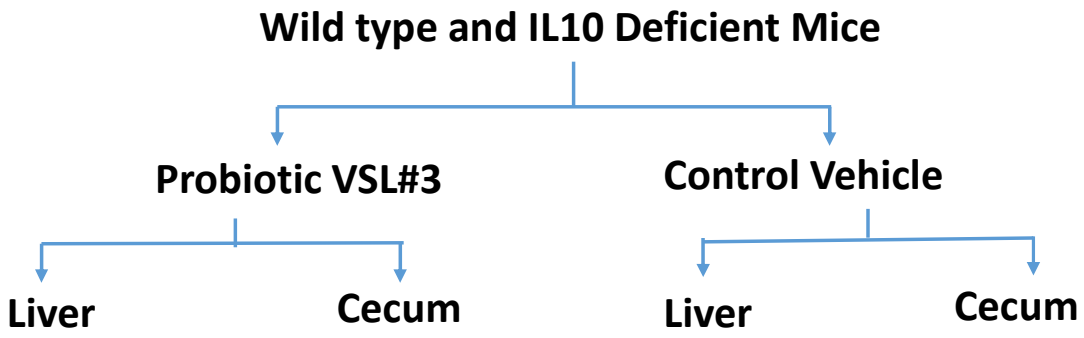

Figure 1: The experiment design for chapter 3.

2.2.2 Experiment for chapter 4 and 5

In this study, wild-type and IL10 deficient mice were treated with probiotic formulation VSL#3 for 14 days and sacrificed. Cytokine in the colon and ileum were

analyzed by electro-chemiluminescent multiplex kit. Cecal Contents and Cecal Mucosa were used for obtaining the Microbiome Abundances Data using the Multi-Tag Pyrosequencing method (Gillevet *et al*., 2010). Thus, there were 4 groups for each tissue in this study: P_WT, P_IL10, C_WT, and C_IL10. We have 4 sub-classes for each class. For example: Cecal Contents microbiome + colon cytokine, Cecal Contents microbiome + ileum cytokine, Cecal Mucosa microbiome + colon cytokine, and Cecal Mucosa microbiome + ileum cytokine.

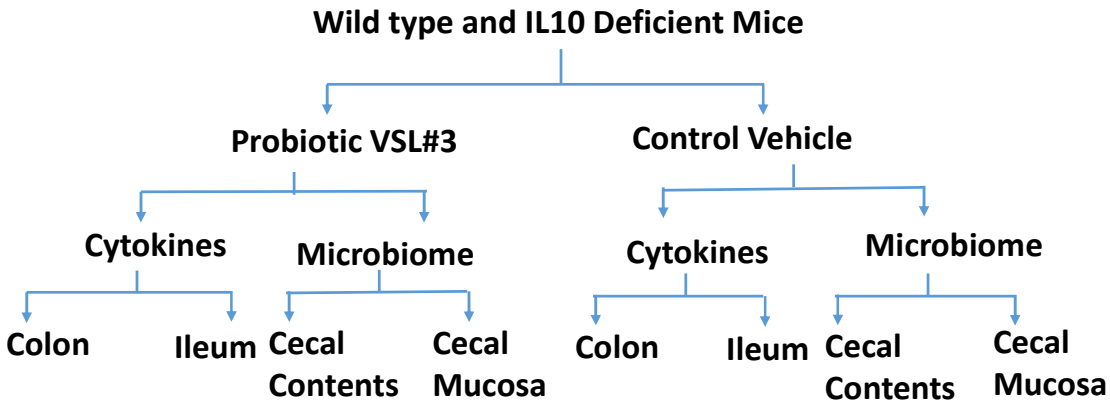

Figure 2: Experimental Design for chapter 4 and 5

2.3. Methods

2.3.1. Analytical Techniques for Obtaining Data

NMR spectroscopy was used to analyze liver and caecal extracts from wild type and IL-10 knock-out mice. Metabolite profile included measurement of specific metabolites. Data consisted of metabolite concentrations as features for each sample. There were 23 features for liver data, and 39 features for the cecum data. Despite the low sensitivity of NMR, it is ideal because of its high reproducibility, and ability to simultaneously quantifying multiple classes of metabolites.

2.3.2. Analytical Tools Used To Analyze Data

In order to analyze chapter 3 (probiotic metabolite) data, we used in- house R scripts (Appendix A) to calculate the correlations, t-values, Z-scores and p-values. A Python (Python 2.6) script was used to generate network and attribute files for uploading to cytoscape 2.8.2. Cytoscape 2.8.2 was used to visualize the networks. For chapter 4 and 5 microbiome and cytokine data sets, the Correlation analysis scripts on MBAC Galaxy server (available at *http://mbac.gmu.edu/mbac/index.php*) were used. The data was split by class, followed by correlation and significant differential correlation calculations. The output files and attribute files were used to visualize networks using Cytoscape 2.8.2.

2.3.2.1. Heatmap Representation of Correlations

Pairwise metabolite correlations are correlated using Pearson correlations. **P**airwise metabolite correlations matrices of the different mice groups were calculated using in-house R-script using the R-statistical package (R Development Core Team, 2010). The resulting correlation matrices were used to generate heat-maps in Konstanz

Information Miner (KNIME, Berthold *et al.*, 2007, available at http://www.knime.org/) for the different treatment groups.

2.3.2.2. Pearson Correlation Coefficient

Pearson's correlation coefficient (*r*) is a statistic used to describe the relationship between two variables. Formally known as the Pearson product-moment correlation coefficient, it ranges from -1.0 to 1.0, where -1.0 indicates the strongest inverse relationship and 1.0 indicates the strongest direct relationship between the two variables. A Pearson *r* of 0.0 indicates absence of relationship. The closer the Pearson *r* is to 0.0, the weaker the relationship, whereas the closer the *r* is to 1.0 or -1.0, the stronger is the relationship.

Pearson correlation coefficient between two variables is the covariance of the two variables divided by the product of their standard deviations. Thus, the Pearson correlation of coefficient of two metabolites *X* and *Y* is given by.

Equation 1: Pearson correlation coefficient

$$r_{xy} = \frac{\mathrm{COV}(X,Y)}{(\sigma_x \sigma_y)}$$

where, r_{xy} is the Pearson correlation, $\mathrm{COV}(X,Y)$ is the covariance of two variables, and $(\sigma_x \sigma_y)$ is their standard deviations.

2.3.2.3. Significance of Correlation Differences between Groups

Pairwise metabolite correlations for each group are first calculated. Then the significance of Pearson Correlations and correlation differences are determined as described below. Metabolite pairs with strong positive correlations (r values between 0.9 and 1.0) and strong negative correlations (r values between –0.9 and –1.0) were selected. For these metabolite pairs below mentioned tests were done.

(i). Statistical Significance of Pearson Correlations:In order to determine the statistical significance of the Pearson correlations, the t-statistic is calculated using the student t-transformation as described in Morgenthal *et al.* (2006), and is given by:

Equation 2: Statistical Significance of Pearson Correlation

$$t = \frac{C\sqrt{(N-2)}}{\sqrt{(1-C^2)}}$$

where,
N = Number of samples for each Metabolite pair
C = Pearson correlation

Two sided p-values are determined for α (alpha) = 0.05. The level of significance was set at $p < 0.05$.

(ii). Statistical Significance of Correlation Differences between two groups:

The correlations from two groups are compared and the statistical significance of the correlation differences is determined by calculating the *z* – statistic using the *z* – transformation as described in Morgenthal et al., (2006), and is given by:

Equation 3: Statistical Significance of Correlation Differences between two groups

$$Z = \frac{1}{2} \frac{\left\{ \text{Log}\left(\frac{1+C_1}{1-C_1}\right) - \text{Log}\left(\frac{1+C_2}{1-C_2}\right) \right\}}{\sqrt{\left(\frac{1}{N_1-3}\right) + \left(\frac{1}{N_2-3}\right)}}$$

where,
C1 and C2 are correlations for the two groups
N1 and N2 are the number of samples for each metabolite pair.

Two sided p-values are then determined for α (alpha) = 0.05. The level of significance was set at $p < 0.05$. This will allow us to determine significantly different metabolite-metabolite correlations between the two groups, indicative of the pathways affected by probiotic treatment.

2.3.2.4. Visualizing Correlation Differential Networks

Cytoscape 2.8.2 was used to visualize the networks (Shannon *et al.*, 2003). A python script (Python 2.6) was used to create the files of desired format, as input to cytoscape. Different nodes attributes were included in the attributes file so that networks with different node names could be visualized. These attributes are the metabolites, KEGG Id, Pathway names, Super-pathway names. This information is obtained from the KEGG database. All the metabolite information is collected from the KEGG Compounds database. The edge attributes were the correlation coefficient values and the correlation difference p-values. One of the strengths of Cytoscape is its ability to incorporate external attributes.

2.4. Sample Sizes

Sample size plays an important role in determining the significant correlation difference between two groups. Based on the equation 3, the denominator becomes infinity when N1 and N2 is <= 3. Z-scores are reported as "NAs" in the correlation difference matrix. Hence we cannot determine the significance of correlation difference between groups. Thus, a minimum sample size of 4 is needed to compute significant differential correlations.

Table 1: Sample Size for Each Class in the Different Studies

	Probiotic Data (ch. 3)		Microbiome-Cytokine Data (ch. 4 and 5)	
Class	**Liver samples**	**Caecal samples**	**Cecal Mucosa**	**Cecal Contents**
C_WT	**9**	**11**	**8**	**7**
P_WT	**20**	**22**	**8**	**8**
C_IL10	**5**	**6**	**4**	**4**
P_IL10	**21**	**22**	**6**	**6**

3: ANALYSIS OF LIVER AND CECUM METABOLOME

In this section we have analysed the data using correlation maps, determined the global properties of the networks, the significant correlations and significant correlation differences. The significant correlations networks are in Appendix B.

3.1. Heat-Maps of Liver Metabolites

The correlation heat maps of liver metabolites for the four groups are shown in figures 3, 4, 5 and 6. In the correlation maps, the dark blue color indicates strong positive correlation and dark red color indicates strong negative correlation. A visual color comparison of the heat maps of liver control wild type (C_WT, Figure 3), probiotic fed wild type mice (P_WT, Figure 4), and probiotic fed IL10 deficient (P_IL10, Figure 6) suggest that P_IL10 mice have similar metabolite-metabolite correlations. When the liver control IL10 deficient mice (C_IL10, Figure 5) and P_IL10 mice (Figure 6) are compared, most of the strong negative correlations (red) in C_IL10 mice become positive correlations (blue) in the P_IL10 mice. This suggests that feeding probiotics to the IL10 deficient mice reverses most of the correlations from negative to positive.

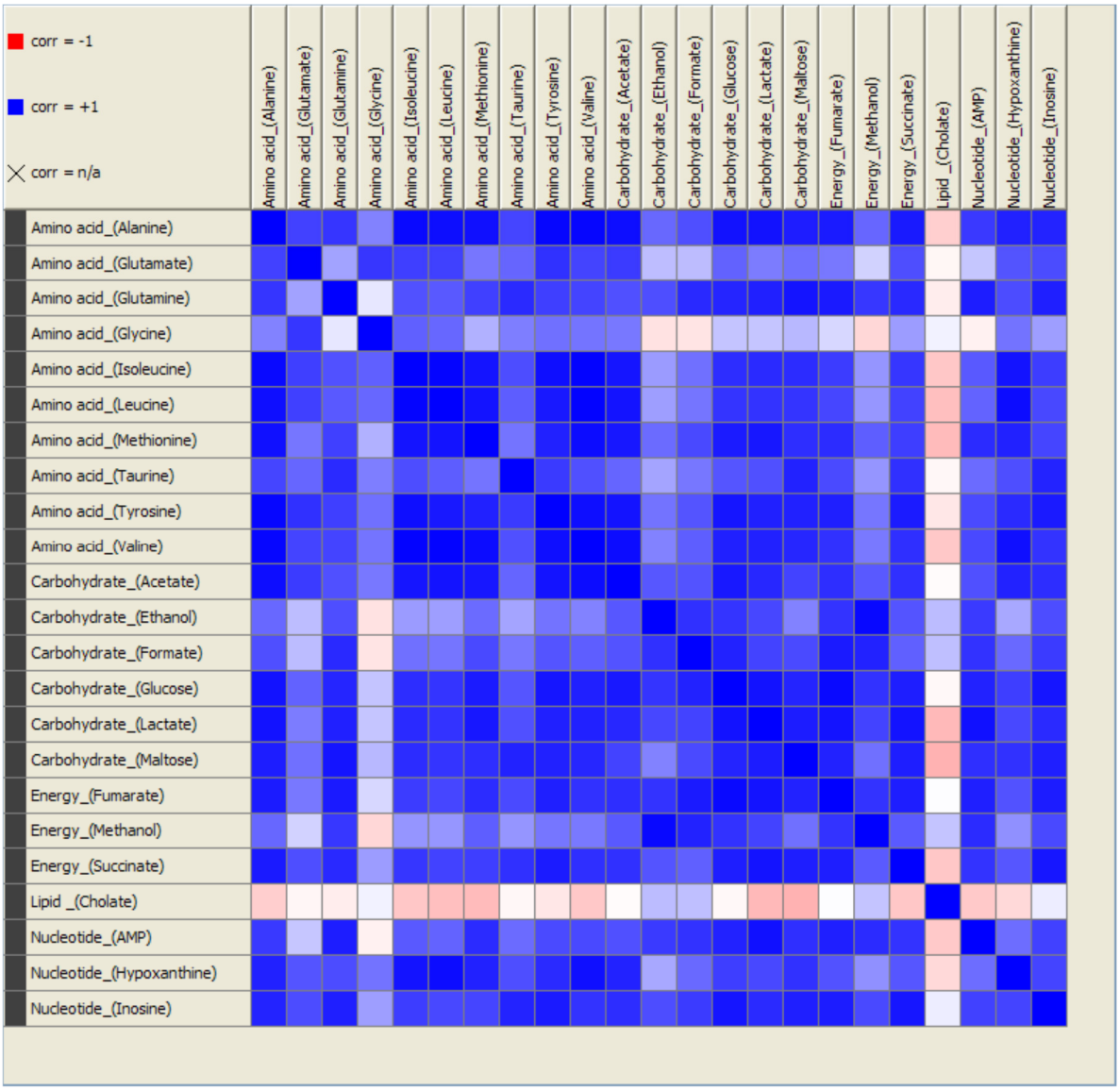

Figure 3: Heat-map of Liver wild-type mice fed with control diet

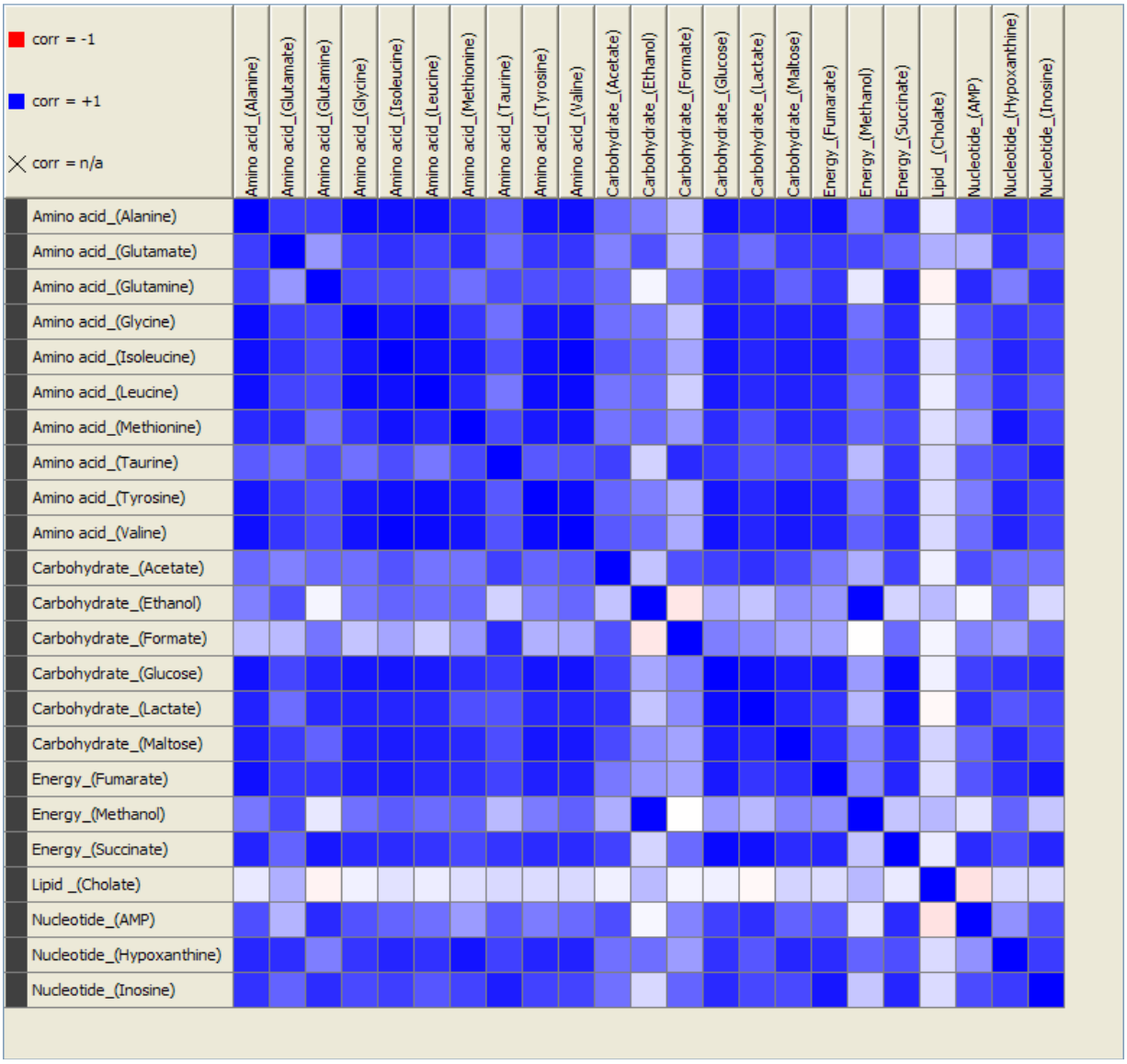

Figure 4: Heat-map of Liver wild-type mice fed with probiotic diet (VSL#3).

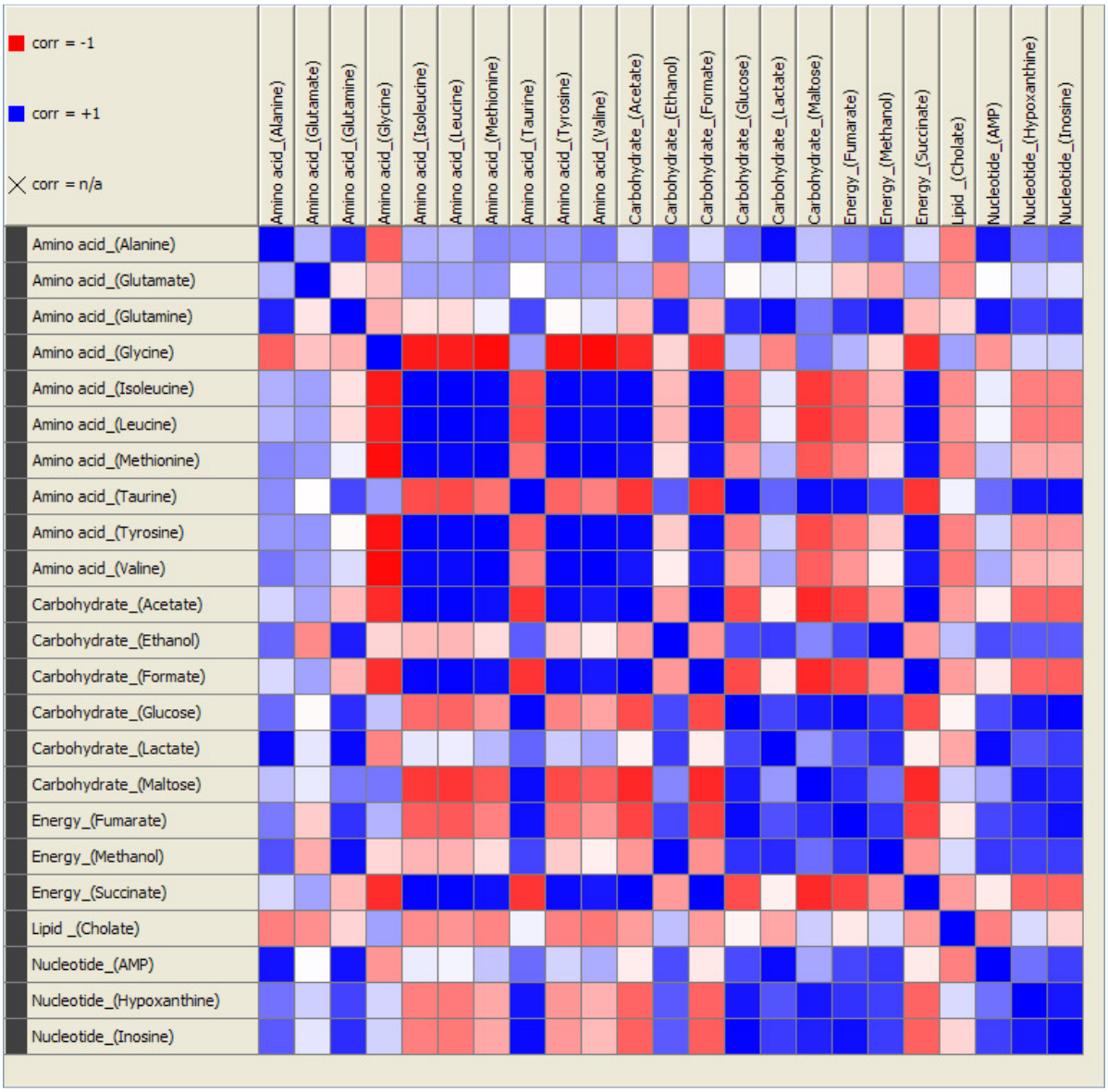

Figure 5: Heat-map of Liver IL10-deficient mice fed with control diet.

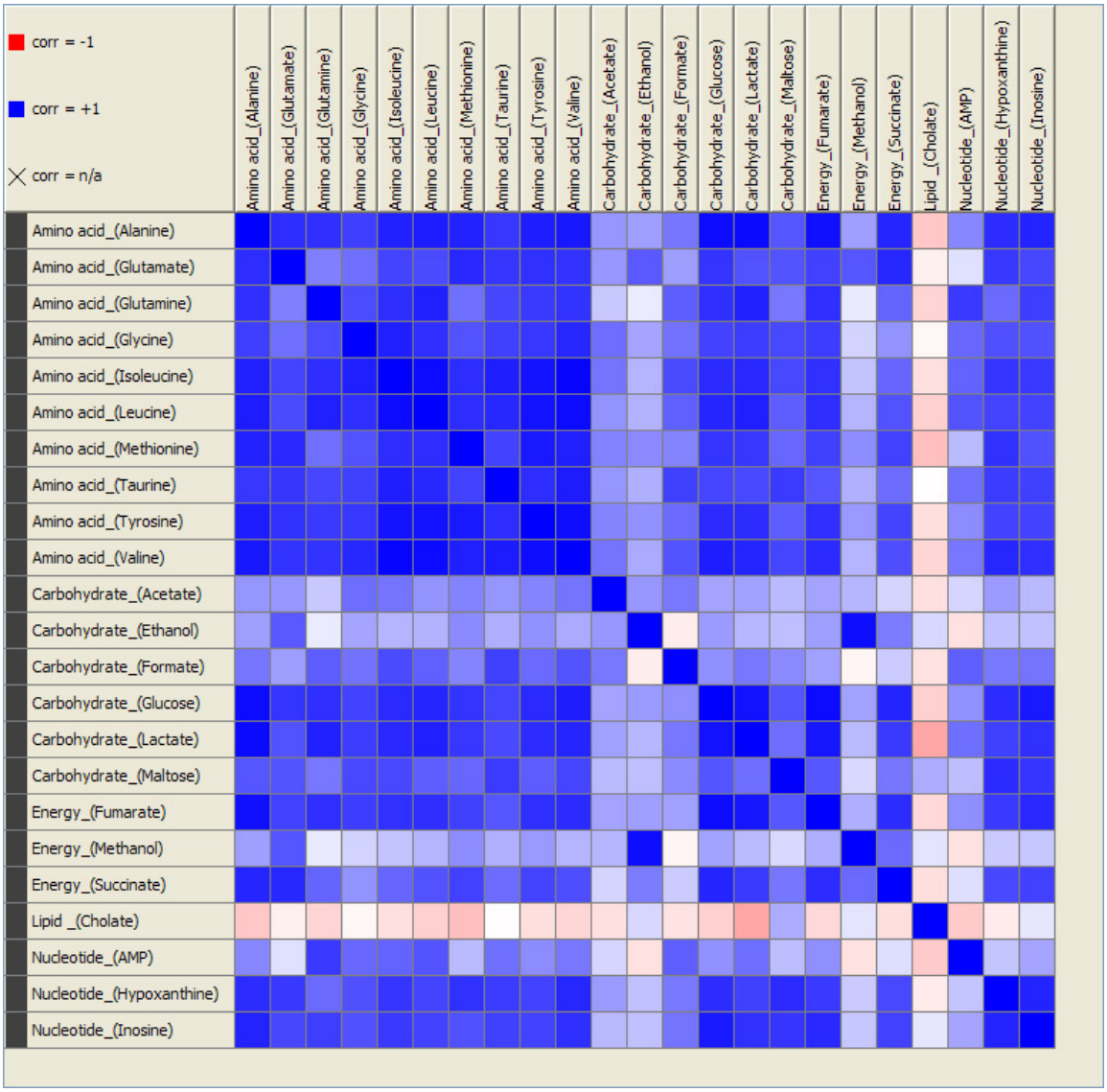

Figure 6: Heat-map of Liver IL10-deficient mice fed with probiotic diet (VSL#3).

3.2. Heat-maps of Caecal Metabolites

The correlation heat-maps of cecal metabolites for the four groups are shown in figure 7, 8, 9, and 10. The results for the cecal data show a few changes in correlations between the groups. A visual color comparison of the heat maps of cecal C_WT (Figure 7), P_WT mice (Figure 8), and P_IL10 mice (Figure 10) suggests that some negative (red) correlations become positive (blue) correlations. The cecal C_WT (Figure 7), P_WT (Figure 8), and P_IL10 mice (Figure 10) have some positively strong correlations that are different from the C_IL10 mice (Figure 9). The differences appear to be subtle.

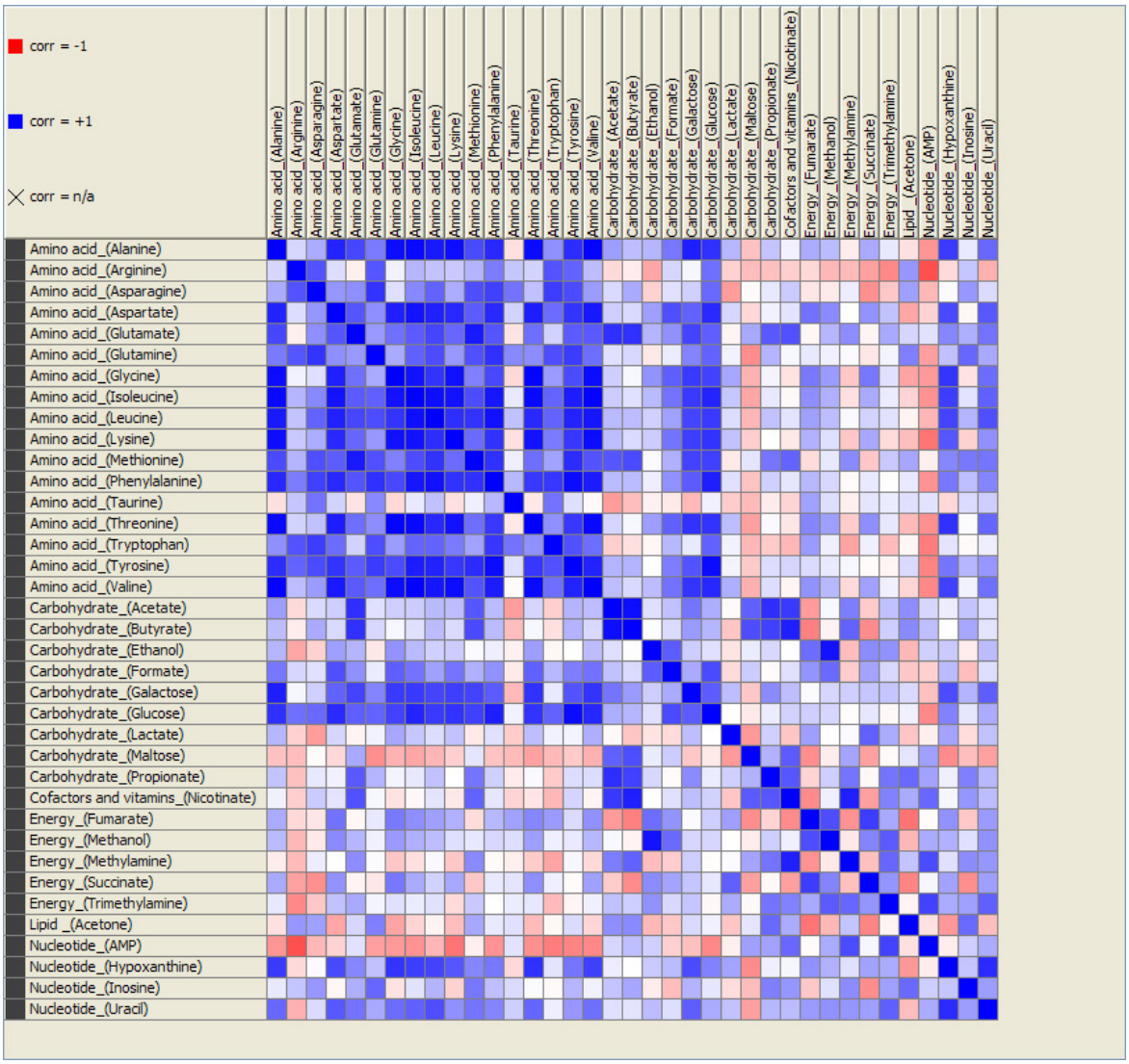

Figure 7: Heat-map of Cecal wild-type mice fed with control diet.

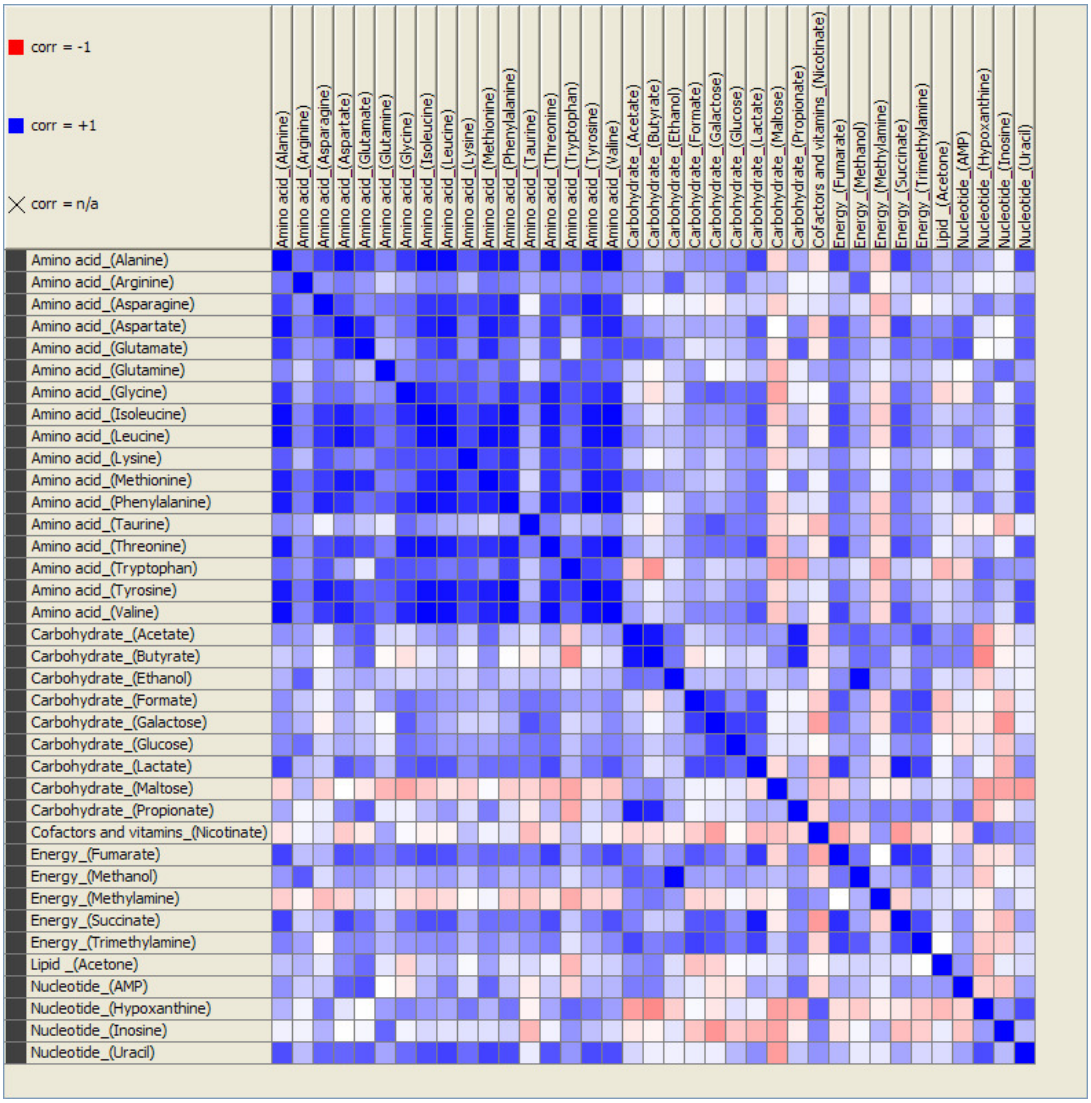

Figure 8: Heat-map of Cecal wild-type mice fed with probiotics (VSL#3).

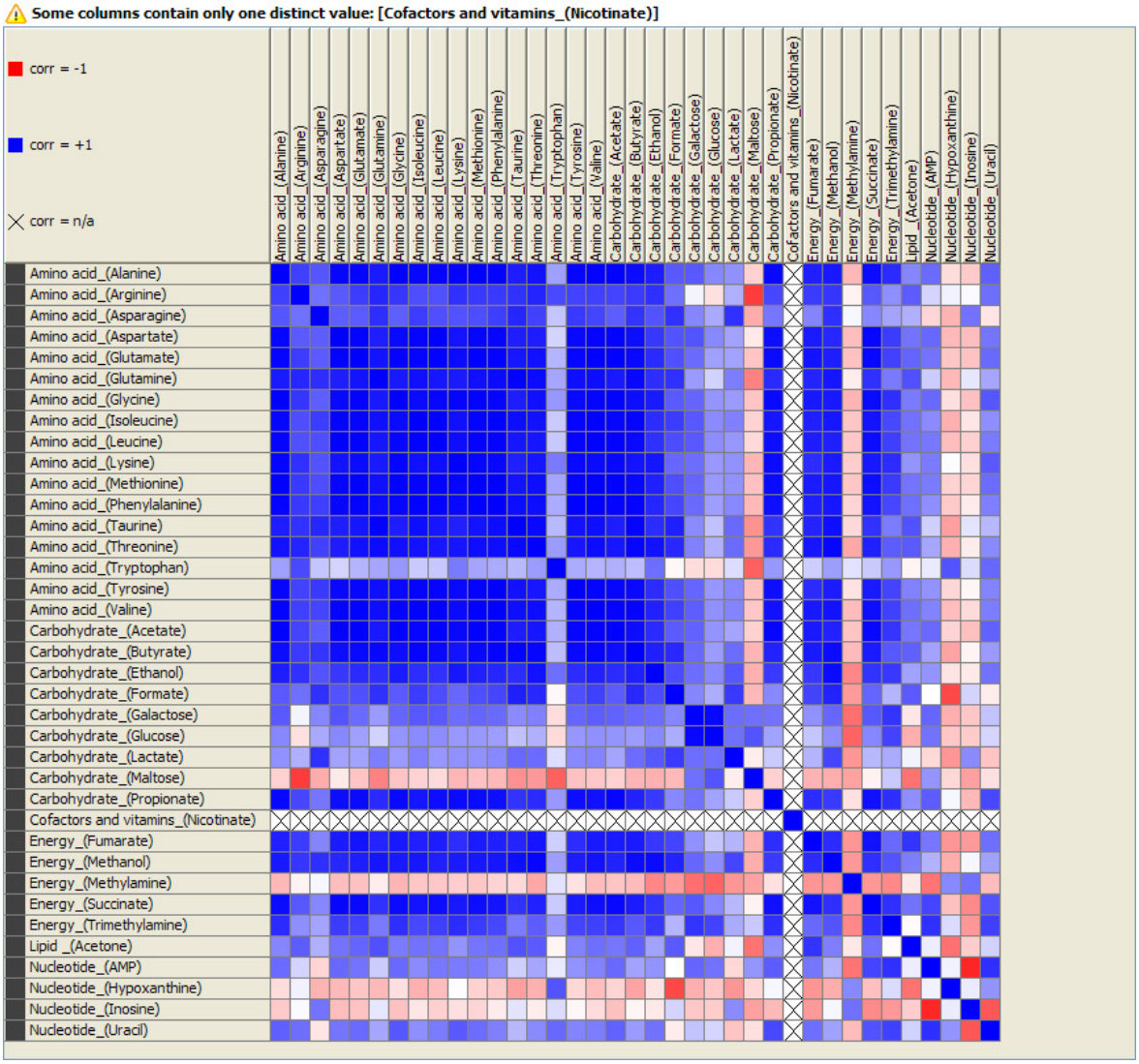

Figure 9: Heat-map of Cecal IL10-deficient mice fed with control diet.

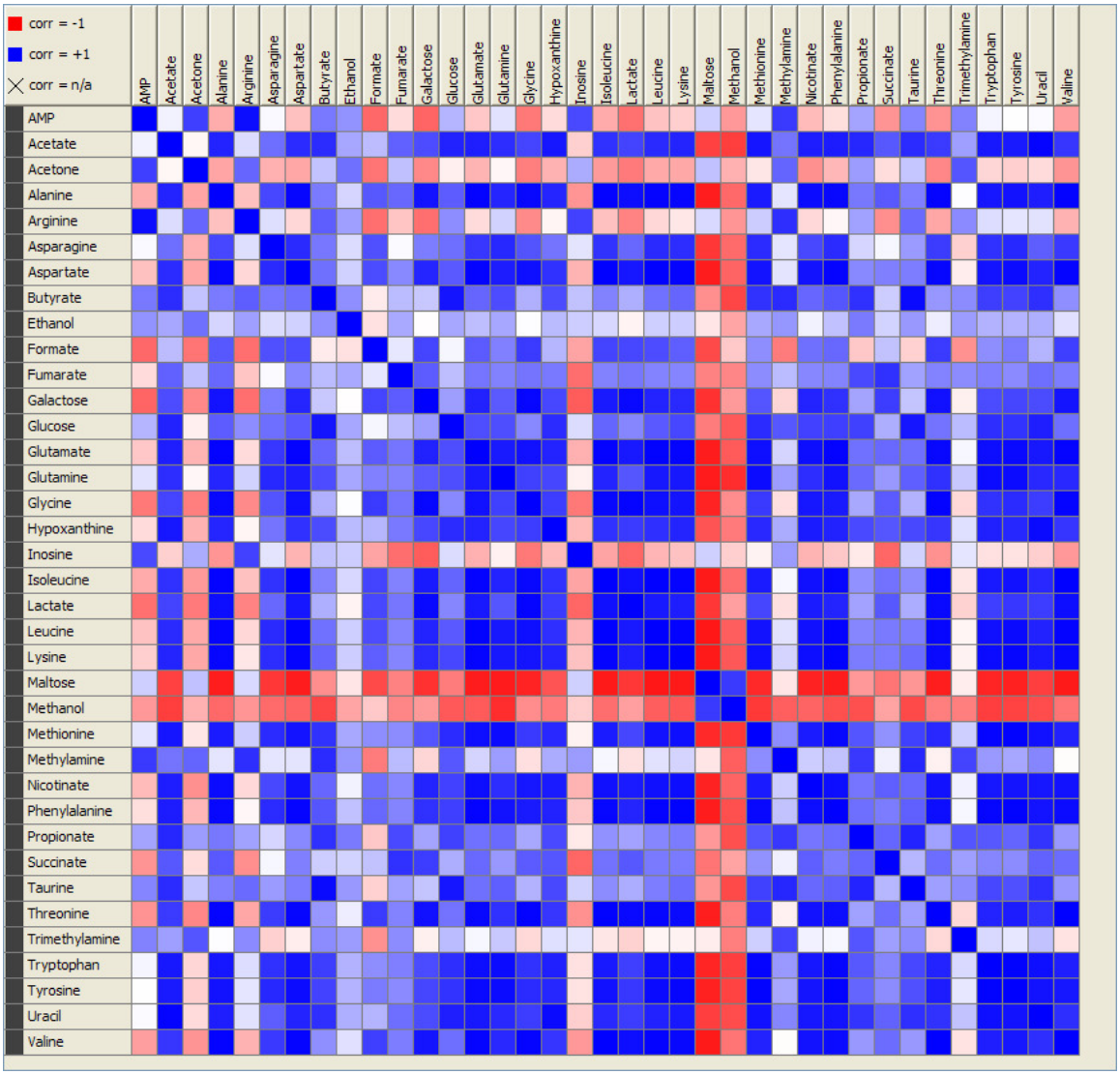

Figure 10: Heat-map of Cecal IL10-deficient mice fed with probiotics (VSL#3).

3.3. Global Properties

Table 2: Basic Global Properties of Networks gives the basic global properties of the significant correlations networks for both liver and cecum groups. In the liver sample classes, 50 significant correlations in Control IL-10 deficient (C_IL10) mice shift to 137 significant correlations when fed with probiotics. Control wild type (C_WT) mice have 166 significant correlations. In the Cecum classes: 234 significant correlations in C_IL10 shift to 202 significant correlations when fed with probiotics. Control wild type has 146 significant correlations. In both the liver and cecum samples, the percent significant correlations in probiotic fed wild type (P_WT) are comparable to that in the wild type.

Table 2: Basic Global Properties of Networks

	Caecum C_WT	Caecum C_IL10	Caecum P_WT	Caecum P_IL10	Liver C_WT	Liver C_IL10	Liver P_WT	Liver P_IL10
Number of Significant Edges	146	234	150	202	166	50	160	137
Number of Nodes	33	31	33	30	22	20	22	21
Percentage of the total number of correlatons	23.2	37.1	23.8	32.1	65.6	19.8	63.2	54.2

Total number of correlations: Cecum = 630, Liver = 253
C_WT= Control Wild type; C_IL10= Control IL10 deficient mice; P_WT = Probiotic fed Wild type; P_IL10 = Probiotic fed IL10 deficient mice

3.4. Significantly Differential Correlation Networks

We compared the correlation networks of treated (probiotic diet fed) and untreated (control diet fed), wild type and Interleukin-10 (IL-10) deficient mice for both liver and cecum tissues. Thus there were 4 groups for each tissue type—Control wild type mice (C_WT), Control IL-10 deficient mice (C_IL10), Probiotic fed wild type mice

(P_WT), and Probiotic fed IL-10 deficient mice (P_IL10). There are six comparisons for each tissue type as shown in Table 3 below.

First the number of significant correlations (r>= 0.6) for each group in a comparison are determined. Then, we determine the number of these correlations that are significantly different in the other group. Lastly, we use Cytoscape 2.8.2 to visualize the correlation networks and determine the number of nodes and edges (interactions).

Table 3: List of Correlation Comparisons

COMPARISON No.	GROUP 1	GROUP 2
COMPARISON 1	Control Wild type mice (C_WT)	Control IL10 deficient mice (C_IL-10)
COMPARISON 2	Probiotic fed Wild type mice (P_WT)	Probiotic fed IL10 deficient mice (P_IL10)
COMPARISON 3	Probiotic fed IL10 deficient mice (P_IL10)	Control IL10 deficient mice (C_IL-10)
COMPARISON 4	Control Wild type mice (C_WT)	Probiotic fed IL10 deficient mice (P_IL10)
COMPARISON 5	Probiotic fed Wild type mice (P_WT)	Control IL10 deficient mice (C_IL-10)
COMPARISON 6	Probiotic fed Wild type mice (P_WT)	Control Wild type mice (C_WT)

Table 3 shows the comparisons that were analyzed in Table 4 and Table 5. Table 4 shows the number of significant correlation differences between the liver classes, and Table 5 shows the number of significant correlation differences between the cecum classes.

Table 4: Significant Correlation Differences between the Liver Classes.

	GROUP 1	GROUP 2	COLUMN A	COLUMN B
COMPARISON No.	Significant Correlations in Group 1 Edges (nodes)	Significant Correlations in Group2 Edges (nodes)	Group1 vs Group2 Edges (nodes)	Group2 vs Group1 Edges (nodes)
COMPARISON 1	C_WT **166 (22)**	C_IL-10 **50 (20)**	C_WT - C_IL10 **48 (17)**	C_IL10 and C_WT **9 (9)**
COMPARISON 2	P_WT **160 (22)**	P_IL10 **137 (21)**	P_WT - P_IL10 **12 (13)**	P_IL10 - P_WT **7 (11)**
COMPARISON 3	P_IL10 **137 (21)**	C_IL-10 **50 (20)**	P_IL10 - C_1L10 **45 (17)**	C_IL10 - P_IL10 **15 (12)**
COMPARISON 4	P_IL10 **137 (21)**	C_WT **166 (22)**	C_WT - P_IL10 **18 (18)**	P_IL10 - C_WT **1 (2)**
COMPARISON 5	P_WT **160 (22)**	C_IL-10 **50 (20)**	P_WT - C_IL10 **51 (18)**	C_IL10 - P_WT **12(9)**
COMPARISON 6	P_WT **160 (22)**	C_WT **166 (22)**	P_WT - C_WT **8 (9)**	C_WT - P_WT **9 (10)**

C_WT: Control wild type **C_IL10: Control IL10 deficient mice**
P_WT: Probiotic fed wild type **P_IL10: Probiotic fed IL10 deficient**

Table 5 shows the number of significant correlation differences between the cecum classes.

Table 5: Significant Correlation Differences between the Cecum Classes

	GROUP 1	GROUP 2	COLUMN A	COLUMN B
COMPARISON No.	Significant Correlations in Group 1 Edges (nodes)	Significant Correlations in Group2 Edges (nodes)	Group1 vs Group2 Edges (nodes)	Group2 vs Group1 Edges (nodes)
COMPARISON 1	C_WT **146 (33)**	C_IL-10 234 **(31)**	C_WT - C_IL10 **21 (15)**	C_IL10 and C_WT **98 (26)**
COMPARISON 2	P_WT **150 (33)**	P_IL10 202 **(30)**	P_WT - P_IL10 **34 (27)**	P_IL10 - P_WT **55 (28)**
COMPARISON 3	P_IL10 202 **(30)**	C_IL-10 234 **(31)**	P_IL10 - C_1L10 21 **(16)**	C_IL10 - P_IL10 78 **(26)**
COMPARISON 4	C_WT **146 (33)**	P_IL10 202 **(30)**	C_WT - P_IL10 **17 (19)**	P_IL10 - C_WT **38 (26)**
COMPARISON 5	P_WT **150 (33)**	C_IL-10 234 **(31)**	P_WT - C_IL10 20 **(15)**	C_IL10 - P_WT **85 (21)**
COMPARISON 6	P_WT **150 (33)**	C_WT **146 (33)**	P_WT - C_WT 10 **(16)**	C_WT - P_WT **9 (11)**

C_WT: Control wild type, C_IL10: Control IL10 deficient mice, P_WT: Probiotic fed wild type, P_IL10: Probiotic fed IL10 deficient

Consider the comparison 1 (C_WT - C_IL10) and Comparison 4 (C_WT - P_IL10) of liver samples in Table 4 column A. Here, the 48 significantly different interactions in C_WT-C_IL10 decreases to 18 interactions in C_WT- P_IL10. Hence, probiotic diet reduces the number of significant differential correlation in P_IL10 mice. In a similar comparison of cecum samples in Table 5 column A, the 21 significantly different interactions in C_WT-C_IL10 is comparable to 17 interactions in C_WT-P_IL10. Thus, feeding probiotic diet to C_IL10, results in fewer significant correlation differences between C_WT and P_IL10. This, reduction in the significant differential correlations between C_WT-P_IL10 mice is more pronounced in the liver samples, than cecum samples.

The number of significantly different correlations between C_WT and C_IL10 mice (comparison 1, liver edges=48, cecum edges=21), P_IL10 and C_IL10 mice (comparison 3, liver edges=45, cecum edges=21), and P_WT and C_IL10 (comparison 5, liver edges=51, cecum edges=20) are similar in liver (Table 4) and cecum (Table 5) samples.

Thus, P_IL10 network, has similar significant differential correlations with C_IL10, as C_WT network and P_WT network. Hence, suggesting that in C_IL10, a probiotic diet establishes a network similar to that in C_WT and P_WT.

3.4.4. Overlapping Significant Correlation Networks

Probiotic fed IL10 deficient mice share more edges with wild type mice than IL10 deficient mice.

Overlapping networks share edges and nodes. Two networks will overlap if they share some nodes and edges. Table 6 shows the number of significant overlapping nodes and edges between two groups.

Table 6: Significant overlapping networks among Liver and Cecum sub-classes.

COMPARISON NUMBER	COMPARISONS	LIVER		CAECAL	
		EDGES	NODES	EDGES	NODES
COMPARISON 1	C_WT - C_IL10	46	20	81	29
COMPARISON 2	P_WT - P_IL10	127	21	106	28
COMPARISON 3	P_IL10 - C_1L10	35	19	104	26
COMPARISON 4	C_WT - P_IL10	106	20	108	29
COMPARISON 5	P_WT - C_IL10	40	20	92	30
COMPARISON 6	P_WT - C_WT	134	22	85	31

Legend: C_WT: Control wild type mice; **C_IL10:** Control IL10 deficient mice; **P_WT:** Probiotic fed wild type mice; and **P_IL10:** Probiotic fed IL10 deficient mice

In liver network (Table 6), 46 edges shared between C_WT and C_IL10 (comparison 1) mice increases to 106 shared edges in C_WT and P_IL10 (comparison 4) network. Similarly, in cecum network, the 81 edges shared between C_WT and C_IL10 mice increases to 108 shared edges between C_WT and P_IL10. The number of nodes are comparable.

Similarly, in the case of liver network, 40 edges shared between P_WT and C_IL10 (comparison 5) increases to 127 in P_WT and P_IL10 (comparison 2) network.

Similarly, in cecum network, the 92 edges shared between P_WT and C_IL10 network increases to 106 for P_WT and P_IL10 (comparison 2). The number of nodes are comparable. Thus, probiotic fed IL10 deficient mice share more edges with wild type mice than IL10 deficient mice.

3.5. Significant Metabolite Differential Correlations between Groups:

Phenotypically, the IL10-deficient mice have severe intestinal inflammation or colitis, as opposed to the WT mice. If any of these significant differential correlations are significantly different between probiotic fed IL10 deficient mice and WT mice we can conclude that probiotics affect the metabolite-metabolite correlations. In other words, that the null hypothesis is rejected.

3.5.1. Significant Liver Metabolite Differential Correlations

Table 7 shows the p-values of the correlation differences between the different treatment groups, and Table 8 is the attributes guide to the metabolite, super-pathway, and pathways. The significant correlation differences between C_IL10 mice and P_IL10 are shown in Table 7 column1. Significant correlation differences of $p < 0.001$ are seen between (i) Formate (pyruvate metabolism) and Acetate (Glycolysis/Gluconeogenesis pathway), (ii) Succinate (Krebs Cycle) and Acetate (Glycolysis/Gluconeogenesis pathway), and (iii) Succinate (Krebs Cycle) and Formate (Pyruvate metabolism).

Other significant differential correlations are between Acetate and Tyrosine, Formate and Isoleucine, Formate and Leucine, Glucose and Taurine, Methanol and Glutamine, Succinate and Isoleucine, Succinate and Leucine, and Valine and Methionine.

Acetate, formate, and glucose are metabolites of the Carbohydrate super-pathway, whereas succinate is a metabolite of the Energy pathway. Amino acid super-pathway metabolites include valine, leucine, isoleucine, tyrosine, glutamine, methionine, and taurine. Thus, interactions between carbohydrate, energy, and amino-acid pathways are significantly different in C_IL10 and P_IL10 mice.

Table 7: Liver Metabolite Differential Correlations

NODE1(PP)NODE2	CIL10 Pil10	PWT CIL10	PWT PIL10	CWT PIL10	CWT CIL10	CWT PWT
Metabolite--Metabolite	pvalue	pvalue	pvalue	pvalue	pvalue	pvalue
Acetate (pp) Alanine				0.00335	0.04280	
Acetate (pp) Isoleucine	**0.00763**			0.04084		
Acetate (pp) Leucine	**0.00163**			0.01284		
Acetate (pp) Methionine	0.09153			0.03588		
Acetate (pp) Tyrosine	0.04196			0.02058		
Acetate (pp) Valine	0.19480			0.00797		
Formate (pp) Acetate	**5.650E-07**					
Formate (pp) Isoleucine	0.03270					
Formate (pp) Leucine	0.01040					
Glucose (pp) Acetate				0.01599	0.00370	
Glucose (pp) Glycine		0.08277	0.08495			**0.00244**
Glucose (pp) Isoleucine		0.00228	0.23586			**0.00612**
Glucose (pp) Leucine		**0.00347**	0.54253			0.34572
Glucose (pp) Taurine	0.04410					
Glucose (pp) Tyrosine		**0.00403**	0.23578	0.43767	0.00960	
Glucose (pp) Valine		0.00651	0.44740			0.76815
Glycine (pp) Alanine		**0.00029**	0.00327			0.03060
Isoleucine (pp) Alanine		0.05249	0.19140	0.15913	0.04065	
Isoleucine (pp) Glycine		**5.008E-05**	0.56096			0.20133
Leucine (pp) Alanine		0.04652	0.28717	0.45896	0.07041	
Leucine (pp) Glycine		**7.860E-06**	0.02344			0.08010
Maltose (pp) Glutamine				0.03264	0.21338	
Maltose (pp) Tyrosine		0.00104	0.01708			0.07435
Maltose (pp) Valine		0.00237	0.07363			0.52640
Methanol (pp) Glutamine	0.0218					
Succinate (pp) Acetate	**2.270E-09**					
Succinate (pp) Formate	**1.480E-11**					
Succinate (pp) Glucose		**0.00013**	0.03667			0.41304
Succinate (pp) Glutamine		0.01591	0.01521			0.52406
Succinate (pp) Isoleucine	0.0132					
Succinate (pp) Lactate		0.01484	0.02958	0.19452	0.03768	
Succinate (pp) Leucine	0.0113					
Tyrosine (pp) Alanine		0.11771	0.54600	0.07585	0.02730	
Tyrosine (pp) Glycine		**2.909E-05**	0.20378			0.53111
Tyrosine (pp) Methionine						**0.00997**
Valine (pp) Alanine		0.11473			0.04570	
Valine (pp) Glycine		**1.115E-06**	0.25430			0.56079
Valine (pp) Methionine	0.027544		0.45795	0.26804		

Red= Significant Difference (p<0.05), Grey = No Significant Difference, Cyan= Interaction not present

Table 8: Liver Data Attributes Guide: Metabolite, Super-Pathway and Pathway Guide

METABOLITE	SUPER PATHWAY	PATHWAY
Alanine	Amino acid	Alanine and aspartate metabolism
Glutamate	Amino acid	Glutamate metabolism
Glutamine	Amino acid	Glutamate metabolism
Glycine	Amino acid	Glycine, serine and threonine metabolism
Isoleucine	Amino acid	Valine, leucine and isoleucine metabolism
Leucine	Amino acid	Valine, leucine and isoleucine metabolism
Methionine	Amino acid	Cysteine and methionine metabolism
Taurine	Amino acid	Cysteine, methionine, SAM, taurine metabolism
Tyrosine	Amino acid	Phenylalanine & tyrosine metabolism
Valine	Amino acid	Valine, leucine and isoleucine metabolism
Acetate	Carbohydrate	Glycolysis / Gluconeogenesis
Ethanol	Carbohydrate	Glycolysis / Gluconeogenesis
Formate	Carbohydrate	Pyruvate metabolism
Glucose	Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism
Lactate	Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism
Maltose	Carbohydrate	Fructose, mannose, galactose, starch, and sucrose metabolism
Fumarate	Energy	Krebs cycle
Methanol	Energy	Methane metabolism
Succinate	Energy	Krebs cycle
Cholate	Lipid	Primary bile acid biosynthesis
AMP	Nucleotide	Purine metabolism
Hypoxanthine	Nucleotide	Purine metabolism, (hypo)xanthine/inosine containing
Inosine	Nucleotide	Purine metabolism, (hypo)xanthine/inosine containing

3.5.2. Significant Cecal Metabolite Differential Correlations

Table 9 shows the p-values of the correlation differences between the different treatment groups, whereas Table 10 is the attributes guide to the metabolite, super-pathway, and pathways. **S**ignificant correlation differences between C_IL10 and P_IL10 are shown in Table 9, column 1. There are significant correlation differences of $p<0.05$between (i). Butyrate (Butanoate metabolism), and isoleucine (Valine, leucine and isoleucine metabolism), (ii). Butyrate and threonine (Glycine, serine and threonine metabolism), (iii). Fumarate (Krebs cycle) and Butyrate, (iv). Butyrate and methanol (Methane metabolism), (v). Methanol (Methane metabolism) and glycine (Glycine, serine and threonine metabolism), (vi) Methanol and threonine, (vii). Methanol and tyrosine

(Phenylalanine and tyrosine metabolism), (viii) Tyrosine (Phenylalanine & tyrosine metabolism) and tryptophan (Tryptophan metabolism). Thus, some of the carbohydrate-energy, energy-amino-acid and carbohydrate-amino-acid pathways are significantly different in C_IL10 and P_IL10 mice.

Table 9: Cecal Significant Metabolite Differential Correlations

NODE1(PP)NODE2	CCIL10_CPIL10	CCWT_CCIL10	CCWT_CPIL10	CCWT_CPWT	CPWT_CCIL10	CPWT_CPIL10
Metabolite--Metabolite	pvalue	pvalue	pvalue	pvalue	pvalue	pvalue
Butyrate(pp)Aspartate	0.0829	0.015047802	0.177	0.581138087	**0.0229**	0.3
Butyrate(pp)Glycine	0.0155	0.00875999	0.521	0.728514274	0.00199	0.199
Butyrate(pp)Isoleucine	0.00352	**7.84E-04**			2.24E-04	
Butyrate(pp)Threonine	0.00449	0.001227687	0.315		8.46E-04	
Butyrate(pp)Tyrosine	0.0705	0.012585253	0.179	0.563613817	0.00186	0.0126
Formate(pp)Butyrate	0.0392	0.107107473	0.652	0.569435671	**0.0322**	0.878
Fumarate(pp)Butyrate	3.09E-02	0.002857969	0.108	0.041707397	0.0617	0.579
Fumarate(pp)Formate	0.0761	0.470397181	0.146	0.393515801	0.835	0.00271
Glycine(pp)Arginine	0.033	0.132899			0.232	
Glycine(pp)Aspartate	0.243	0.342915816	0.844	0.085310529	**0.0277**	0.0477
Hypoxanthine(pp)Butyrate	0.0651	0.577317878	0.0682	0.238279528	0.847	9.59E-05
Hypoxanthine(pp)Threonin	0.115	0.031650083	0.26		0.272	0.36
Lactate(pp)Leucine	0.369	0.660910392	0.0424	0.11959436	0.563	0.539
Maltose(pp)Arginine	0.0849				0.0383	
Methanol(pp)Arginine	**0.0219**	**0.046131874**		**0.011915301**	0.64	**4.77E-04**
Methanol(pp)Aspartate	0.0141	0.267566165			0.189	**2.88E-02**
Methanol(pp)Butyrate	**2.73E-04**	**0.003596148**			**0.0368**	**0.00297**
Methanol(pp)Formate	0.0181		**0.0486**		0.145	
Methanol(pp)Fumarate	0.0181	0.610395955	**0.00766**		0.345	**0.00656**
Methanol(pp)Glycine	0.00688	0.117302261			0.0478	
Methanol(pp)Maltose	0.0442		**0.0187**		0.335	**0.0447**
Methanol(pp)Threonine	**6.26E-05**	**0.006556898**			**0.00341**	**0.0397**
Methanol(pp)Tyrosine	0.00291	0.047273586			0.079	**0.0194**
Methylamine(pp)Butyrate	0.0348				0.164	
Succinate(pp)Butyrate	**0.0322**	**0.004416954**			**0.0463**	
Succinate(pp)Isoleucine	0.0396	0.057084491			0.2	
Succinate(pp)Lactate	0.814				0.0491	**9.07E-04**
Taurine(pp)Phenylalanine	0.0561	0.022851546			**0.0176**	
Threonine(pp)Arginine	0.0244	0.100670766			0.188	
Trimethylamine(pp)Format	0.482		0.49	0.052857318	0.281	6.43E-04
Trimethylamine(pp)Fumara	0.588		0.975		0.605	0.0425
Tyrosine(pp)Tryptophan	3.05E-02				0.331	
Uracil(pp)Butyrate	0.293	0.648863462	0.0225	0.920142289	0.573	0.00198

Red= Significant Difference, Grey = No Significant Difference, Cyan= Interaction not present

Table 10: Cecum Data Attribute Guide: Metabolite, Super-Pathway and Pathway

METABOLITE	SUPERPATHWAY	PATHWAY
Alanine	Amino acid	Alanine and aspartate metabolism
Arginine	Amino acid	Urea cycle; arginine-, proline-, metabolism
Asparagine	Amino acid	Alanine, aspartate and glutamate metabolism
Aspartate	Amino acid	Alanine and aspartate metabolism
Glutamate	Amino acid	Glutamate metabolism
Glutamine	Amino acid	Glutamate metabolism
Glycine	Amino acid	Glycine, serine and threonine metabolism
Isoleucine	Amino acid	Valine, leucine and isoleucine metabolism
Leucine	Amino acid	Valine, leucine and isoleucine metabolism
Lysine	Amino acid	Lysine metabolism
Methionine	Amino acid	Cysteine and methionine metabolism
Phenylalanine	Amino acid	Phenylalanine & tyrosine metabolism
Taurine	Amino acid	Cysteine, methionine, SAM, taurine metabolism
Threonine	Amino acid	Glycine, serine and threonine metabolism
Tryptophan	Amino acid	Tryptophan metabolism
Tyrosine	Amino acid	Phenylalanine & tyrosine metabolism
Valine	Amino acid	Valine, leucine and isoleucine metabolism
Acetate	Carbohydrate	Glycolysis / Gluconeogenesis
Butyrate	Carbohydrate	Butanoate metabolism
Ethanol	Carbohydrate	Glycolysis / Gluconeogenesis
Formate	Carbohydrate	Pyruvate metabolism
Galactose	Carbohydrate	Fructose, mannose, galactose, starch, and sucrose metabolism
Glucose	Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism
Lactate	Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism
Maltose	Carbohydrate	Fructose, mannose, galactose, starch, and sucrose metabolism
Propionate	Carbohydrate	Propanoate metabolism
Nicotinate	Cofactors and vitamins	Nicotinate and nicotinamide metabolism
Fumarate	Energy	Krebs cycle
Methanol	Energy	Methane metabolism
Methylamine	Energy	Methane metabolism
Succinate	Energy	Krebs cycle
Trimethylamine	Energy	Methane metabolism
Acetone	Lipid	Synthesis and degradation of ketone bodies
AMP	Nucleotide	Purine metabolism
Hypoxanthine	Nucleotide	Purine metabolism, (hypo)xanthine/inosine containing
Inosine	Nucleotide	Purine metabolism, (hypo)xanthine/inosine containing
Uracil	Nucleotide	Pyrimidine metabolism, uracil containing

3.7. Conclusions

Correlation Heat-Map analysis indicates a reversal of most of the liver metabolite-metabolite correlations (negative to positive) in C_IL10 mice. The differences are subtle for the cecal metabolite-metabolite correlations. Basic global properties analysis shows that probiotic diet shifts the number of significant correlations in C_IL10 to that in the C_WT Significant Correlations network. Feeding probiotic diet to C_IL10, results in fewer significant correlation differences between C_WT and P_IL10. The P_IL10 network, has similar significant differential correlations with C_IL10, as with C_WT network and P_WT network, hence, suggesting that in C_IL10, a probiotic diet establishes a network similar to that in C_WT and P_WT. Probiotic fed IL10 knock-out mice (P_IL10) mice share more edges with C_WT mice than with IL10 deficient (C_IL10) mice. Metabolite-Metabolite significant differential correlations analysis suggests that the interactions between carbohydrate, energy, and amino-acid pathways are significantly different between C_IL10 and P_IL10 mice.

4: ANALYSIS OF CECAL CONTENT AND CECAL MUCOSA MICROBIOMES

In this chapter the effect of probiotic treatment on correlation networks of cecum content and cecum mucosa microbiomes is described. Does the treatment change the number of nodes and edges of the classes? What are the phyla represented in the four main classes? The four classes are control-wild type mice (C_WT), control-IL10 - deficient mice (C_IL10), probiotic-wild type (P_WT) mice and probiotic- IL10- deficient mice. The networks are in Appendix C.

4.1. Cecal Content Microbiome (Significant Correlations)

The number of significant correlations ($p<0.05$) in each of the four classes of Cecal Content Microbiome is shown in Table 11 below. The number of nodes and edges in C_IL10 (nodes=3, edges=3) increases in P_IL10 (nodes=24, edges=42). The number of nodes and edges in C_WT (nodes=24, edges= 28) is almost similar to that in P_WT (nodes=23, edges= 28).

Table 11: Number of Significant Correlations in Cecal Contents **Microbiome Classes**

	C-WT	C-IL10	P-WT	P-IL10
Number of Nodes	24	3	23	24
Number of Edges	28	3	28	42

Legend: C_WT: Control wild type mice; C_IL10: Control IL10 deficient mice; P_WT: Probiotic fed wild type mice; and P_IL10: Probiotic fed IL10 deficient mice

Thus feeding probiotics increases the number of significant correlations in C_IL10 mice, but not in C_WT mice.

The number of phyla nodes in Cecal Contents microbiome correlation networks, are listed in Table 12. Table 12 result shows that the (i). Proteobacteria is present in the C_WT network, and absent in the C_IL10, P_WT and P_IL10 networks. (ii). C_IL10 network has only Firmicutes (iii). The P_WT and P_IL10 networks have only Bacteroidetes and Firmicutes.

Table 12: Number of Phyla Nodes in Cecal Contents **Microbiome Significant Correlation Network.**

PHYLA	C_WT	C_IL10	P_WT	P_IL10
Proteobacteria	1	0	0	0
Bacteroidetes	12	0	9	12
Firmicutes	11	3	14	12

Legend: C_WT: Control wild type mice; C_IL10: Control IL10 deficient mice; P_WT: Probiotic fed wild type mice; and P_IL10: Probiotic fed IL10 deficient mice

The effect of probiotic treatment on the number of interactions for each phylum in the significant correlations in Cecal Contents microbiome networks is shown in Table 13. Table 13 shows that (i). P_IL10mice show a well-established significant correlations network with Bacteroidetes (29 edges) and Firmicutes (12 edges), as opposed to (ii). P_WT mice which show a decrease in Bacteroidetes (21 edges) and 12 Firmicutes (23 edges) in C_WT to Bacteroidetes (13 edges) and 12 Firmicutes (7 edges) in P_WT.

Table 13: Number of Phyla Edges in Cecal Contents **Microbiome Significant Correlation Network**

PHYLA	C_WT	C_IL10	P_WT	P_IL10
Proteobacteria	1	0	0	0
Bacteroidetes	21	0	13	29
Firmicutes	23	3	7	12

Legend: C_WT: Control wild type mice; C_IL10: Control IL10 deficient mice; P_WT: Probiotic fed wild type mice; and P_IL10: Probiotic fed IL10 deficient mice

Thus, probiotic treatment of C_WT decreases the Bacteroidetes and Firmicutes interactions in P_WT, whereas probiotic treatment of IL-10 deficient mice increases the interactions in P_IL10.

4.2. Cecal Mucosa Microbiome (Significant Correlations)

The number of significant correlations ($p<0.05$) in each of the four classes of Cecal Mucosa Microbiome is shown in Table 14. The number of significant nodes and edges in C_IL10 (nodes=0, edges =0) increases in P_IL10 (nodes=25, edges=49). The number of significant nodes and edges in C_WT (nodes=18, edges =12) increases in P_WT (nodes=21, edges=31). Thus, probiotic diet establishes significant correlations network in IL-10 deficient mice and increases the network in wild type mice.

Table 14: Number of Significant Correlations in Cecal Mucosa **Microbiome Classes Networks.**

	C-WT	C-IL10	P-WT	P-IL10
Number of Nodes	18	0	21	25
Number of Edges	12	0	31	49

Legend: C_WT: Control wild type mice; C_IL10: Control IL10 deficient mice; P_WT: Probiotic fed wild type mice; and P_IL10: Probiotic fed IL10 deficient mice

The effect of probiotic treatment on the number of interactions for each phylum in the significant correlations Cecal Mucosa microbiome networks is shown in Table 15.

We can see from Table 15 that (i). Probiotic diet establishes Proteobacteria, Bacteroidetes, and Firmicutes in _IL10. (ii). There are no Proteobacteria, Bacteroidetes, and Firmicutes in the C_IL10 network, and (iii). Proteobacteria are present in C_WT, P_WT, and P_IL10 networks.

Table 15: Number of Phyla Nodes in Cecal Mucosa Microbiome Significant Correlation Network

PHYLA	C_WT	C_IL10	P_WT	P_IL10
Proteobacteria	3	0	3	3
Bacteroidetes	5	0	7	9
Firmicutes	10	0	11	13

Legend: C_WT: Control wild type mice; C_IL10: Control IL10 deficient mice; P_WT: Probiotic fed wild type mice; and P_IL10: Probiotic fed IL10 deficient mice

The effect of probiotic treatment on the number of phylum interactions in the significant correlations in Cecal Mucosa microbiome networks is shown in Table 16. We observe that (i). Probiotic treatment of IL10 deficient mice (P_IL10) establishes a large number of interactions for Proteobacteria, Bacteroidetes, and Firmicutes. (ii). P_WT network has increased number of significant correlation edges for Proteobacteria, Bacteroidetes, and Firmicutes, than the C_WT network. (iii). There are no significant

nodes and edges in C_IL10 network. Thus, probiotic treatment of C_WT and C_IL10, increases the interactions in each phyla group in Cecal Mucosa microbiome.

Table 16: Number of Phyla Edges in Cecal Mucosa Microbiome Significant Correlations Networks

PHYLA	C_WT	C_IL10	P_WT	P_IL10
Proteobacteria	5	0	10	13
Bacteroidetes	6	0	16	34
Firmicutes	10	0	24	38

Legend: C_WT: Control wild type mice; C_IL10: Control IL10 deficient mice; P_WT: Probiotic fed wild type mice; and P_IL10: Probiotic fed IL10 deficient mice

4.3. Comparison of Cecal Content and Cecal Mucosa Microbiomes data

When comparing the phyla nodes in Cecal Contents (Table 12) with those in Cecal Mucosa (Table 15), we find that (i). C_IL10 Cecal Contents microbiome network has only Firmicutes (Table 12), that are absent in the C_IL10 Cecal Mucosa microbiome network (Table 15). (ii). Cecal Contents Microbiome network lacks Proteobacteria in C_IL10, P_WT, and P_IL10 (Table 12). However, Proteobacteria are present in C_WT, P_WT and P_IL10 Cecal Mucosa microbiome network (Table 15).

(iii). Proteobacteria are present in C_WT and absent in P_WT (Table 12) cecal content microbiome network, and (iv). Proteobacteria are present in C_WT and P_WT Cecal Mucosa microbiome networks (Table 15).

When comparing phyla edges in Cecal Contents (Table 13) and Cecal Mucosa (Table 16) we find that, (i). Probiotic treatment of C_IL10 does not establish Proteobacteria in Cecal Contents microbiome network (Table 13). (ii). Probiotic

treatment of C_WT increases Proteobacteria in Cecal Mucosa microbiome, network (Table 16).

(iii). Probiotic treatment of C_WT decreases the number of significant edges in Cecal Contents microbiome network (Table 13, compare C_WT with P_WT), and is in contrast to the increase observed in P_WT in Cecal Mucosa microbiome network (Table 16, compare C_WT and P_WT).

4.4. Cecal Contents Microbiome (Significant Differential Correlations)

In this section, we determine the interactions which are significantly different in the sub-networks. We look at the signifnicant differential correlations in Cecal Content Microbiomes, between C_IL10-P_IL10 (Table 17) and C_WT-P_WT (Table 18). The attribute guide for Microbiome-Cytokine networks is given in Table 21.

The significant differential correlations ($p<0.05$) between C_IL10-P_IL10 Cecal Contents Microbiome network are given in Table 17. The C_IL10-P_IL10 significant differential network consists of Firmicutes (*Howardella, Lactobacterium, Roseburia, Sporobacterium, Robinsoniella*, and *Butyricicoccus*) and Bacteroidetes (*Bacteroides*, and *Parabacteroides*). The significant interactions are between (i). Bacteroidetes and Firmicutes, (ii). Firmicutes and Firmicutes, The most significant differential correlation is between *Sporobacterium* and *Parabacteroides* ($p=0.0015$).

Table 17: Signifnicant Differential Correlations in Cecal Content Microbiome between C_IL10 - P_IL10.

Node 1 (Family-Genus)	Node 2 (Family-Genus)	Correlation Difference p-value	Positive/ Negative
Lachnospiraceae_Sporobacterium	Porphyromonadaceae_Parabacteroides	0.00151009	Pos to Neg
Incertae Sedis XIV_ Howardella	Lachnospiraceae_Sporobacterium	0.013507234	Neg to Pos
Lachnospiraceae_Sporobacterium	Ruminococcaceae_Butyricicoccus	0.013938388	Neg to Pos
Lachnospiraceae_Lachnobacterium	Lachnospiraceae_Robinsoniella	0.017268091	Neg to Pos
Incertae Sedis XIV_Howardella	Lachnospiraceae_Roseburia	0.019568826	Pos to Neg
Lachnospiraceae_Roseburia	Porphyromonadaceae_Parabacteroides	0.01979261	Neg to Pos
Bacteroidaceae_Bacteroides	Lachnospiraceae_Roseburia	0.048107144	Neg to Pos

The significant differential correlations ($p<0.05$) between C_WT-P_WT Cecal Contents Microbiome network are given in Table 18. The C_WT-P_WT significant differential network consists of Firmicutes (*Howardella, Acetitomaculum, Lactobacterium, Roseburia, Sporobacterium, Robinsoniella*, and *Butyricicoccus*) and Bacteroidetes (*Anerophaga, Hallela, Paraprevotella, Prevotella, Bacteroides*, and *Parabacteroides*). The significant interactions are between (i). Bacteroidetes and Firmicutes, (ii). Firmicutes and Firmicutes, and (iii). Bacteroidetes and Firmicutes. The most significant differential correlations are between (i). *Howardella* and *Sporobacterium* ($p=0.00025$), and (ii). *Robinsoniella* and *Anerophaga* ($p=0.00033$)

Table 18: Signifnicant Differential Correlations in Cecal Content Microbiome between C_WT - P_WT

Node 1 (Family-Genus)	Node 2 (Family-Genus)	Correlation Difference p-value	Positive/ Negative
Incertae Sedis XIV_Howardella	Lachnospiraceae_Sporobacterium	0.00025593300	Neg to Pos
Lachnospiraceae_Robinsoniella	Marinilabiaceae_Anaerophaga	0.00033091400	Neg to Pos
Lachnospiraceae_Acetitomaculum	Lachnospiraceae_Robinsoniella	0.00218302400	Pos to Neg
Bacteroidaceae_Bacteroides	Marinilabiaceae_Anaerophaga	0.00482564400	Pos to Neg
Lachnospiraceae_Sporobacterium	Ruminococcaceae_Oscillibacter	0.00540013100	Neg to Pos
Incertae Sedis XIV_Howardella	Ruminococcaceae_Oscillibacter	0.00812882200	Pos to Pos
Incertae Sedis XIV_Howardella	Ruminococcaceae_Butyricicoccus	0.00818976900	Neg to Pos
Lachnospiraceae_Acetitomaculum	Lachnospiraceae_Lachnobacterium	0.00891845900	Neg to Pos
Lachnospiraceae_Sporobacterium	Marinilabiaceae_Anaerophaga	0.00968088100	Pos to Neg
Prevotellaceae_Hallella	Prevotellaceae_Paraprevotella	0.01366791100	Neg to Pos
Bacteroidaceae_Bacteroides	Lachnospiraceae_Sporobacterium	0.01567186700	Pos to Neg
Prevotellaceae_Paraprevotella	Prevotellaceae_Prevotella	0.03217483300	Neg to Pos
Lachnospiraceae_Roseburia	Rikenellaceae_Rikenella	0.03629591000	Neg to Pos
Lachnospiraceae_Lachnobacterium	Ruminococcaceae_Butyricicoccus	0.03858525900	Pos to Neg
Lachnospiraceae_Robinsoniella	Lachnospiraceae_Syntrophococcus	0.04244934000	Neg to Pos
Lachnospiraceae_Robinsoniella	Ruminococcaceae_Butyricicoccus	0.04254444500	Neg to Pos
Lachnospiraceae_Lachnobacterium	Prevotellaceae_Hallella	0.04586071900	Pos to Neg

4.5. Cecal Mucosa Microbiome (Significant Differential Correlations)

The significant differential correlations ($p<0.05$) between C_IL10-P_IL10 Cecal Mucosa Microbiome network are given in Table 19. The C_IL10-P_IL10 significant differential correlations network consists of Firmicutes (*Roseburia, Oscillibacter,* and *Robinsoniella*) and Bacteroidetes (*Anerophaga, Bacteroides, Rikenella, Filomonas, Barnesiella*, and *Paraprevotella*). The significant interactions are between (i). Bacteroidetes and Bacteroidetes, and (ii). Bacteroidetes and Firmicutes. The most significant differential correlation is between *Roseburia* and *Barnesiella* ($p=0.00157$).

Table 19: Signifnicant Differential Correlations in Cecal Mucosa **Microbiome between C_IL10 - P_IL10**

Node 1 (Family-Genus)	Node 2 (Family-Genus)	Correlation Difference p-value	Positive/ Negative
Lachnospiraceae_Roseburia	Porphyromonadaceae_Barnesiella	0.001571262	Neg to Pos
Marinilabiaceae_Anaerophaga	Porphyromonadaceae_Barnesiella	0.015316806	Pos to Neg
Bacteroidaceae_Bacteroides	Prevotellaceae_Paraprevotella	0.028767953	Pos to Neg
Rikenellaceae_Rikenella	Ruminococcaceae_Oscillibacter	0.031997812	Neg to Pos
Chitinophagaceae_Filimonas	Prevotellaceae_Paraprevotella	0.039993738	Neg to Pos
Lachnospiraceae_Robinsoniella	Rikenellaceae_Rikenella	0.048107144	Neg to Pos

The significant differential correlations ($p<0.05$) between C_WT−P_WT Cecal Mucosa Microbiome network are given in Table 20. The C_WT− P_WT differential correlations network includes (i). Firmicutes (*Howardella, Robinsoniella, Oscillibacter* and *Lachnobacterium*), (ii) Bacteroidetes (*Filomonas, Bacteroides*, and *Butyricimonas*), and (iii). Proteobacteria (*Wolinella*). The most significant differential correlations is between *Filomonas* and *Oscillibacter* ($p=0.0185$).

Table 20: Signifnicant Differential Correlations in Cecal Mucosa Microbiome between C_WT – P_WT

Node 1 (Family-Genus)	Node 2 (Family-Genus)	Correlation Difference p-value	Positive/ Negative
Chitinophagaceae_Filimonas	Ruminococcaceae_Oscillibacter	0.01852002000	Pos to Neg
Bacteroidaceae_Bacteroides	Lachnospiraceae_Robinsoniella	0.02447198000	Neg to Pos
Incertae Sedis XIV_Howardella	Ruminococcaceae_Oscillibacter	0.03286808000	Neg to Pos
Lachnospiraceae_Robinsoniella	Porphyromonadaceae_Butyricimonas	0.03713553600	Neg to Pos
Helicobacteraceae_Wolinella	Lachnospiraceae_Robinsoniella	0.03960400900	Pos to Neg
Helicobacteraceae_Wolinella	Incertae Sedis XIV_Howardella	0.04223019000	Pos to Neg
Lachnospiraceae_Lachnobacterium	Ruminococcaceae_Oscillibacter	0.04762159300	Neg to Pos

Thus, significant differential correlations between Proteobacteria and Firmicutes are observed in only C_WT− P_WT Cecal Mucosa Microbiome differential network.

4.6. Conclusions

The results indicate that probiotic diet increases the number of significant correlations of Bacteroidetes and Firmicutes in the C_IL10 Cecal Contents network. However, probiotic diet decreases the number of significant correlations of Bacteroidetes and Firmicutes in C_WT Cecal Contents network. In C_IL10 Cecal Mucosa, probiotic diet establishes a significant correlations network consisting of Proteobacteria, Bacteroidetes, and Firmicutes.

The C_IL10-P_IL10, Cecal Contents and Cecal Mucosa significant differential correlations networks, contain *Roseburia, Robinsoniella, Bacteroides* and *Paraprevotella*. The C_WT-P_WT, Cecal Contents and Cecal Mucosa significant differential correlations networks, contain *Howardella, Robinsoniella, Bacteroides* and *Paraprevotella*. The C_WT-P_WT Cecal Mucosa network contains Proteobacteria (Wolinella) but Cecal Contents network does not

The gut microbiome like any other microbial community, is a diverse, stable, trophic web (flow of energy and nutrients), exhibits nutritional interdependency among its members, and is a networks with structural properties (Foster *et al.,* 2008). In a microbial network, cross-feeding due to positive feedback loop (where nutrients produced by one population are required and used up by another population) or negative feedback loop (where metabolites produced by one population disrupts a cellular pathway in another population) results in control of the population size of different microbial species, and their activities (Foster *et al.,* 2008). Therefore, any change in the population size and activity, or the addition or removal of a species in a microbial network will result in a change the population density of the microbial species.

In any microbial system, the interactions are determined by their spatial relationship in a habitat, and their metabolic relationship in a niche. Hence, there will be patterns of microbe-microbe and microbe-host relationships (Foster *et al.,* 2008). In the gastrointestinal tract, the microbial density increases along the spatial axis, the proximal (small intestine)-distal (colon) axis. The radial axis of the gut includes the lumen and mucosa. Thus, host factors influence the interactions between the microbes and the host cells (Foster *et al.,* 2008).

The microbe-microbe interactions or communication is mediated by quorum sensing, defined as the process by which microbes monitor and regulate their cell population, through chemical signaling (Federle and Bassler, 2003). The signaling molecules are secreted by microbes. Bacterial quorum sensing includes intraspecies signaling, interspecies signaling and interkingdom signaling. Acetylate homoserine lactones (AHL) are the major type of signal for bacterial intraspecies communication. The AHL autoinducer binds to the LuxR protein and regulates the transcription of genes of the LuxI/LuxR systems in Gram negative bacteria, which includes the α, β, and γ subclasses of Proteobacteria (Eberl, 1999). The Gram positive bacteria use autoinducing peptides (AIPs) consisting of 5-17 amino acids. The cell-cell signaling is mediated by the agr signaling system (Lyon *et al.*, 2000).

Shenderov (2011) suggests that probiotic and gut bacteria can synthesize, sense and release the low weight bio-active signaling molecules that can trigger regulation of structural and regulatory genes, microbe-microbe and microbe-host cell communication and metabolic processes.The biological significance of these findings needs to be

discussed and possible mechanisms. What does all this mean? How can probiotics change microbial networks? How is a microbial network determined?

Table 21: Attribute Guide for Microbiome-Cytokine Networks

Index	Name	Phylum/Cytokine	Class	Order	Family	Genus
3	IFNg	ProInflammatory-Cytokine	Class II Cytokines-Type II Interferons	Th1 cytokine profile		
4	IL_10	AntiInflammatory-Cytokine	Class II Cytokines-IL10 family	Th2 cytokine profile		
5	IL_12	ProInflammatory-Cytokine	Class I Cytokines- IL6 Receptor family	Th1 cytokine profile		
6	IL_1B	ProInflammatory-Cytokine	IL1 family	Th17 cytokine profile		
7	IL_2	ProInflammatory-Cytokine	Class I Cytokines- IL2 Receptor family	Th1 cytokine profile		
8	IL_4	AntiInflammatory-Cytokine	Class I Cytokines- IL2 Receptor family	Th2 cytokine profile		
9	IL_5	AntiInflammatory-Cytokine	Class I Cytokines- IL3 Receptor family	Th2 cytokine profile		
10	IL_8	ProInflammatory-Cytokine	Chemokines: CXC Chemokine family	macrophages, epithelial cells		
11	TNF_a	ProInflammatory-Cytokine	TNF family	Th1 cytokine profile		
12	Aerococcaceae_Abiotrophia	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Abiotrophia
13	Alcaligenaceae_Parasutterella	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Parasutterella
14	Anaeroplasmataceae_Anaeroplasma	Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	Anaeroplasma
15	Bacteroidaceae_Bacteroides	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
16	Bacteroidales_incertae_sedis_Phocaeicola	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidales	incertae sedis Phocaeicola
17	Bradyrhizobiaceae_Bradyrhizobium	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium
18	Burkholderiaceae_Ralstonia	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Ralstonia
19	Burkholderiaceae_Wautersia	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Wautersia
20	Chitinophagaceae_Filimonas	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Chitinophagaceae	Filimonas
21	Chitinophagaceae_Sediminibacterium	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Chitinophagaceae	Sediminibacterium
22	Clostridiaceae_Clostridium	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium
23	Enterobacteriaceae_Citrobacter	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter
24	Erysipelotrichaceae_Allobaculum	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Allobaculum
25	Erysipelotrichaceae_Catenibacterium	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Catenibacterium
26	Erysipelotrichaceae_Coprobacillus	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Coprobacillus
27	Erysipelotrichaceae_Turicibacter	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Turicibacter
28	Eubacteriaceae_Eubacterium	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Eubacterium
29	Flammeovirgaceae_Limibacter	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Flammeovirgaceae	Limibacter
30	Helicobacteraceae_Helicobacter	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Helicobacter
31	Helicobacteraceae_Wolinella	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	Wolinella
32	Incertae Sedis XIV_Blautia	Firmicutes	Clostridia	Clostridiales	Incertae Sedis XIV	Blautia
33	Incertae Sedis XIV_Howardella	Firmicutes	Clostridia	Clostridiales	Incertae Sedis XIV	Howardella
34	Incertae Sedis XI_Finegoldia	Firmicutes	Clostridia	Clostridiales	Incertae Sedis XI	Finegoldia
35	Lachnospiraceae_Acetitomaculum	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Acetitomaculum
36	Lachnospiraceae_Anaerostipes	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Anaerostipes
37	Lachnospiraceae_Butyrivibrio	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio
38	Lachnospiraceae_Catonella	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Catonella
39	Lachnospiraceae_Coprococcus	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus
40	Lachnospiraceae_Dorea	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Dorea
41	Lachnospiraceae_Lachnobacterium	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnobacterium
42	Lachnospiraceae_Marvinbryantia	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Marvinbryantia
43	Lachnospiraceae_Parasporobacterium	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Parasporobacterium
44	Lachnospiraceae_Robinsoniella	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Robinsoniella
45	Lachnospiraceae_Roseburia	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia
46	Lachnospiraceae_Sporobacterium	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Sporobacterium
47	Lachnospiraceae_Syntrophococcus	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Syntrophococcus
48	Lactobacillaceae_Lactobacillus	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus
49	Marinilabiaceae_Anaerophaga	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiaceae	Anaerophaga
50	Porphyromonadaceae_Barnesiella	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Barnesiella
51	Porphyromonadaceae_Butyricimonas	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Butyricimonas
52	Porphyromonadaceae_Paludibacter	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter
53	Porphyromonadaceae_Parabacteroides	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
54	Porphyromonadaceae_Proteiniphilum	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Proteiniphilum
55	Porphyromonadaceae_Tannerella	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Tannerella
56	Prevotellaceae_Hallella	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Hallella
57	Prevotellaceae_Paraprevotella	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Paraprevotella
58	Prevotellaceae_Prevotella	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella
59	Propionibacteriaceae_Propionibacterium	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium
60	Rhodobacteraceae_Pelagibaca	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Pelagibaca
61	Rikenellaceae_Alistipes	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Alistipes
62	Rikenellaceae_Rikenella	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenella
63	Ruminococcaceae_Acetanaerobacterium	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Acetanaerobacterium
64	Ruminococcaceae_Anaerotruncus	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerotruncus
65	Ruminococcaceae_Butyricicoccus	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Butyricicoccus
66	Ruminococcaceae_Hydrogenoanaerobacterium	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Hydrogenoanaerobacterium
67	Ruminococcaceae_Lactonifactor	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Lactonifactor
68	Ruminococcaceae_Oscillibacter	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillibacter
69	Ruminococcaceae_Papillibacter	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Papillibacter
70	Ruminococcaceae_Ruminococcus	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
71	Ruminococcaceae_Sporobacter	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Sporobacter
72	Sphingobacteriaceae_Pseudosphingobacterium	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Sphingobacteriaceae	Pseudosphingobacterium
73	TM7_TM7_genera_incertae_sedis	TM7	TM7	genera	incertae	sedis

5: ANALYSIS OF MICROBIOME –CYTOKINES DATA

In this chapter we first analyze the basic properties of the microbiome-cytokine networks for all the classes (Table 22). Then, the effect of probiotic treatment on the Cecal Contents and cecal microbiome networks in C_IL10 - P_IL10 and C_WT - P_WT is analyzed. Next we compare the significant correlations, and finally the significant differential correlations between classes. The networks are in Appendix D.

5.1. Basic Properties of Microbiome-Cytokine Significant Correlation Subnetworks.

In Table 22 the number of nodes and edges for the 4 classes is given. In C_WT, the number of edges in Cecal Mucosa (colon cytokine edges=32 and ileum cytokine edges=28), is about half of that in Cecal Contents (65 and 61 respectively) even though the number of nodes is about the same in the range of 28 to36.

Table 22: Number of Nodes (Edges) in Microbiome-Cytokine Significant Correlation Sub-networks

	Classes			
Subnetwork	**C-WT**	**C-IL10**	**P-WT**	**P-IL10**
Col_CC_M_ColCyt	36 (65)	31 (72)	36 (56)	38 (94)
Col_CM_M_ColCyt	28 (32)	40 (75)	33 (59)	43 (100)
Ile_CC_M_IleCyt	35 (61)	26 (68)	37 (66)	35 (66)
Ile_CM_M_IleCyt	30 (28)	35 (71)	32 (69)	42 (72)

Legend: Col_CC_M_ColCyt: Cecal Content Microbiome_Colon Cytokines, Col_CM_M_ColCyt: Cecal Mucosa Microbiome_Colon Cytokines, Ile_CC_M_IleCyt: Cecal Content Microbiome_Ileum Cytokines, and CM_M_IleCyt: Cecal Mucosa Microbiome_Ileum Cytokines

A comparison of the C_WT and P_WT Cecal Mucosa colon cytokines in row 2 and ileum cytokines row 4 of Table 22 shows that probiotic diet doubles the number of edges in Cecal Mucosa, (C_WT range = 32 and 28; P_WT range= 59 and 69). The number of edges in C_IL10 are comparable (ranging from 68-75) for all sub-networks even though nodes are variable (ranging from 26-40). Feeding probiotics to C_IL10, results in an increase in the number of edges in Cecal Mucosa colon cytokine subnetworks only.

5.2. The Effect of Probiotic Treatment on Microbiome (Significant Correlation) Networks in IL10-Deficient Mice.

In order to understand the effect of treatment on microbiome networks in IL10 deficient mice, we compared the microbiome-cytokine sub-networks of C_IL10 mice fed with control and probiotic diet.

5.2.1. Comparing C_IL10 and P_IL10 Cecal Content Colon Cytokines

In Figure 11A, Actinobacteria, Proteobacteria, Tenericutes, TM7, and Anti-inflammatory cytokine nodes are absent in C_IL10 cecal content colon cytokine network. Probiotic diet establishes Actinobacteria, Proteobacteria and Anti-inflammatory cytokine nodes and edges in P_IL10 network, however, Tenericutes and TM7 are not.

5.2.2. Comparing C_IL10 and P_IL10 Cecal Content Ileum Cytokines

In Figure 11B, Actinobacteria, Proteobacteria, Tenericutes, TM7, Anti-inflammatory and Pro-inflammatory cytokine nodes absent in C_IL10 cecal content colon cytokine sub-network. Probiotic diet establishes Actinobacteria, Proteobacteria, and Anti-

inflammatory and Pro-inflammatory cytokine in the P_IL10 network, however, Tenericutes and TM7 are not.

.

A

	CECAL CONTENTS	
	Colon Cytokines	
Group Name	**C_IL10**	**P_IL10**
Actinobacteria		1(5)
Bacteroidetes	11(51)	12(40)
Firmicutes	15 (57)	15(54)
Proteobacteria		2(9)
Tenericutes		
TM7		
Anti-Inflammatory Cytokines		2(14)
Pro-Inflammatory Cytokines	5(4)	6(32)

B

	CECAL CONTENTS	
	Ileum Cytokines	
Group Name	**C_IL10**	**P_IL10**
Actinobacteria		1(3)
Bacteroidetes	11(51)	11(38)
Firmicutes	15(57)	15(57)
Proteobacteria		2(5)
Tenericutes		
TM7		
Anti-Inflammatory Cytokines		2(5)
Pro-Inflammatory Cytokines		4(7)

C

	CECAL MUCOSA	
	Colon Cytokines	
Group Name	**C_IL10**	**P_IL10**
Actinobacteria		1(4)
Bacteroidetes	13(38)	12(48)
Firmicutes	19(59)	18(59)
Proteobacteria	3(6)	
Tenericutes		
TM7		
Anti-Inflammatory Cytokines		2(18)
Pro-Inflammatory Cytokines	5(4)	6(27)

D

	CECAL MUCOSA	
	Ileum Cytokines	
Group Name	**C_IL10**	**P_IL10**
Actinobacteria		1(4)
Bacteroidetes	13(38)	12(40)
Firmicutes	19(59)	18(54)
Proteobacteria	3(6)	4(16)
Tenericutes		
TM7		
Anti-Inflammatory Cytokines		2(4)
Pro-Inflammatory Cytokines		5(7)

Figure 11: Effect of before and after probiotic treatment of C_IL10 mice on microbiome-cytokine sub-networks. Legend: Red bar: Absence of phylum in a sub-network. If the phylum node is present in a sub-network, its nodes(edges) are indicated.

5.2.3. Comparing C_IL10 and P_IL10 Cecal Mucosa Colon Cytokines

The Figure 11C, shows that Actinobacteria, and Anti-inflammatory cytokine nodes absent in C_IL10 Cecal Mucosa colon cytokine sub-network. Probiotic diet establishes Actinobacteria, and Anti-Inflammatory cytokines in the P_IL10 network.

However, Tenericutes and TM7 are not established in the P_IL10 network. Furthermore, Proteobacteria are present in C_IL10 Cecal Mucosa and absent in the P_IL10 significant correlations network.

5.2.4. Comparing C_IL10 and P_IL10 Cecal Mucosa Ileum Cytokines

In Figure 11D, Actinobacteria, Tenericutes, TM7, Anti-inflammatory and Pro-inflammatory cytokine nodes absent in C_IL10 Cecal Mucosa ileum cytokine sub-network. Probiotic diet establishes Actinobacteria, Proteobacteria, Anti-inflammatory and Pro-inflammatory cytokine in the P_IL10 network, however, Tenericutes and TM7 are not established in P_IL10 Significant Correlation Network.

5.3. The Effect of Probiotic Treatment on Microbiome (Significant Correlation) Networks Wild Type Mice.

We compared the microbiome-cytokine subnetworks of C_IL10 mice fed with control and probiotic diet.

5.3.1. Comparing C_WT and P_WT Cecal Content Colon Cytokines

Figure 12 A shows that Tenericutes and TM7 are absent in C_WT Cecal Contents colon cytokine SCN. Probiotic treatment establishes only TM7 in the P_WT SCN.

5.3.2. Comparing C_WT and P_WT 10 Cecal Content Ileum Cytokines

The Figure 12B shows that Tenericutes and TM7 are absent in the C_WT Cecal Contents colon cytokine SCN. Probiotic treatment establishes only TM7 in the P_WT SCN.

5.3.3. Comparing C_WT and P_WT Cecal Mucosa Colon Cytokines

The Figure 12C shows that Actinobacteria, Tenericutes and TM7 are absent in Cecal Mucosa of C_WT. Probiotic treatment establishes only Tenericutes in the P_WT.

A

	CECAL CONTENTS	
	Colon Cytokines	
Group Name	C_WT	P_WT
Actinobacteria	1(6)	1(7)
Bacteroidetes	10(29)	9(23)
Firmicutes	14(30)	14(35)
Proteobacteria	3(13)	2(3)
Tenericutes		
TM7		1(2)
Anti-Inflammatory Cytokines	3(18)	3(8)
Pro-Inflammatory Cytokines	5(17)	6(13)

B

	CECAL CONTENTS	
	Ileum Cytokines	
Group Name	C_WT	P_WT
Actinobacteria	1(5)	1(5)
Bacteroidetes	9(34)	9(25)
Firmicutes	14(33)	15(31)
Proteobacteria	3(9)	2(3)
Tenericutes		
TM7		1(2)
Anti-Inflammatory Cytokines	3(11)	3(18)
Pro-Inflammatory Cytokines	5(15)	6(23)

C

	CECAL MUCOSA	
	Colon Cytokines	
Group Name	C_WT	P_WT
Actinobacteria		
Bacteroidetes	10(14)	7(17)
Firmicutes	6(6)	13(43)
Proteobacteria	3(4)	3(14)
Tenericutes		1(3)
TM7		
Anti-Inflammatory Cytokines	3(10)	3(8)
Pro-Inflammatory Cytokines	6(17)	6(12)

D

	CECAL MUCOSA	
	Ileum Cytokines	
Group Name	C_WT	P_WT
Actinobacteria		
Bacteroidetes	9(10)	7(15)
Firmicutes	8(12)	12(41)
Proteobacteria	3(3)	3(14)
Tenericutes	1(2)	1(3)
TM7		
Anti-Inflammatory Cytokines	3(5)	3(19)
Pro-Inflammatory Cytokines	6(13)	6(21)

Figure 12: Effect of before and after probiotic treatment of C_WT mice on microbiome-cytokine sub-networks.

5.3.4. Comparing C_WT and P_WT Cecal Mucosa Ileum Cytokines

Figure 12D shows that Actinobacteria, and TM7 are absent in the C_WTand are not established in P_WT upon probiotic treatment.

Thus, Tenericutes are absent in the Cecal Contents SCN of C_WT and P_WT. Actinobacteria and TM7 are absent in Cecal Mucosa SCN of both C_WT and P_WT.

5.4. Tissue Differences between Cecal Content and Cecal Mucosa Significant Correlation Networks in IL10-Deficient Mice

In order to determine the tissue differences, Cecal Contents and Cecal Mucosa from C_IL10, P_IL10, C_WT and P_WT are compared.

A

	C_IL10	
	Colon Cytokines	
Group Name	**C.Contents**	**C.Mucosa**
Actinobacteria		
Bacteroidetes	11(51)	13(38)
Firmicutes	15 (57)	19(59)
Proteobacteria		3(6)
Tenericutes		
TM7		
Anti-Inflammatory Cytokines		
Pro-Inflammatory Cytokines	5(4)	5(4)

B

	C_IL10	
	Ileum Cytokines	
Group Name	**C.Contents**	**C.Mucosa**
Actinobacteria		
Bacteroidetes	11(51)	13(38)
Firmicutes	15(57)	19(59)
Proteobacteria		3(6)
Tenericutes		
TM7		
Anti-Inflammatory Cytokines		
Pro-Inflammatory Cytokines		

C

	P_IL10	
	Colon Cytokines	
Group Name	**C.Contents**	**C.Mucosa**
Actinobacteria	1(5)	1(4)
Bacteroidetes	12(40)	12(48)
Firmicutes	15(54)	18(59)
Proteobacteria	2(9)	
Tenericutes		
TM7		
Anti-Inflammatory Cytokines	2(14)	2(18)
Pro-Inflammatory Cytokines	6(32)	6(27)

D

	P_IL10	
	Ileum Cytokines	
Group Name	**C.Contents**	**C.Mucosa**
Actinobacteria	1(3)	1(4)
Bacteroidetes	11(38)	12(40)
Firmicutes	15(57)	18(54)
Proteobacteria	2(5)	4(16)
Tenericutes		
TM7		
Anti-Inflammatory Cytokines	2(5)	2(4)
Pro-Inflammatory Cytokines	4(7)	5(7)

Figure 13: Tissue Differences between Cecal Content and Cecal Mucosa **Significant Correlation Networks in C_IL10 and P_IL10 mice.**

5.4.1. Comparing C_IL10 Cecal Content and Cecal Mucosa (Colon Cytokines)

Figure 13A shows that both Cecal Contents and Cecal Mucosa colon cytokine lack Actinobacteria, Tenericutes, TM7 and Anti-inflammatory cytokines. Proteobacteria nodes are absent in Cecal Contents, but present in Cecal Mucosa C_IL10.

5.4.2. Comparing C_IL10 Cecal Content and Cecal Mucosa (Ileum Cytokines)

In Figure 13B the Cecal Contents and Cecal Mucosa ileum cytokine networks lack phyla nodes from Actinobacteria, Tenericutes, TM7, Anti-inflammatory and Pro-inflammatory cytokines. Here also, Proteobacteria are absent in the cecal content ileum cytokine correlations network and present in Cecal Mucosa correlations network.

5.4.3. Comparing P_IL10 Cecal Content and Cecal Mucosa (Colon Cytokines)

Figure 13C shows that Cecal Contents and Cecal Mucosa colon cytokines correlation networks of P_IL10 lack Tenericutes and TM7. Proteobacteria are absent in Cecal Contents correlation network, but present in Cecal Mucosa correlations network.

5.4.4. Comparing P_IL10 Cecal Content and Cecal Mucosa (Ileum Cytokines)

In Figure 13D, the Cecal Contents and Cecal Mucosa ileum cytokine correlation networks of P_IL10 lack Tenericutes and TM7.

Thus, Proteobacteria are absent C_IL10 Cecal Contents, but present in C_IL10 Cecal Mucosa correlations networks. However, in the case of P_IL10, Proteobacteria are present in Cecal Contents and absent in Cecal Mucosa correlation networks. Furthermore, both anti-inflammatory cytokines and pro- inflammatory cytokines are absent in C_IL10 ileum cytokine correlations network. However, anti-inflammatory cytokines are absent and pro- inflammatory cytokines are present in C_IL10 Colon cytokine correlations networks. Finally, both are present in P_IL10 Cecal Contents and Cecal Mucosa correlation networks.

5.5. Tissue Differences between Cecal Content and Cecal Mucosa Significant Correlation Networks in Wild Type Mice

In this section the significant correlation networks of Cecal Contents and Cecal Mucosa of C_WT and P_WT are compared for tissue differences.

A

	C_WT	
	Colon Cytokines	
Group Name	**C.Contents**	**C.Mucosa**
Actinobacteria	1(6)	
Bacteroidetes	10(29)	10(14)
Firmicutes	14(30)	6(6)
Proteobacteria	3(13)	3(4)
Tenericutes		
TM7		
Anti-Inflammatory Cytokines	3(18)	3(10)
Pro-Inflammatory Cytokines	5(17)	6(17)

B

	C_WT	
	Ileum Cytokines	
Group Name	**C.Contents**	**C.Mucosa**
Actinobacteria	1(5)	
Bacteroidetes	9(34)	9(10)
Firmicutes	14(33)	8(12)
Proteobacteria	3(9)	3(3)
Tenericutes		1(2)
TM7		
Anti-Inflammatory Cytokines	3(11)	3(5)
Pro-Inflammatory Cytokines	5(15)	6(13)

C

	P_WT	
	Colon Cytokines	
Group Name	**C.Contents**	**C.Mucosa**
Actinobacteria	1(7)	
Bacteroidetes	9(23)	7(17)
Firmicutes	14(35)	13(43)
Proteobacteria	2(3)	3(14)
Tenericutes		1(3)
TM7	1(2)	
Anti-Inflammatory Cytokines	3(8)	3(8)
Pro-Inflammatory Cytokines	6(13)	6(12)

D

	P_WT	
	Ileum Cytokines	
Group Name	**C.Contents**	**C.Mucosa**
Actinobacteria	1(5)	
Bacteroidetes	9(25)	7(15)
Firmicutes	15(31)	12(41)
Proteobacteria	2(3)	3(14)
Tenericutes		1(3)
TM7	1(2)	
Anti-Inflammatory Cytokines	3(18)	3(19)
Pro-Inflammatory Cytokines	6(23)	6(21)

Figure 14: Tissue differences between Cecal Contents **and** Cecal Mucosa **significant correlation networks, in C_WT and P_WT.**

5.5.1. Comparing C_WT Cecal Content and Cecal Mucosa (Colon Cytokines)

In Figure 14A Tenericutes and TM7 are absent in the Cecal content colon cytokine and Cecal Mucosa colon cytokine correlations networks C_WT. However, Actinobacteria are present in Cecal Contents and absent in Cecal Mucosa correlations networks.

5.5.2. Comparing C_WT Cecal Content and Cecal Mucosa (Ileum Cytokines)

In Figure 14B, TM7 is absent in both Cecal Contents ileum cytokine and Cecal Mucosa ileum cytokine correlations networks of C_WT. Tenericutes are absent in Cecal Content, but present in Cecal Mucosa, whereas, Actinobacteria are present in Cecal Content and absent in Cecal Mucosa.

5.5.3. Comparing P_WT Cecal Content and Cecal Mucosa (Colon Cytokines)

Figure 14C shows that Actinobacteria and TM7 are absent in P_WT Cecal Mucosa colon cytokine correlations networks, whereas Tenericutes are absent in P_WT Cecal Content correlations networks.

5.5.4. Comparing P_WT Cecal Content and Cecal Mucosa (Ileum Cytokines)

In Figure 14D, Actinobacteria and TM7 are absent in the P_WT Cecal Mucosa ileum cytokine correlations networks, while Tenericutes are absent in P_WT Cecal Content Ile cytokine correlations network.

Thus, Actinobacteria and TM7 are absent in the Cecal Mucosa correlations network of C_WT and P_WT, whereas Tenericutes are absent in Cecal Contents correlations networks of C_WT and P_WT.

5.6. Significant Differential Correlations of Microbiome-Cytokine Sub-networks.

Figure 15 summarizes the number of nodes and edges for the microbiome-cytokine significant differential correlations sub-networks. The number of nodes and edges for all C_IL10 -P_IL10 differential sub-networks ranges from 15-20 and 12-15 respectively. The number of nodes and edges in the C_WT -P_WT Cecal Mucosa

significant differential correlations networks (figure 15 B and Figure15 D) are less than those in Cecal Contents (Fig 15A and Figure 15 C).

Thus there are fewer significant differential correlations between C_WT and P_WT in the Cecal Mucosa than Cecal Contents.

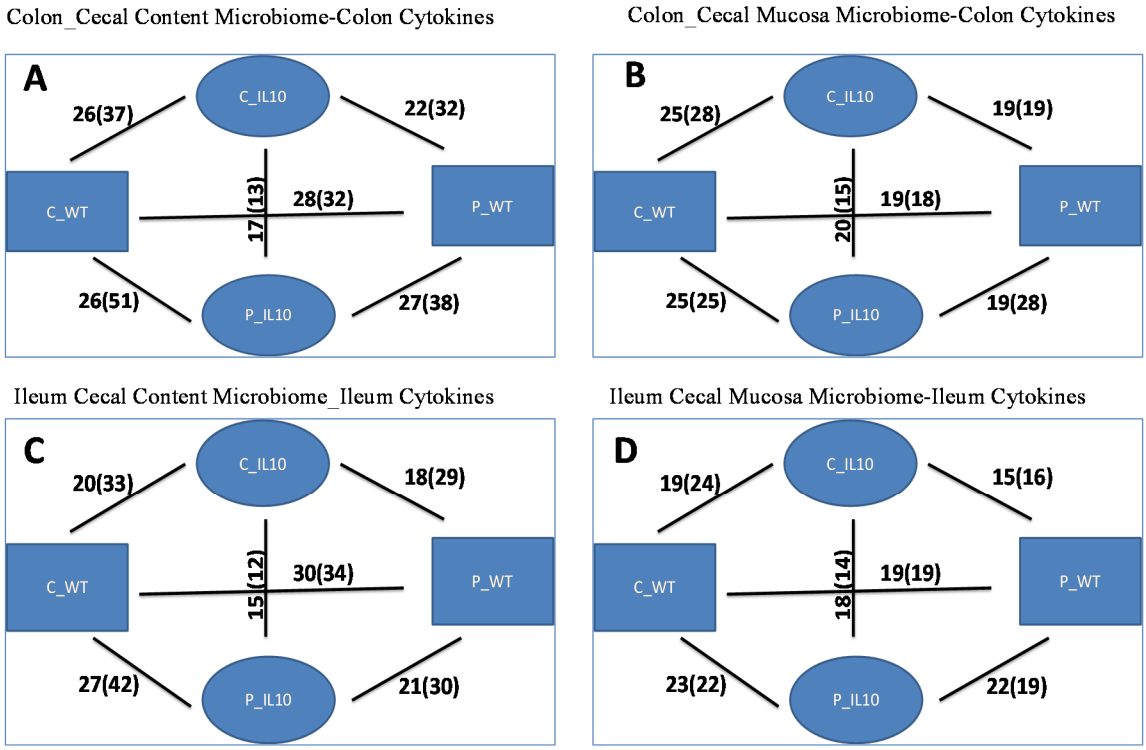

Figure 15: Significant differential correlation nodes (edges) between Microbiome-Cytokine SubNetwroks

5.7. C_IL10 – P_IL10.Cecal Contents Microbiome –Colon and Ileum Cytokines, Significant Differential Correlations Networks

The Cecal Content microbiome-colon and Cecal Content microbiome-ileum cytokines significant differential correlations are in Table 23 and Table 24 respectively.

Both the networks contain interactions between (i). Bacteroiodetes–Bacteroidetes, (ii). Firmicutes–Firmicutes, and (iii). Bacteroidetes–Firmicutes,

The Bacteroidetes include *Barnesiella, Butyricimonas, Anerophaga, Prevotella, Parabacteroides, Limibacter, Phocaeicola*, and *Paludibacter*. The Firmicutes include *Sporobacterium, Syntrophococcus, Roseburia, Catenbacterium, Turicibacter, Acetanaerobacterium,* and *Hydrogenoanaerobacterium*.

The interaction between *IFN-*$\gamma$and *TNF-*α(p=0.04364, pro-inflammatory cytokines) is present only in the Cecal Contents Colon cytokines network. The most significantly differential interaction is between *Barnesiella* and *Butyricimonas* with p= 0.000292.

Table 23: Significant Differential Correlations in C_IL10 – P_IL10 Cecal Content microbiome –Colon Cytokine Network

Node 1 (Family-Genus)	Node 2 (Family-Genus)	Correlation Difference p-value	Positive/ Negative
Porphyromonadaceae_Barnesiella_CC	Porphyromonadaceae_Butyricimonas_CC	0.00292797	Pos to Pos
Lachnospiraceae_Sporobacterium_CC	Marinilabiaceae_Anaerophaga_CC	0.00880563	Neg to Pos
Lachnospiraceae_Syntrophococcus_CC	Prevotellaceae_Prevotella_CC	0.01693391	Pos to Neg
Lachnospiraceae_Roseburia_CC	Porphyromonadaceae_Butyricimonas_CC	0.0185843	Neg to Neg
Porphyromonadaceae_Butyricimonas_CC	Porphyromonadaceae_Parabacteroides_CC	0.02022618	Neg to Pos
Lachnospiraceae_Sporobacterium_CC	Porphyromonadaceae_Butyricimonas_CC	0.02435667	Neg to Pos
Erysipelotrichaceae_Catenibacterium_CC	Flammeovirgaceae_Limibacter_CC	0.02986967	Pos to Pos
Erysipelotrichaceae_Turicibacter_CC	Porphyromonadaceae_Barnesiella_CC	0.03522428	Neg to Pos
Bacteroidales_incertae_sedis_Phocaeicola_CC	Ruminococcaceae_Acetanaerobacterium_CC	0.03813148	Pos to Neg
Porphyromonadaceae_Butyricimonas_CC	Ruminococcaceae_Acetanaerobacterium_CC	0.04046276	Neg to Pos
IFNg_colon	TNF_a_colon	0.04364608	Neg to Pos
Lachnospiraceae_Syntrophococcus_CC	Ruminococcaceae_Hydrogenoanaerobacterium_CC	0.04809008	Pos to Pos
Erysipelotrichaceae_Turicibacter_CC	Porphyromonadaceae_Paludibacter_CC	0.049896	Neg to Neg

Table 24: Significant Differential Correlations in C_IL10 – P_IL10 Cecal Content microbiome –Ileum Cytokine Network

Node 1 (Family-Genus)	Node 2 (Family-Genus)	Correlation Difference p-value	Positive/ Negative
Porphyromonadaceae_Barnesiella_CC	Porphyromonadaceae_Butyricimonas_CC	0.00292797	Pos to Pos
Lachnospiraceae_Sporobacterium_CC	Marinilabiaceae_Anaerophaga_CC	0.00880563	Neg to Pos
Lachnospiraceae_Syntrophococcus_CC	Prevotellaceae_Prevotella_CC	0.01693391	Pos to Neg
Lachnospiraceae_Roseburia_CC	Porphyromonadaceae_Butyricimonas_CC	0.0185843	Neg to Neg
Porphyromonadaceae_Butyricimonas_CC	Porphyromonadaceae_Parabacteroides_CC	0.02022618	Neg to Pos
Lachnospiraceae_Sporobacterium_CC	Porphyromonadaceae_Butyricimonas_CC	0.02435667	Neg to Pos
Erysipelotrichaceae_Catenibacterium_CC	Flammeovirgaceae_Limibacter_CC	0.02986967	Pos to Pos
Erysipelotrichaceae_Turicibacter_CC	Porphyromonadaceae_Barnesiella_CC	0.03522428	Neg to Pos
Bacteroidales_incertae_sedis_Phocaeicola_CC	Ruminococcaceae_Acetanaerobacterium_CC	0.03813148	Pos to Neg
Porphyromonadaceae_Butyricimonas_CC	Ruminococcaceae_Acetanaerobacterium_CC	0.04046276	Neg to Pos
Lachnospiraceae_Syntrophococcus_CC	Ruminococcaceae_Hydrogenoanaerobacterium_C	0.04809008	Pos to Pos
Erysipelotrichaceae_Turicibacter_CC	Porphyromonadaceae_Paludibacter_CC	0.049896	Neg to Neg

5.8. C_IL10 – P_IL10.Cecal Mucosa Microbiome –Colon and Ileum Cytokines, Significant Differential Correlations Networks

The Cecal Mucosa microbiome colon cytokines and ileum cytokine significant differential correlations are in Table 25 and Table 26 respectively. Both the networks contain interactions between (i). Bacteroiodetes–Bacteroidetes, (ii). Firmicutes–Firmicutes, (iii). Bacteroidetes–Firmicutes, (iv). Proteobacteria-Bacteroidetes, and (v). Proteobacteria-Firmicutes.

Bacteroidetes include *Barnesiella, Filomonas, Bacteroides, Butyricimonas, Parabacteroides, Rikenella, Tannerella, Paraprevotella*, and *Paludibacter*. The Firmicutes include *Howardella, Sporobacter, Syntrophococcus, Roseburia, Lachnobacterium, Coprobacillus,* and *Robinsoniella.* The Proteobacteria included are *Parasutterella* and *Ralstonia*. The interaction between *IFN-*$\gamma$and *TNF-*α(p=0.04364, pro-inflammatory cytokines) is present only in the Cecal Mucosa Colon cytokines network. The most significantly differential interaction is between *Roseburia* and

Butyricimonas with p= $4.26x10^{-6}$. The Beta-Proteobacteria, *Parasutterella* and *Ralstonia* interact with *Lachnobacterium* (p=0.0081) and *Rikenella* (p=0.0323) respectively.

Table 25: Significant Differential Correlations in C_IL10 – P_IL10 Cecal Mucosa microbiome –Colon Cytokines Network

Node 1 (Family-Genus)	Node 2 (Family-Genus)	Correlation Difference p-value	Positive/ Negative
Lachnospiraceae_Roseburia_CM	Porphyromonadaceae_Barnesiella_CM	4.26E-06	Neg to Neg
Alcaligenaceae_Parasutterella_CM	Lachnospiraceae_Lachnobacterium_CM	0.0081697	Pos to Neg
Chitinophagaceae_Filimonas_CM	Ruminococcaceae_Sporobacter_CM	0.01162446	Neg to Pos
Incertae Sedis XIV_Howardella_CM	Porphyromonadaceae_Parabacteroides_CM	0.01258841	Neg to Neg
Bacteroidaceae_Bacteroides_CM	Ruminococcaceae_Sporobacter_CM	0.01326089	Pos to Neg
Bacteroidaceae_Bacteroides_CM	Chitinophagaceae_Filimonas_CM	0.02478544	Pos to Neg
Bacteroidaceae_Bacteroides_CM	Erysipelotrichaceae_Coprobacillus_CM	0.02836947	Pos to Pos
Bacteroidaceae_Bacteroides_CM	Porphyromonadaceae_Tannerella_CM	0.02966346	Pos to Neg
Lachnospiraceae_Roseburia_CM	Porphyromonadaceae_Paludibacter_CM	0.03234908	Pos to Neg
Burkholderiaceae_Ralstonia_CM	Rikenellaceae_Rikenella_CM	0.0346748	Neg to Pos
Incertae Sedis XIV_Howardella_CM	Lachnospiraceae_Robinsoniella_CM	0.03555088	Pos to Pos
Lachnospiraceae_Syntrophococcus_CM	Ruminococcaceae_Sporobacter_CM	0.03702528	Pos to Pos
Bacteroidaceae_Bacteroides_CM	Lachnospiraceae_Robinsoniella_CM	0.03898686	Pos to Neg
Porphyromonadaceae_Butyricimonas_CM	Prevotellaceae_Paraprevotella_CM	0.04016996	Neg to Pos
IFNg_colon	TNF_a_colon	0.04364608	Neg to Pos

Table 26: Significant Differential Correlations in C_IL10 – P_IL10 Cecal Mucosa microbiome –Ileum Cytokine Network

Node 1 (Family-Genus)	Node 2 (Family-Genus)	Correlation Difference p-value	Positive/ Negative
Lachnospiraceae_Roseburia_CM	Porphyromonadaceae_Barnesiella_CM	4.26E-06	Neg to Neg
Alcaligenaceae_Parasutterella_CM	Lachnospiraceae_Lachnobacterium_CM	0.0081697	Pos to Neg
Chitinophagaceae_Filimonas_CM	Ruminococcaceae_Sporobacter_CM	0.01162446	Neg to Pos
Incertae Sedis XIV_Howardella_CM	Porphyromonadaceae_Parabacteroides_CM	0.01258841	Neg to Neg
Bacteroidaceae_Bacteroides_CM	Ruminococcaceae_Sporobacter_CM	0.01326089	Pos to Neg
Bacteroidaceae_Bacteroides_CM	Chitinophagaceae_Filimonas_CM	0.02478544	Pos to Neg
Bacteroidaceae_Bacteroides_CM	Erysipelotrichaceae_Coprobacillus_CM	0.02836947	Pos to Pos
Bacteroidaceae_Bacteroides_CM	Porphyromonadaceae_Tannerella_CM	0.02966346	Pos to Neg
Lachnospiraceae_Roseburia_CM	Porphyromonadaceae_Paludibacter_CM	0.03234908	Pos to Neg
Burkholderiaceae_Ralstonia_CM	Rikenellaceae_Rikenella_CM	0.0346748	Neg to Pos
Incertae Sedis XIV_Howardella_CM	Lachnospiraceae_Robinsoniella_CM	0.03555088	Pos to Pos
Lachnospiraceae_Syntrophococcus_CM	Ruminococcaceae_Sporobacter_CM	0.03702528	Pos to Pos
Bacteroidaceae_Bacteroides_CM	Lachnospiraceae_Robinsoniella_CM	0.03898686	Pos to Neg
Porphyromonadaceae_Butyricimonas_CM	Prevotellaceae_Paraprevotella_CM	0.04016996	Neg to Pos

5.9. C_WT – P_WT Cecal Contents Microbiome –Colon and Ileum Cytokines, Significant Differential Correlations Networks

The Cecal Contents microbiome colon cytokines and ileum cytokine significant differential correlations are in Table 27 and Table 28 respectively. Both the networks contain interactions between (i). Bacteroiodetes–Bacteroidetes, (ii). Firmicutes–Firmicutes, (iii). Bacteroidetes–Firmicutes, (iv). Pro-inflammatory cytokine–Firmicutes, (vi). Pro-inflammatory cytokine–Bacteroidetes, (vii). Anti-Inflammatory cytokine–Firmicutes, (viii). Actinobacteria– Bacteroidetes, (ix). Actinobacteria–Firmicutes, and (x). Anti-Inflammatory –Pro-Inflammatory cytokines.

Table 27: Significant Differential Correlations in C_WT – P_WT Cecal Content microbiome –Colon Cytokines Network

Node 1 (Family-Genus)	Node 2 (Family-Genus)	Correlation Difference p-value	Positive/ Negative
IL_1B_colon	Porphyromonadaceae_Barnesiella_CC	3.35E-04	Neg to Pos
Porphyromonadaceae_Barnesiella_CC	Propionibacteriaceae_Propionibacterium_CC	0.001326633	Pos to Neg
Porphyromonadaceae_Parabacteroides_CC	Ruminococcaceae_Anaerotruncus_CC	0.00482623	Neg to Pos
Incertae Sedis XI_Finegoldia_CC	Ruminococcaceae_Hydrogenoanaerobacterium_CC	0.005715892	Pos to Neg
Incertae Sedis XI_Finegoldia_CC	Lachnospiraceae_Syntrophococcus_CC	0.005898415	Pos to Neg
IL_5_colon	Incertae Sedis XI_Finegoldia_CC	0.007397944	Neg to Pos
Lachnospiraceae_Sporobacterium_CC	Ruminococcaceae_Acetanaerobacterium_CC	0.007480358	Pos to Neg
Porphyromonadaceae_Barnesiella_CC	Prevotellaceae_Paraprevotella_CC	0.008967234	Neg to Neg
IL_2_colon	Helicobacteraceae_Wolinella_CC	0.010526153	Pos to Neg
Lachnospiraceae_Anaerostipes_CC	Lachnospiraceae_Marvinbryantia_CC	0.011169079	Pos to Neg
Lachnospiraceae_Catonella_CC	Prevotellaceae_Paraprevotella_CC	0.014777876	Neg to Pos
Lachnospiraceae_Marvinbryantia_CC	Marinilabiaceae_Anaerophaga_CC	0.015000334	Pos to Neg
IL_5_colon	Porphyromonadaceae_Barnesiella_CC	0.016729598	Pos to Neg
TNF_a_colon	Propionibacteriaceae_Propionibacterium_CC	0.018372316	Pos to Neg
IL_12_colon	Prevotellaceae_Paraprevotella_CC	0.024291324	Pos to Neg
IL_12_colon	Ruminococcaceae_Papillibacter_CC	0.026253347	Pos to Neg
Lachnospiraceae_Anaerostipes_CC	Lachnospiraceae_Roseburia_CC	0.028769355	Neg to Pos
IL_8_colon	Helicobacteraceae_Wolinella_CC	0.02938161	Neg to Neg
Lachnospiraceae_Marvinbryantia_CC	Ruminococcaceae_Anaerotruncus_CC	0.033854001	Neg to Pos
IL_1B_colon	Incertae Sedis XI_Finegoldia_CC	0.034744549	Pos to Neg
Prevotellaceae_Hallella_CC	Ruminococcaceae_Anaerotruncus_CC	0.035096505	Neg to Pos
IL_12_colon	Porphyromonadaceae_Parabacteroides_CC	0.039507425	Neg to Pos
IL_10_colon	Ruminococcaceae_Hydrogenoanaerobacterium_CC	0.039657146	Pos to Neg
Prevotellaceae_Paraprevotella_CC	Ruminococcaceae_Hydrogenoanaerobacterium_CC	0.039975028	Pos to Neg
IL_4_colon	IL_8_colon	0.041975543	Pos to Pos
IL_10_colon	Ruminococcaceae_Oscillibacter_CC	0.044149146	Pos to Neg
Lachnospiraceae_Roseburia_CC	Porphyromonadaceae_Barnesiella_CC	0.044316166	Pos to Neg
Lachnospiraceae_Catonella_CC	Lachnospiraceae_Roseburia_CC	0.048083722	Neg to Pos
Lachnospiraceae_Marvinbryantia_CC	Porphyromonadaceae_Tannerella_CC	0.049197249	Pos to Neg
Lachnospiraceae_Catonella_CC	Porphyromonadaceae_Barnesiella_CC	0.049599864	Neg to Neg
Propionibacteriaceae_Propionibacterium_CC	Ruminococcaceae_Hydrogenoanaerobacterium_CC	0.049743195	Pos to Neg
IL_5_colon	Lachnospiraceae_Anaerostipes_CC	0.049999173	Neg to Pos

Table 28: Significant Differential Correlations in C_WT – P_WT Cecal Content microbiome –Ileum Cytokine Network

Node 1 (Family-Genus)	Node 2 (Family-Genus)	Correlation Difference p-value	Positive/ Negative
TNF_a_ileum	Ruminococcaceae_Papillibacter_CC	0.001008784	Neg to Pos
Porphyromonadaceae_Barnesiella_CC	Propionibacteriaceae_Propionibacterium_CC	0.001326633	Pos to Neg
Porphyromonadaceae_Parabacteroides_CC	Ruminococcaceae_Anaerotruncus_CC	0.00482623	Neg to Pos
Incertae Sedis XI_Finegoldia_CC	Ruminococcaceae_Hydrogenoanaerobacterium_C	0.005715892	Pos to Neg
Incertae Sedis XI_Finegoldia_CC	Lachnospiraceae_Syntrophococcus_CC	0.005898415	Pos to Neg
IL_4_ileum	Prevotellaceae_Prevotella_CC	0.006981833	Pos to Neg
Lachnospiraceae_Sporobacterium_CC	Ruminococcaceae_Acetanaerobacterium_CC	0.007480358	Pos to Neg
Porphyromonadaceae_Barnesiella_CC	Prevotellaceae_Paraprevotella_CC	0.008967234	Neg to Neg
IL_1B_ileum	Prevotellaceae_Prevotella_CC	0.009678007	Pos to Neg
Lachnospiraceae_Anaerostipes_CC	Lachnospiraceae_Marvinbryantia_CC	0.011169079	Pos to Neg
IL_5_ileum	Prevotellaceae_Hallella_CC	0.011974364	Neg to Pos
IL_8_ileum	Lachnospiraceae_Anaerostipes_CC	0.012029181	Neg to Pos
IL_5_ileum	Prevotellaceae_Prevotella_CC	0.012168665	Pos to Neg
IL_10_ileum	IL_2_ileum	0.014026979	Pos to Pos
IL_10_ileum	Prevotellaceae_Prevotella_CC	0.014413928	Pos to Neg
Lachnospiraceae_Catonella_CC	Prevotellaceae_Paraprevotella_CC	0.014777876	Neg to Pos
Lachnospiraceae_Marvinbryantia_CC	Marinilabiaceae_Anaerophaga_CC	0.015000334	Pos to Neg
IL_10_ileum	TNF_a_ileum	0.016430561	Pos to Neg
IL_8_ileum	Lactobacillaceae_Lactobacillus_CC	0.019804967	Pos to Neg
IL_10_ileum	Prevotellaceae_Hallella_CC	0.022089466	Neg to Pos
IL_4_ileum	Prevotellaceae_Hallella_CC	0.023131121	Neg to Pos
IL_10_ileum	Ruminococcaceae_Hydrogenoanaerobacterium_C	0.023669063	Neg to Neg
IL_1B_ileum	Lactobacillaceae_Lactobacillus_CC	0.024414393	Neg to Neg
Lachnospiraceae_Anaerostipes_CC	Lachnospiraceae_Roseburia_CC	0.028769355	Neg to Pos
IFNg_ileum	Prevotellaceae_Prevotella_CC	0.031230146	Pos to Neg
Lachnospiraceae_Marvinbryantia_CC	Ruminococcaceae_Anaerotruncus_CC	0.033854001	Neg to Pos
Prevotellaceae_Hallella_CC	Ruminococcaceae_Anaerotruncus_CC	0.035096505	Neg to Pos
IL_12_ileum	Ruminococcaceae_Oscillibacter_CC	0.036979347	Pos to Neg
Prevotellaceae_Paraprevotella_CC	Ruminococcaceae_Hydrogenoanaerobacterium_C	0.039975028	Pos to Neg
Lachnospiraceae_Roseburia_CC	Porphyromonadaceae_Barnesiella_CC	0.044316166	Pos to Neg
Lachnospiraceae_Catonella_CC	Lachnospiraceae_Roseburia_CC	0.048083722	Neg to Pos
Lachnospiraceae_Marvinbryantia_CC	Porphyromonadaceae_Tannerella_CC	0.049197249	Pos to Neg
Lachnospiraceae_Catonella_CC	Porphyromonadaceae_Barnesiella_CC	0.049599864	Neg to Neg
Propionibacteriaceae_Propionibacterium_CC	Ruminococcaceae_Hydrogenoanaerobacterium_C	0.049743195	Pos to Neg

The Bacteroidetes include *Barnesiella, Butyricimonas, Parabacteroides, Hallela, Tannerella, Paraprevotella*, and *Anaerophaga*. The Firmicutes include *Sporobacterium, Anaerostipes, Catonella, Marvinbryantia, Roseburia, Anaerotruncus, Lactobacillus, Hydrogenoanaerobacterium, Syntrophococcus, Finegoldia, Acetanaerobacterium, Papillibacter,* and *Oscillibacter.* The Actinobacteria includes *Propionibacterium.* The pro-inflammatory cytokines include *IL-1β, IL-2, IL-8, IL-12*, and *TNF-α.* The Anti-inflammatory cytokines are *IL-4, IL-5*, and *IL-10*.

The significant differential interactions between Epsilon-Proteobacteria (*Wolinella*) and Pro-inflammatory cytokines (*IL-2* and *IL-8)* is present in the Cecal Contents Colon cytokine network (Table 27), and absent in the Cecal Contents Ileum cytokine network (Table 28). The most significantly differential correlation Significant Differential Correlations in C_WT – P_WT Cecal Content microbiome –Colon Cytokines Network (Table 27) is between *Barnesiella* and *IL-1β* ($p=3.35x10^{-4}$). The most significantly differential correlation Significant Differential Correlations in C_WT – P_WT Cecal Content microbiome –Colon Cytokines Network (Table 28) is between *TNF-α* and *Papillibacter* (p=0.0010).

5.10. C_WT – P_WT.Cecal Mucosa Microbiome –Colon and Ileum Cytokines, Significant Differential Correlations Networks

The Cecal Mucosa microbiome colon cytokines and ileum cytokine significant differential correlations are in Table 29 and Table 30 respectively. Both the networks contain interactions between (i). Firmicutes–Firmicutes, (ii). Bacteroidetes–Firmicutes, (iii). Pro-inflammatory cytokine–Firmicutes, (iv). Pro-inflammatory cytokine–Bacteroidetes, (v). Anti-Inflammatory cytokine–Firmicutes, and (vi).Epsilon-Proteobacteria–Firmicutes.

The Bacteroidetes include *Bacteroides,* and *Paludibacter.* The Firmicutes include *Butyricicoccus, Howardella, Roseburia, Robinsoniella, Sporobacter, Acetitomaculum, Acetanaerobacterium,* and *Oscillibacter.* The Epsilon-Proteobacteria include *Wolinella* and *Helicobacter*. The pro-inflammatory cytokines include *IFN-γ, IL-1β, IL-2, IL-8,*

IL-12, and *TNF-α*. The Anti-inflammatory cytokines are *IL-4, IL-5*, and *IL-10*.

The significant differential interactions between (i) *IL-5* and *Butyricicoccus* (p=0.0018), (ii). *IL-4* and *IL-8* (p=0.0419), are present in the Cecal Mucosa Colon cytokine network (Table 29). The significant differential interactions between (i). *IL-2* and *Robinsoniella* (p=0.0019), (ii). *IL-10* and *IL-2* (p=0.0140), and (iii). *IL-10* and *TNF-α* (p=0.0164) is present in the Cecal Mucosa Ileum cytokine network (Table 30).

Table 29: Significant Differential Correlations in C_WT – P_WT Cecal Mucosa microbiome –Colon Cytokine Network

Node 1 (Family-Genus)	Node 2 (Family-Genus)	Correlation Difference p-value	Positive/ Negative
IL_5_colon	Ruminococcaceae_Butyricicoccus_CM	0.001834131	Pos to Neg
Helicobacteraceae_Wolinella_CM	Incertae Sedis XIV_Howardella_CM	0.002432176	Neg to Pos
IL_12_colon	Lachnospiraceae_Roseburia_CM	0.003186453	Neg to Pos
Helicobacteraceae_Helicobacter_CM	Incertae Sedis XIV_Howardella_CM	0.003186553	Neg to Pos
IL_10_colon	Ruminococcaceae_Butyricicoccus_CM	0.003305419	Pos to Neg
Incertae Sedis XIV_Howardella_CM	Ruminococcaceae_Oscillibacter_CM	0.008067748	Pos to Neg
IL_4_colon	Ruminococcaceae_Butyricicoccus_CM	0.008136924	Pos to Neg
IL_8_colon	Lachnospiraceae_Robinsoniella_CM	0.010650732	Pos to Neg
IL_1B_colon	Bacteroidaceae_Bacteroides_CM	0.010810063	Neg to Pos
IL_8_colon	Ruminococcaceae_Sporobacter_CM	0.011468741	Pos to Neg
Helicobacteraceae_Wolinella_CM	Lachnospiraceae_Robinsoniella_CM	0.016269269	Neg to Pos
Lachnospiraceae_Acetitomaculum_CM	Porphyromonadaceae_Paludibacter_CM	0.018681031	Pos to Neg
IL_8_colon	Incertae Sedis XIV_Howardella_CM	0.024306605	Pos to Neg
Ruminococcaceae_Acetanaerobacterium_CM	Ruminococcaceae_Sporobacter_CM	0.025777558	Pos to Neg
IFNg_colon	Ruminococcaceae_Butyricicoccus_CM	0.02770989	Pos to Neg
IL_4_colon	IL_8_colon	0.041975543	Pos to Pos
Helicobacteraceae_Helicobacter_CM	Lachnospiraceae_Robinsoniella_CM	0.048745552	Neg to Pos
IL_12_colon	Porphyromonadaceae_Paludibacter_CM	0.049208301	Neg to Pos

Table 30: Significant Differential Correlations in C_WT – P_WT Cecal Mucosa microbiome –Ileum Cytokine Network

Node 1 (Family-Genus)	Node 2 (Family-Genus)	Correlation Difference p-value	Positive/ Negative
IL_2_ileum	Lachnospiraceae_Robinsoniella_CM	0.001984556	Neg to Pos
Helicobacteraceae_Wolinella_CM	Incertae Sedis XIV_Howardella_CM	0.002432176	Neg to Pos
Helicobacteraceae_Helicobacter_CM	Incertae Sedis XIV_Howardella_CM	0.003186553	Neg to Pos
IL_2_ileum	Incertae Sedis XIV_Howardella_CM	0.003973868	Neg to Pos
Incertae Sedis XIV_Howardella_CM	Ruminococcaceae_Oscillibacter_CM	0.008067748	Pos to Neg
IL_2_ileum	Lachnospiraceae_Acetitomaculum_CM	0.010052679	Neg to Pos
IL_10_ileum	IL_2_ileum	0.014026979	Pos to Pos
IL_10_ileum	Lachnospiraceae_Acetitomaculum_CM	0.014724619	Neg to Pos
IL_8_ileum	Lachnospiraceae_Acetitomaculum_CM	0.015157785	Neg to Pos
IL_2_ileum	Lachnospiraceae_Lachnobacterium_CM	0.01518739	Neg to Pos
Helicobacteraceae_Wolinella_CM	Lachnospiraceae_Robinsoniella_CM	0.016269269	Neg to Pos
IL_10_ileum	TNF_a_ileum	0.016430561	Pos to Neg
Lachnospiraceae_Acetitomaculum_CM	Porphyromonadaceae_Paludibacter_CM	0.018681031	Pos to Neg
Ruminococcaceae_Acetanaerobacterium_CM	Ruminococcaceae_Sporobacter_CM	0.025777558	Pos to Neg
IL_4_ileum	Ruminococcaceae_Butyricicoccus_CM	0.031803168	Pos to Neg
IL_4_ileum	Lachnospiraceae_Acetitomaculum_CM	0.037865773	Neg to Pos
IL_1B_ileum	Lachnospiraceae_Acetitomaculum_CM	0.040300686	Neg to Pos
IFNg_ileum	Bacteroidaceae_Bacteroides_CM	0.047693522	Pos to Neg
Helicobacteraceae_Helicobacter_CM	Lachnospiraceae_Robinsoniella_CM	0.048745552	Neg to Pos

5.11. Conclusions

In chapter 5, the effect of probiotics on microbiome-cytokine significant correlation networks (in C_IL10 and C_WT), and the tissue differences between Cecal Contents and Cecal Mucosa (in C_IL10, P_IL10, C_WT, and P_WT) is analyzed. The significant differential correlations between C_IL10-P_IL10, and C_WT-P_WT is also investigated.

Probiotics establish Actinobacteria, and Proteobacteria in Cecal Contents and Cecal Mucosa networks in C_IL10, whereas, Tenericutes and TM7 are absent in all the C_IL10 networks.. In a similar analysis with C_WT, Tenericutes are absent in Cecal Contents significant correlation networks of C_WT and P_WT. Actinobacteria and TM7 are absent in Cecal Mucosa and present in Cecal Contents significant correlation networks.

Tissue differences between Cecal Contents and Cecal Mucosa of all C_IL10 networks suggests that Proteobacteria are absent in C_IL10 Cecal Contents, present in C_IL10 Cecal Mucosa, present in P_IL10 Cecal Contents, and absent in P_IL10 Cecal Mucosa significant correlations networks. Tissue differences between Cecal Contents and Cecal Mucosa of C_WT indicates that Actinobacteria and TM7 are absent in Cecal Mucosa of C_WT and P_WT, while Tenericutes are absent in Cecal Contents of C_WT and P_WT.

Analysis of significant differential correlations between C_IL10−P_IL10 indicates that *Barnesiella, Butyricimonas, Parabacteroides, Paludibacter, Syntrophococcus, and Roseburia* have significantly differential correlations in both Cecal Contents and Cecal Mucosa networks. Proteobacteria (*Parasutterella* and *Ralstonia*) are absent in Cecal Contents and present in Cecal Mucosa significant correlation differential networks.

Analysis of significant differential correlations between C_WT−P_WT suggests that *Roseburia, Acetanaerobacterium, Oscillibacter, Wolinella,* and cytokines *IL-1β, IL-2, IL-8, IL-12*, *TNF-α, IL-4, IL-5*, and *IL-10* are significantly different in C_WT−P_WT networks. *Propionibacterium* (Actinobacteria) is present in Cecal Contents and absent in Cecal Mucosa C_WT−P_WT significantly differential network.

Finally, Beta-Proteobacteria (*Parasutterella* and *Ralstonia*) are significantly different in C_IL10 −P_IL10 Cecal Mucosa network, whereas, Epsilon-Proteobacteria (*Wolinella* and *Helicobacter*) are significantly different in C_WT−P_WT Cecal Mucosa network.

6: DISCUSSION AND CONCLUSION

In the present study, the goal is to determine the effect of probiotics on (i).metabolite-metabolite correlations (chapter 3), (ii). Microbiome-microbiome interactions (chapter 4), and (iii). Microbiome-cytokine (chapter 5) interactions in IL10 deficient mice.

The correlation maps from heat-map analysis indicated a striking reversal of the liver metabolite-metabolite correlations from negative to positive, when C_IL10 mice are fed the probiotic VSL#3. The differences are subtle for the cecal metabolite-metabolite correlations. Basic global properties analysis shows that probiotic diet shifts the number of significant correlations in C_IL10 to that in the C_WT correlations network. Feeding probiotic diet to C_IL10, results in fewer significant correlation differences between C_WT and P_IL10. The P_IL10 network, has similar significant differential correlations with C_IL10, as with C_WT network and P_WT network, hence, suggesting that in C_IL10, a probiotic diet establishes a network similar to that in C_WT and P_WT. Analysis of overlapping significant differential correlations suggests that probiotic fed IL10 knock-out mice (P_IL10) mice share more edges with C_WT mice than with IL10 deficient (C_IL10) mice. Metabolite-Metabolite significant differential correlations suggest that the interactions between carbohydrate, energy, and amino-acid pathways are significantly different between C_IL10 and P_IL10 mice.

The results indicate that probiotic diet increases the significant correlation network in C_IL10, with the increase occurs in the interactions in Bacteroidetes and Firmicutes. However, probiotic diet shows a decreases the significant correlations network in C_WT with decrease occurring in the Bacteroidetes and Firmicutes. In Cecal Mucosa, probiotic diet establishes a significant correlations network in C_IL10, consisting of Proteobacteria, Bacteroidetes, and Firmicutes.

The Cecal Contents and Cecal Mucosa significant differential correlations networks of C_IL10-P_IL10, contain *Roseburia, Robinsoniella, Bacteroides* and *Paraprevotella*. The Cecal Contents and Cecal Mucosa significant differential correlations networks of C_WT-P_WT, contain *Howardella* and *Robinsoniella*, *Bacteroides* and *Paraprevotella*. The C_WT-P_WT network contains Proteobacteria (Wolinella) but Cecal Contents does not.

In chapter 5, the effect of probiotics on microbiome-cytokine significant correlation networks (in C_IL10 and C_WT), and the tissue differences between Cecal Contents and Cecal Mucosa (in C_IL10, P_IL10, C_WT, and P_WT) is analyzed. The significant differential correlations between C_IL10-P_IL10, and C_WT-P_WT is also investigated.

Probiotics establish Actinobacteria, and Proteobacteria in Cecal Contents and Cecal Mucosa networks in C_IL10, whereas, Tenericutes and TM7 are absent in all the C_IL10 networks. In a similar analysis with C_WT, Tenericutes are absent in Cecal Contents significant correlation networks of C_WT and P_WT. Actinobacteria and TM7 are absent in Cecal Mucosa and present in Cecal Contents significant correlation

networks. Fermentation of the undigested carbohydrates (dietary fiber and resistant starch) results in propionic acid production by Actinobacteria (*Propionibacterium*). Actinobacteria produce a wide range of functional bio-moleucles such as B-vitamins, propionic acid, bacteriocins, and conjugated linoleic acid (Poonam *et al.,* 2012). What is the significance of this? What are some possible mechanisms?

Tissue differences between Cecal Contents and Cecal Mucosa of all C_IL10 networks suggests that Proteobacteria are absent in C_IL10 Cecal Contents, present in C_IL10 Cecal Mucosa, present in P_IL10 Cecal Contents, and absent in P_IL10 Cecal Mucosa significant correlations networks. Tissue differences between Cecal Contents and Cecal Mucosa of C_WT indicates that Actinobacteria and TM7 are absent in Cecal Mucosa of C_WT and P_WT, while Tenericutes are absent in Cecal Contents of C_WT and P_WT.

Analysis of significant differential correlations between C_IL10−P_IL10 indicates that *Barnesiella, Butyricimonas, Parabacteroides, Paludibacter, Syntrophococcus, and Roseburia* have significantly differential correlations in both Cecal Contents and Cecal Mucosa networks. Proteobacteria (*Parasutterella* and *Ralstonia*) are absent in Cecal Contents and present in Cecal Mucosa significant correlation differential networks.

Analysis of significant differential correlations between C_WT−P_WT suggests that *Roseburia, Acetanaerobacterium, Oscillibacter, Wolinella,* and cytokines *IL-1β, IL-2, IL-8, IL-12*, *TNF-α, IL-4, IL-5*, and *IL-10* are significantly different in C_WT−P_WT

networks. *Propionibacterium* (actinobacteria) is present in Cecal Contents and absent in Cecal Mucosa C_WT−P_WT significantly differential network.

Finally, Beta-Proteobacteria (*Parasutterella* and *Ralstonia*) are significantly different in C_IL10 −P_IL10 Cecal Mucosa network, whereas, Epsilon-Proteobacteria (*Wolinella* and *Helicobacter*) are significantly different in C_WT−P_WT Cecal Mucosa network.

Based on significant differential analysis between C_IL10 and P_IL10 Cecal metabolites, Butyrate has significant differential correlations with isoleucine (p=0.0035), threonine (p=0.0044), fumarate (p=0.003) and methanol (p=0.000027). A correlation does not determine causality, and simply describes the strength of a relationship. Research suggests that most of the short chain fatty acids (SCFA) such as Butyrate is produced in the cecum and the colon, and it plays an important role in the maintenance of healthy colonic epithelium (Blottière et al., 2003). Butyrate produced by commensal bacteria, modulates many signaling pathways in the intestinal epithelial cells.

Propionic acid produced in the colon is absorbed and passes the colon and viscera and drains into the portal vein. About 90% of the propionic acid is metabolized in the liver by gluconeogenesis pathway. Propionic acid has anti- inflammatory properties and anti-microbial activity. It lowers plasma fatty acid levels by inhibiting lipolysis, and decreases fatty acid synthesis in the liver resulting in the decreased fatty acid related inflammation (Wong *et al.,* 2006).

Acetate, though produced in the colon, is mostly absorbed in the blood stream and transported to the liver. It is the main SCFA in the blood, and is therefore used to monitor

colonic events. It is the main substrate for cholesterol synthesis in the adipose tissue, and liver. Hence, it affects the liver metabolism (Wong *et al.,* 2006).

The significant differential correlations analysis of the C_IL10-P_IL10 Cecal Content network (section 5.7), shows that *Butyricimonas* has significant differential interactions with *Barnesiella* (p=0.0029). *Roseburia* (p=0.018), *Parabacteroides* (p=0.0202), *Sporobacterium* (p=0.024) and *Acetanaerobacterium* (p=0.040). Butyricimonas, are mainly butyric and iso-butyric acid producing bacteria (Sakamoto et al., 2009). Wong *et al.* (2005) showed that butyrate is involved in suppressing early carcinogenic events in rats. Butyrate produced from dietary fiber, by the commensal bacteria provides protection against large bowel cancer in the rat model.

In conclusion, probiotics affect the metabolome and microbiome correlation networks in IL10 deficient mice, and that there are tissue specific microbiome differences in the gut.

APPENDIX A

R-Script to calculate significant correlations and significant differential correlations

```
##Data analysis of correlations from Paper: Metabolomic networks in plants: Transitions
## from pattern recognition to biological interpretation by K. Morgenthal , W.
Weckwerth, and R.Steur . Biosystems 83 (2006) pg. 108-117
## by Sugandha Patibanda  June 2010

#  INSTALL PACKAGES : GPLOTS, MASS
# There are 6 functions:
# ttfn; does the t transformation
# t2pvalues: gets the pvalues for the calculated t values from ttfn (2 sided test)
# corrsignificance: Finds the correlations, tvalues of the correlations, and the pvalues.
# ztfn: does the z transformation
# z2pvalues: calculates the p values for the z values (2 sided test)
#corrdifference: Finds the correlations for 2 data files, the z scores and the corresponding
pvalues.
#student t transformation function

ttfn <- function( c, n ) {

numerator = c * sqrt(n-2)
denominator = sqrt( 1 - c^2)
t = numerator / denominator
#output = rbind(c, n, t)

return (t)
}


t2pvalues <-function(t,n,a)
{

        t <- abs(t)  # makes the t positive
        a <- a/2
        a2use <- (1-a)
```

```
        df <- n-1
        tcrit=qt(p=a2use,df= df)
        pvalue <- 2*(1-(pt(q=t,df= df)))
        return(pvalue)
}


z2pvalue <-function(z,a)
{
        z<- abs(z)  # makes the z positive
        b <- a/2
        #a2use <- 1-b

        zcrit= qnorm(1-b)

        pvalue <- 2*(1-(pnorm(z)))
        #z_out <- cbind(z, zcrit, pvalue)
        #print (z_out)
        return(pvalue)
}


#Z- TRANSFORMATION - TESTING DIFFERENCES BETWEEN CORRELATIONS
FROM 2 DIFFERENT TISSUES     ########################

# c1 and c2 are correlations for the 2 distinct tissues, and n1 and n2 are the number of
samples for each metabolite pair

# a = is the significance level to test for
ztfn <- function (c1, c2, n1, n2) {
r1 <- (1 + c1) / (1- c1)
numerator1 <- log ( r1)
r2 <- (1 + c2)/ (1- c2)
numerator2 <- log ( r2)
Numerator <- numerator1 - numerator2

r3 <-  1/ (n1-3)
print ("********* PRINTING R3********")
print (r3)
r4 <-  1/ (n2-3)
print ("********* PRINTING R4********")
print (r4)
Denominator <-  sqrt( (1/ (n1-3)) + (1/ (n2-3)) )
print ("********* PRINTING denominator ********")
```

```
print (Denominator)
zscore <-   Numerator /  ( 2 * Denominator)
return (zscore)
}
### CORRSIGNIFICANCE function takes following arguments --
# (1) file path: data file path
#(2) FirstDataColNum: First column with the data.
#(3) alpha: alpha value for which the pvalues are needed
#(4) corrFilePath: 1st output file is the correlations text file
#(5) tValuesFilePath: 2nd output file is the tvalues
# (6) p4tValuesFilePath: 3rd output file is the pvalues

corrsignificance <- function (filepath, FirstDataColNum, alpha, corrFilePath,
tValuesFilePath, p4tValuesFilePath )
{
        fileName <- read.table(filepath , sep= "\t", header= T)

        ## Array dimensions
        fileSize <- dim(fileName)
        rowNum <- fileSize[1]
        colNum <- fileSize[2]

        ### Pick the cols for analysis :
        newfileName <- fileName [ FirstDataColNum : colNum]
#####  MAY HAVE TO SELECT THIS ACCORDING TO YOUR DATA
        fileNamematrix <- data.matrix(newfileName)

        #calculate correlations

        corMatrix <- cor(fileNamematrix, method= "pearson", use="pairwise")

        ## Get Header from the Correlations Matrix to be inserted later into the t-matrix
and p-matrix
        header <- corMatrix[0,]
        ## write matrix to a file Please Put in the filepath
        #  write.matrix(x, file = "", sep = " ", blocksize)
        library(MASS)
        write.matrix(corMatrix, file = corrFilePath, sep = "\t")

        ### Plotting the HeatMap of the Correlations
        library(gplots)
        graf1 <- heatmap.2(corMatrix)

        ## Different sizes of the matrix to be used in loops
```

```
	Msize <- dim(corMatrix)
	newcorvals <- c(corMatrix)
	#print (newcorvals)
	L <- length(newcorvals)

	### Creating matrices for storing  t_values and p_values
	tmatrix <- matrix(1:L)# creates  nrows x 1 col matrix for storing t values
	pmatrix <- matrix(1:L)  # creates  nrows x 1 col matrix for storing p values

	k=1
	a <- alpha

	while (k<= L)
	{
		#print (newcorvals[k])
		corvalue <- newcorvals[k]
		n <- rowNum
		t <- ttfn(corvalue,n)
		tmatrix[k] <- ttfn(corvalue,n)
		p <- tsigfun (t, n, a)
		pmatrix[k] <- t2pvalues (t, n, a)

		k= k+1
	}

	tvals <- c(tmatrix)
	#print (tvals)
	newtmatrix <- matrix(c(tvals), nrow=Msize[1], byrow=T)
	newtmatrix <- rbind(header, newtmatrix)
	write.matrix(newtmatrix, file = tValuesFilePath, sep = "\t")

	pvals <- c(pmatrix)
	#print (pvals)
	newpmatrix <- matrix(c(pvals), nrow=Msize[1], byrow=T)
	newpmatrix <- rbind(header, newpmatrix)
	write.matrix(newpmatrix, file = p4tValuesFilePath, sep = "\t")

	return()
}
```

```
# CORRDIFFERENCE Function  takes following arguments --
# (1) filepath1: data file 1 path
# (2) filepath2: data file 2 path
# (3) FirstDataColNum1: First column with the data for file 1
# (4) FirstDataColNum2: First column with the data for file 2
# (5) alpha: alpha value for which the pvalues are needed
# (6) zValuesFilePath: 1st output file is the z scores obtained by the z-transformation
# (7) pzValuesFilePath: 2nd output file is the p-values for the z scores.

corrdifference <- function (filepath1, filepath2, FirstDataColNum1,FirstDataColNum2,
alpha,zValuesFilePath, pzValuesFilePath )
{
        file1 <- read.table(filepath1 , sep= "\t", header= T)
        file2 <- read.table(filepath2 , sep= "\t", header= T)

        ############  For data file 1
        file1_size <- dim(file1)
        #print ("FILE 1 SIZE")
        #print (file1_size)
        rowNum1 <- file1_size[1]
        colNum1 <- file1_size[2]

        ############ For data file 2
        file2_size <- dim(file2)
        #print ("FILE 2 SIZE")
        #print (file2_size)
        rowNum2 <- file2_size[1]
        colNum2 <- file2_size[2]

        ############### Pick the cols for analysis :
        ## For file 1
        newfile1 <- file1 [FirstDataColNum1:colNum1]
#####  first data col number for file 1
        file1_matrix <- data.matrix(newfile1)

        ##For file 2
        newfile2 <- file2 [FirstDataColNum2:colNum2]
#####  first data col number for file 2
        file2_matrix <- data.matrix(newfile2)

        ##############CALCULATES THE CORRELATIONS
        #For file1
        corMatrix1 <- cor(file1_matrix, method= "pearson", use="pairwise")
        #For file2
```

```
corMatrix2 <- cor(file2_matrix, method= "pearson", use="pairwise")

## Get Header from the Correlations Matrix to be inserted later

header <- corMatrix[0,]

## Get the size of correlation matrix to be used later for while loops and reshaping
the matrices
Msize <- dim(corMatrix1)

cor_vals1 <- c(corMatrix1)
cor_vals2 <- c(corMatrix2)

L <- length(cor_vals1)

### Creating matrices for storing  t_values and p_values
zmatrix <- matrix(1:L)          # creates  nrows x 1 col matrix for storing z values
pzmatrix <- matrix(1:L)  # creates  nrows x 1 col matrix for storing pz values

k=1
a <- alpha

while (k<= L)
{

        cor1 <- cor_vals1[k]
        cor2 <- cor_vals2[k]
        n1 <- rowNum1
        n2 <- rowNum2

        z <- ztfn(cor1, cor2, n1, n2)
        print ("****** Z ****")
        print (z)

        zmatrix[k] <- ztfn(cor1, cor2, n1, n2)
        p <- z2pvalue(z,a)
        print ("**** P****")
        print (p)
        pzmatrix[k] <- z2pvalue (z,a)

        in_out <- cbind(cor1, cor2, n1,n2, z, p)
        print (in_out)

        k= k+1
```

```
        }

        zvals <- c(zmatrix)
        #print (zvals)
        newzmatrix <- matrix(c(zvals), nrow=Msize[1], byrow=T)
        newzmatrix <- rbind(header, newzmatrix)

        library(MASS)
        write.matrix(newzmatrix, file = zValuesFilePath, sep = "\t")

        pzvals <- c(pzmatrix)
        #print (pzvals)
        newpzmatrix <- matrix(c(pzvals), nrow=Msize[1], byrow=T)
        newpzmatrix <- rbind(header, newpzmatrix)
        write.matrix(newpzmatrix, file = pzValuesFilePath, sep = "\t")

        return()
}
```

APPENDIX B

Correlation Networks for Metabolome Data

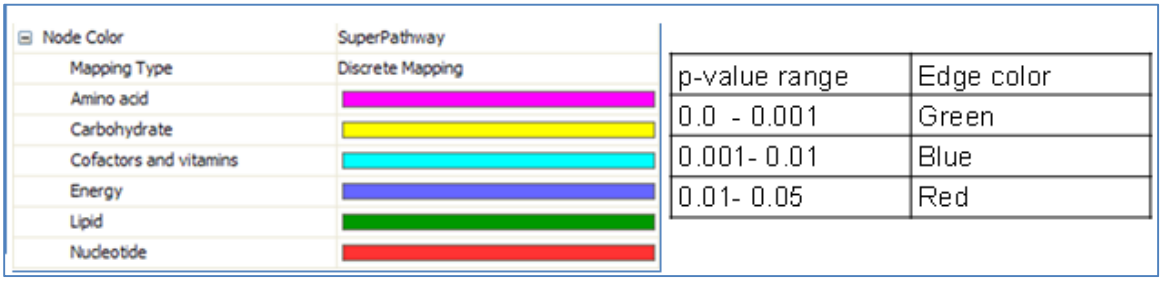

Figure 16: Legend for Metabolome Networks

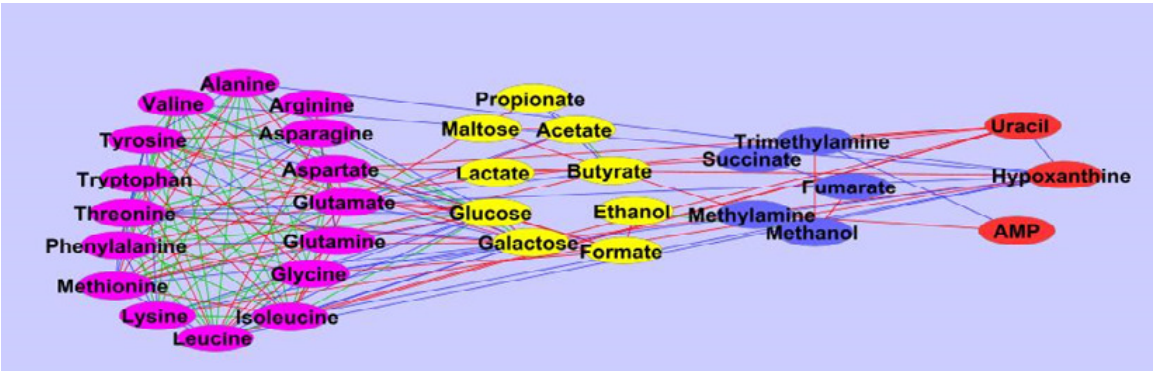

Figure 17: Cecum Control Wild Type Correlations Network

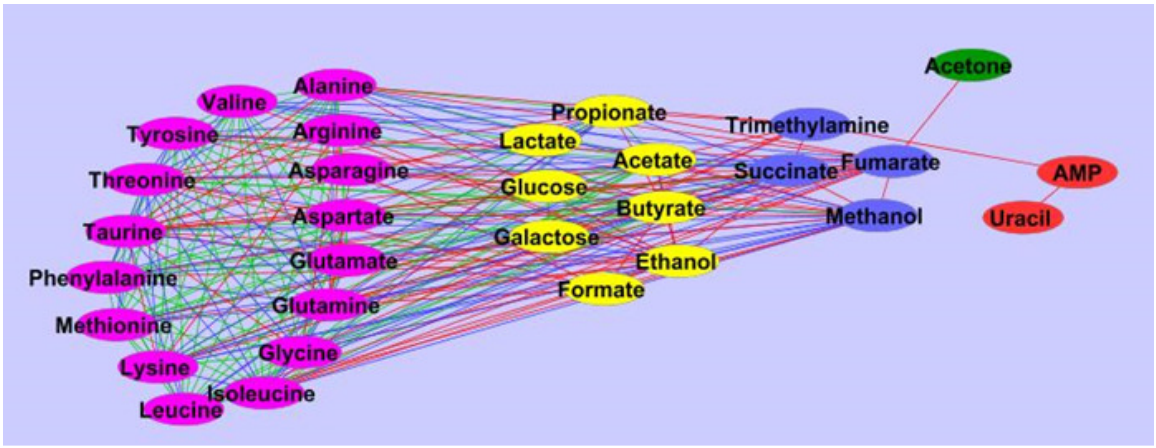

Figure 18: Cecum Control IL10 Knock Out Correlations Network

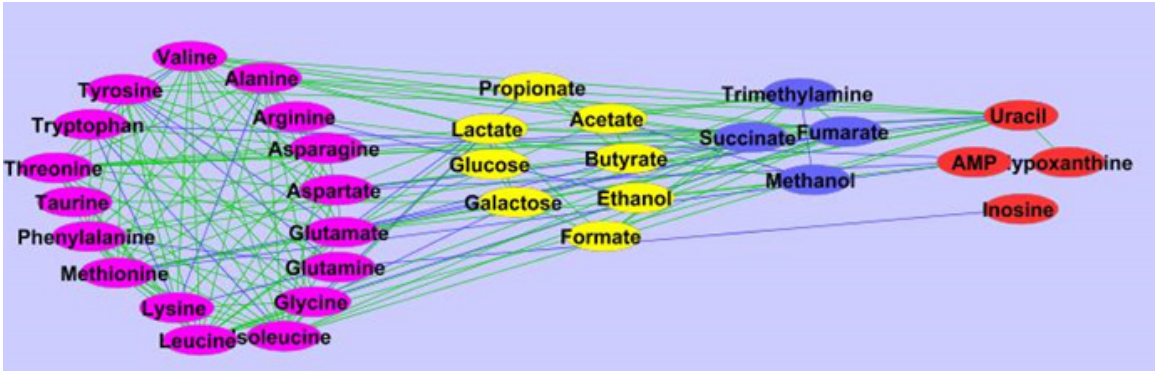

Figure 19: Cecum ProbioticWild Type Correlations Network

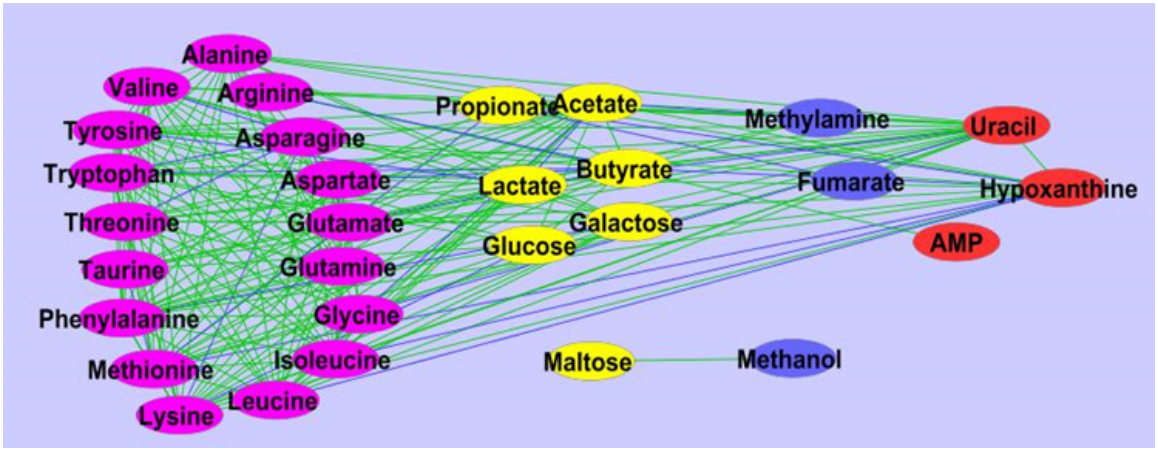

Figure 20: Cecum Probiotic IL10 Correlations Network

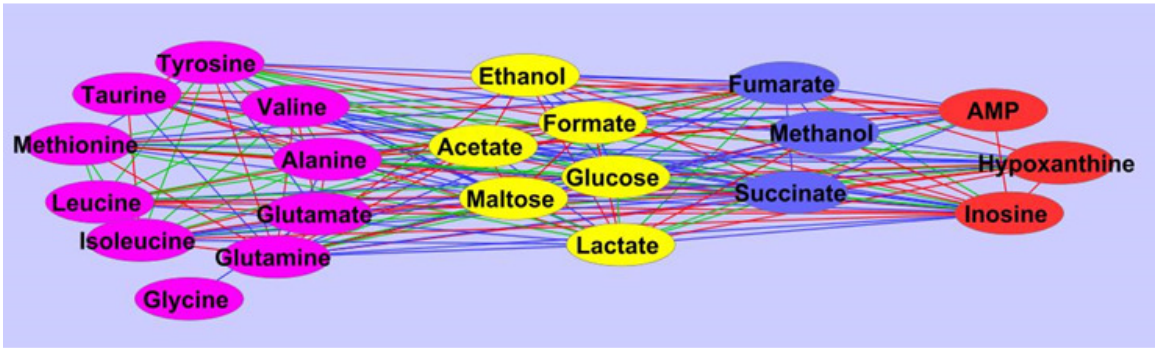

Figure 21: Liver Control Wild Type Correlations Network

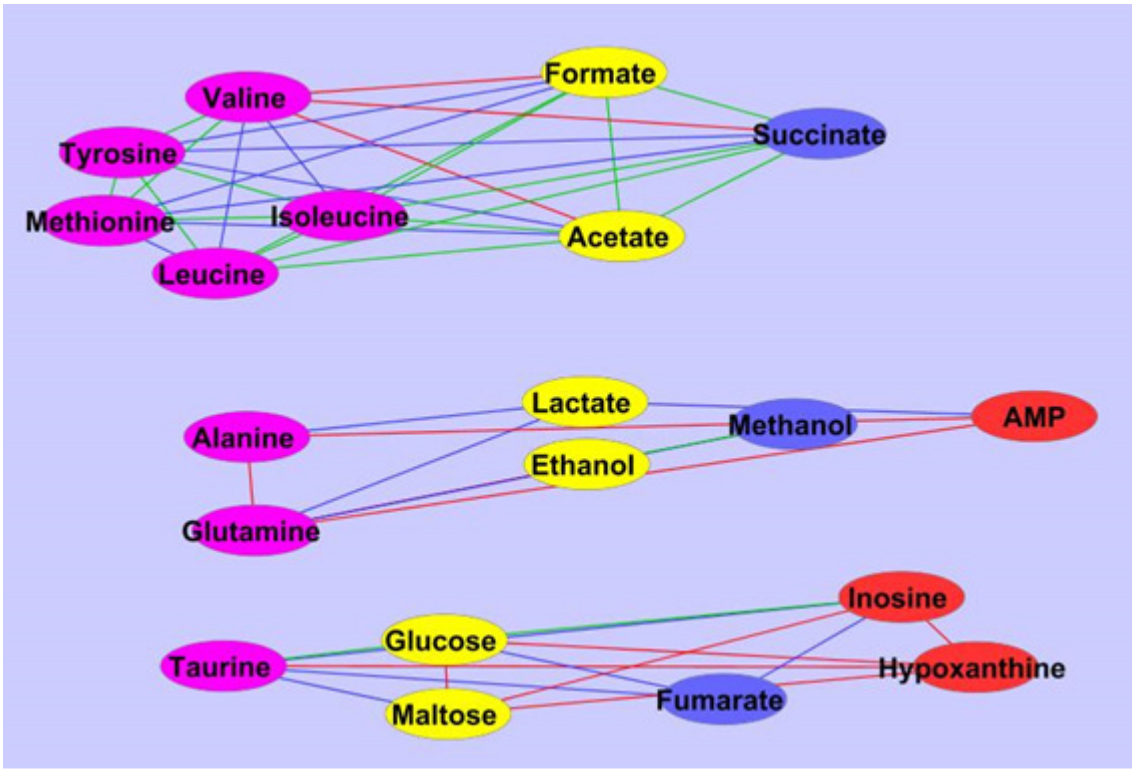

Figure 22: Liver Control IL10 Correlations Network

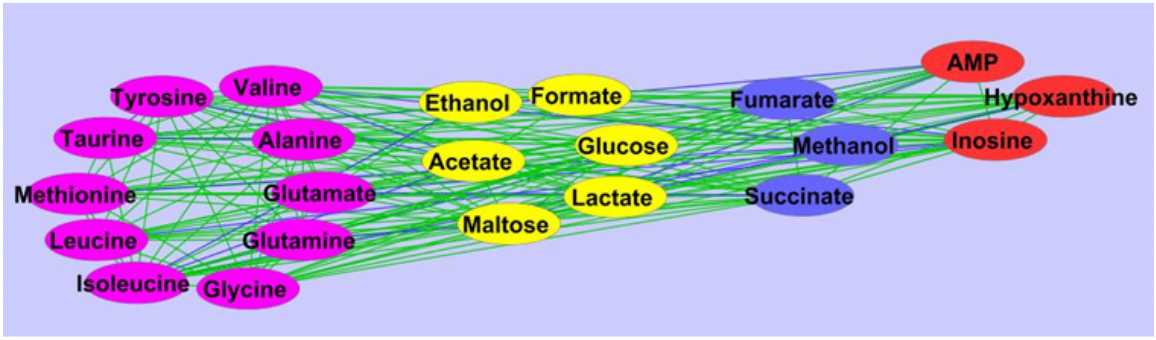

Figure 23: Liver Probiotic Wild Type Correlations Network

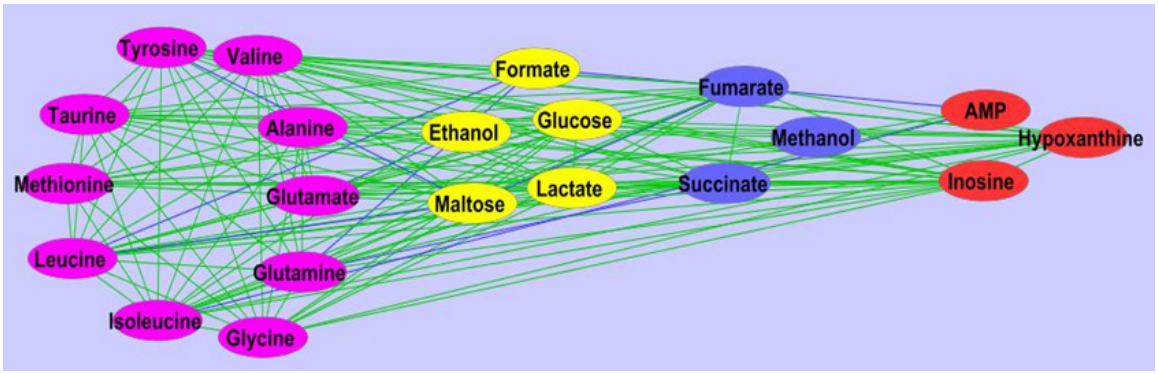

Figure 24: Liver Probiotic IL10 Correlations Network

APPENDIX C

Correlation Networks for Microbiome Data

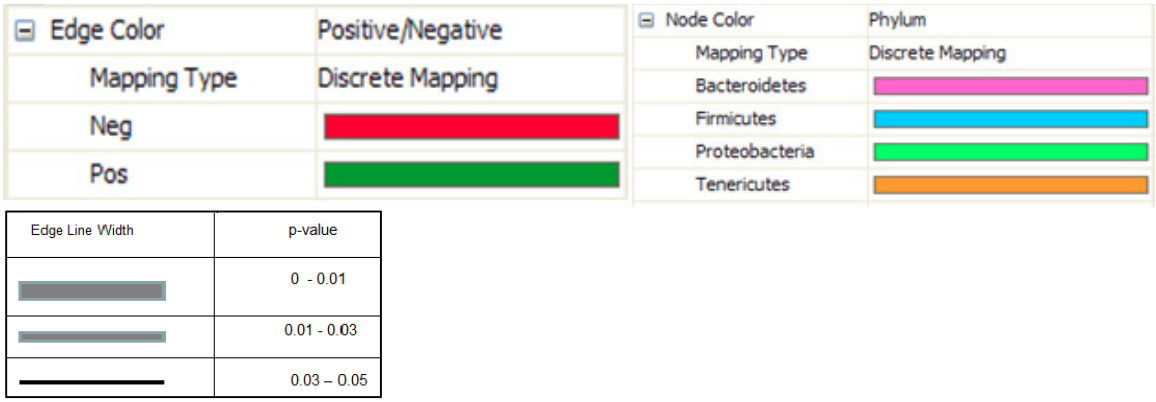

Figure 25: Legend for Microbiome Networks

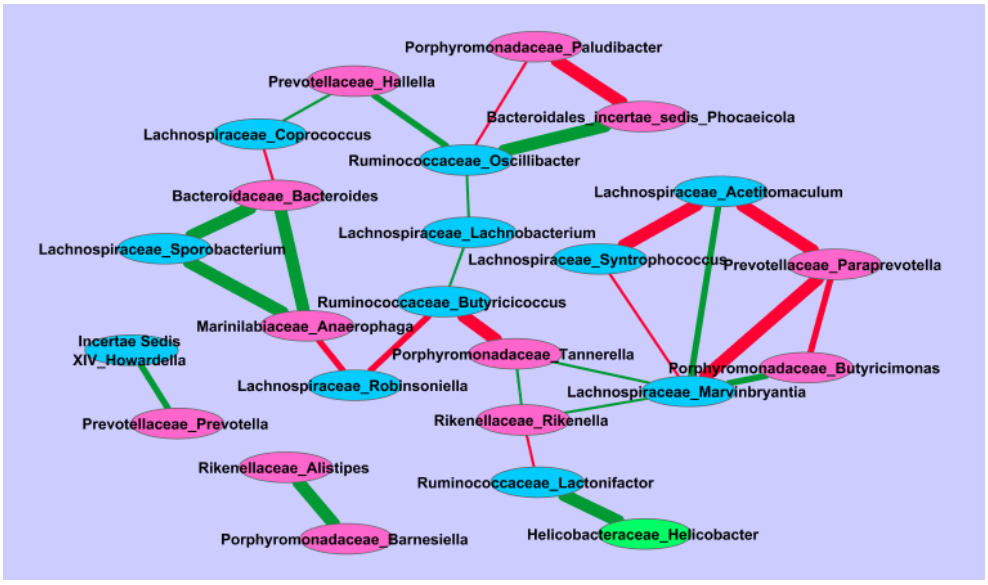

Figure 26: Cecal Content C_WT Correlation Network

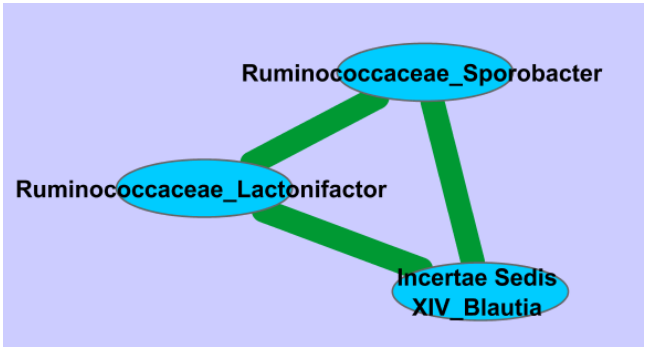

Figure 27: Cecal Content C_ IL10 Correlations Network

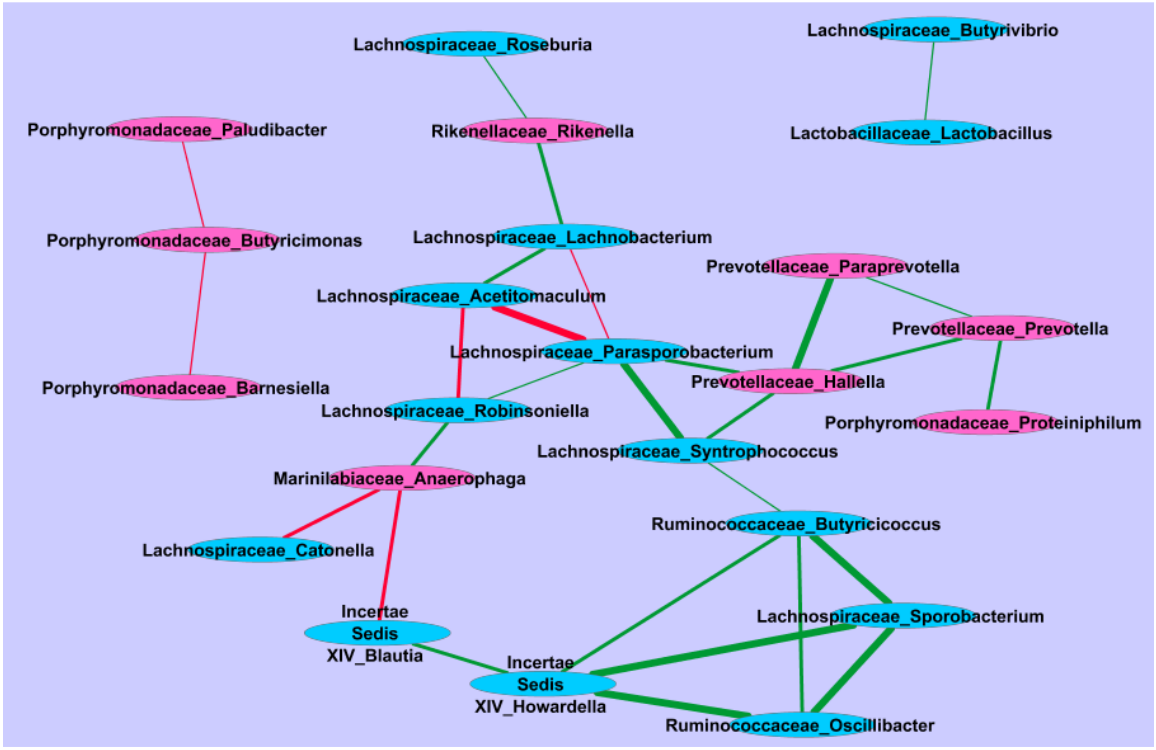

Figure 28: Cecal Content P_WT Correlations Network

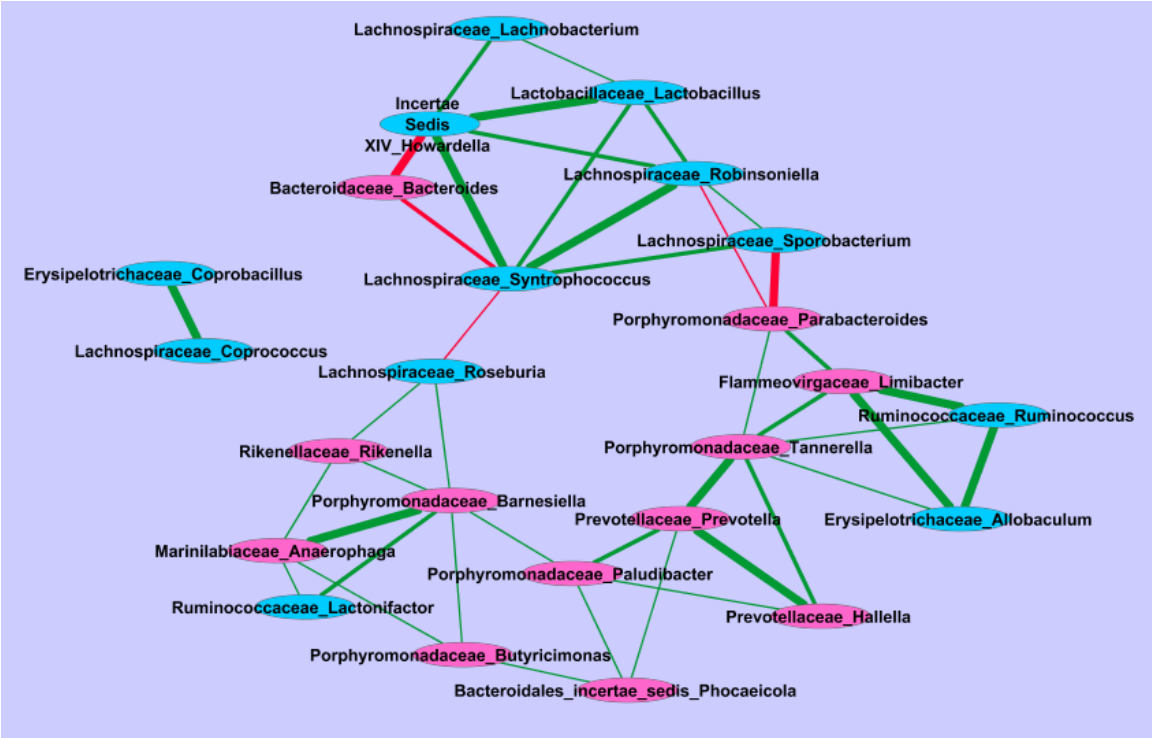

Figure 29: Cecal Content P_IL10 Correlations Network

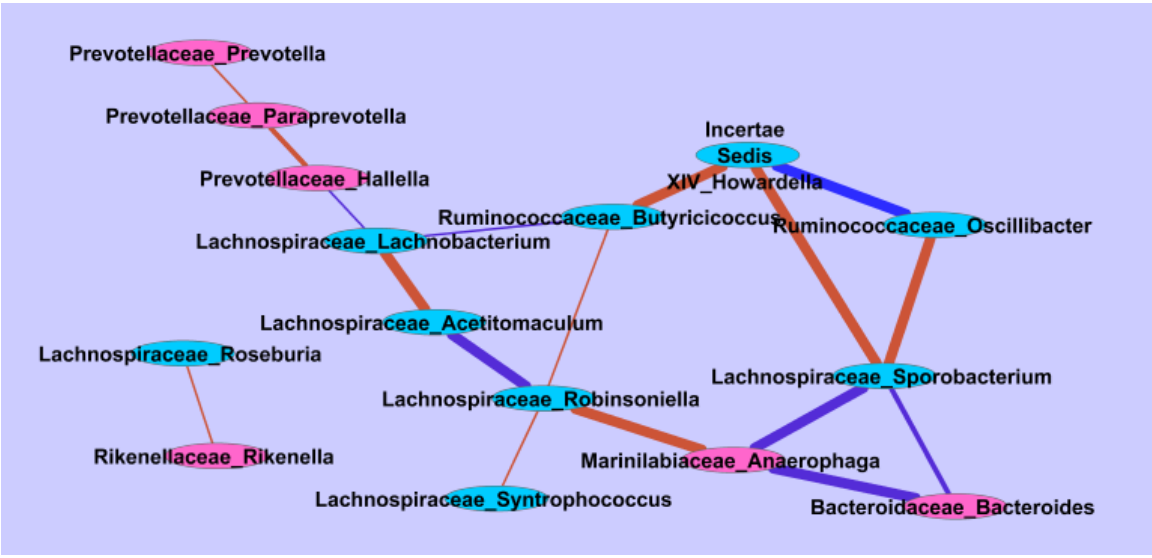

Figure 30: Cecal Content C_WT-P_WT Differential Correlations Network

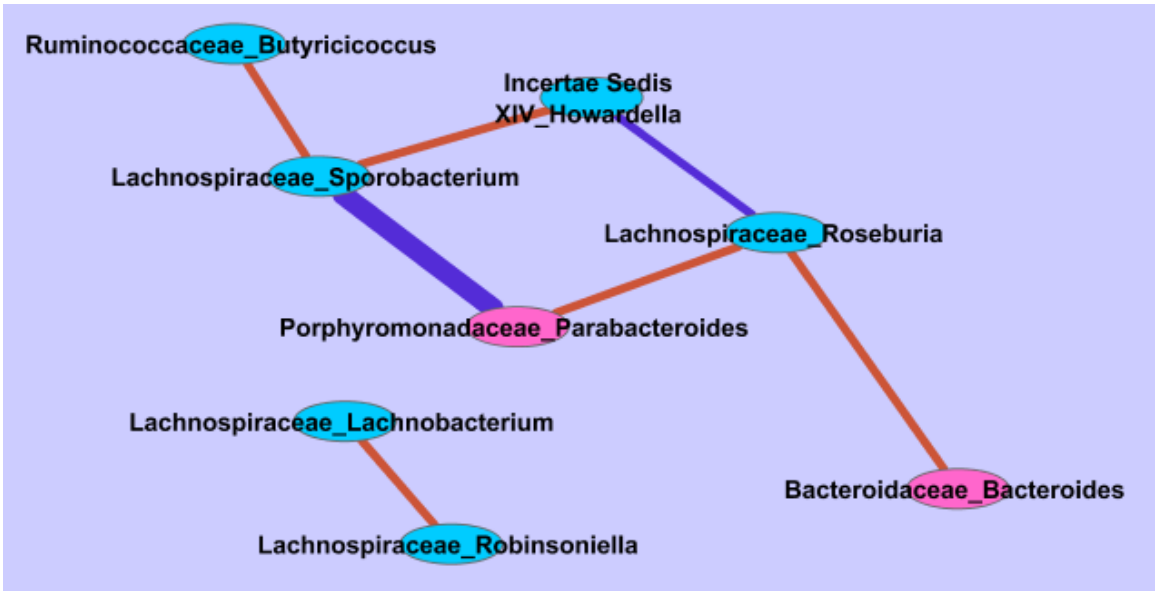

Figure 31: Cecal Content C_IL10-P_IL10 Differential Correlations Network

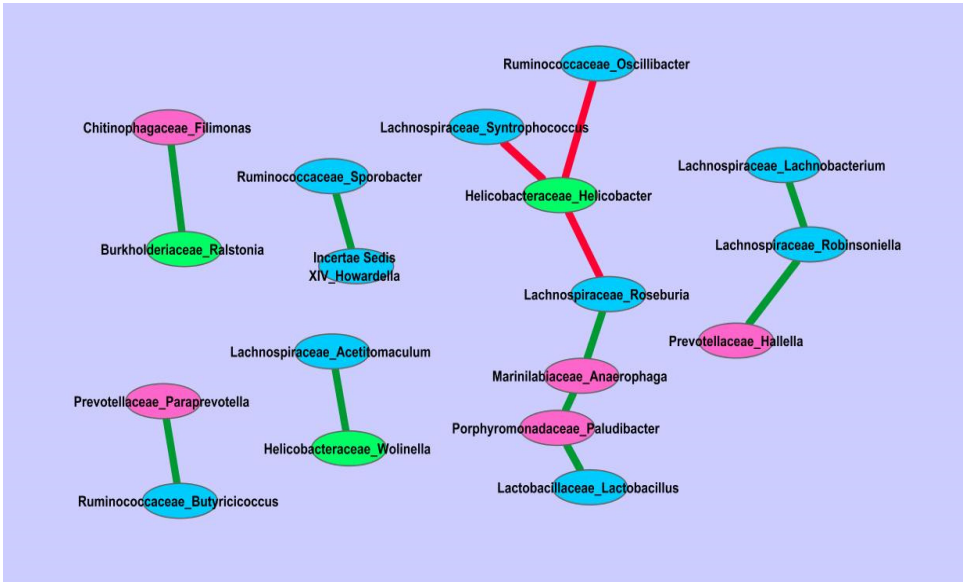

Figure 32: Cecal Mucosa C_WT Correlations Network

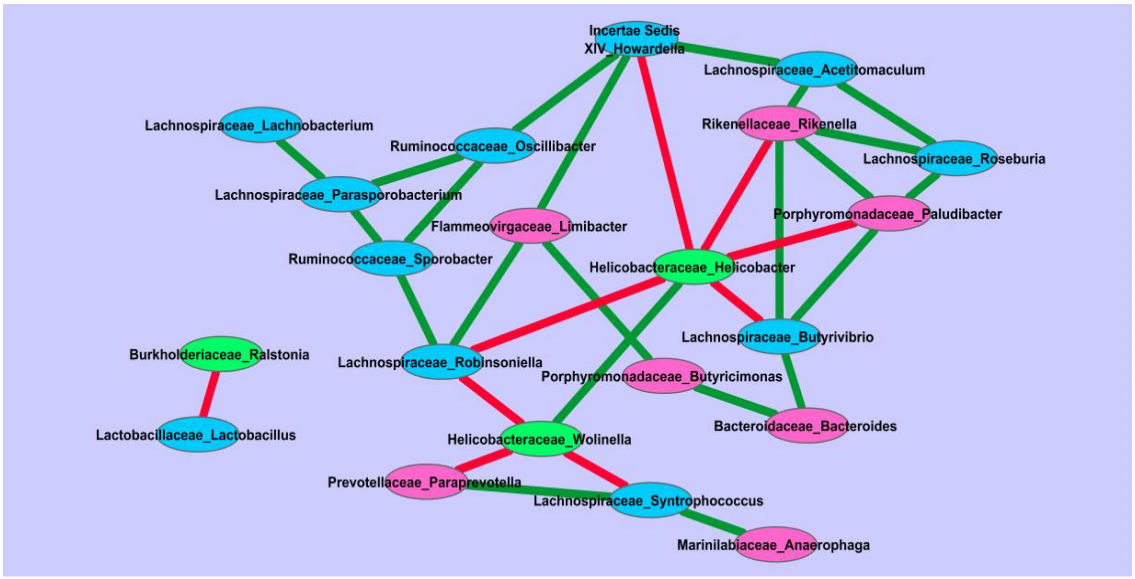

Figure 33: Cecal Mucosa P_WT Correlations Network

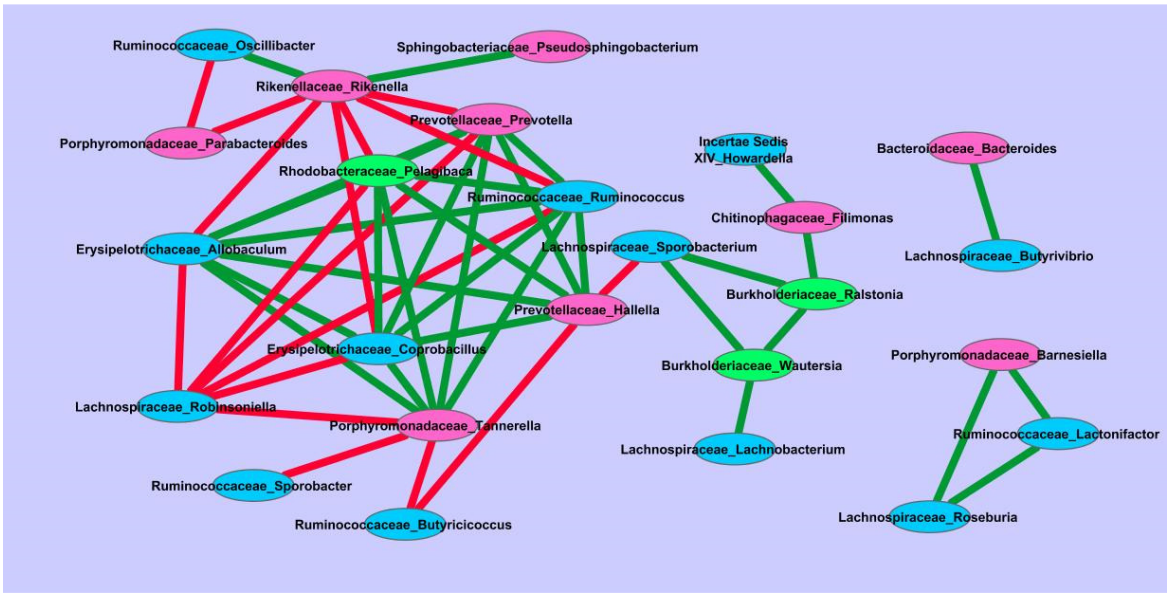

Figure 34: Cecal Mucosa P_IL10 Correlations Network

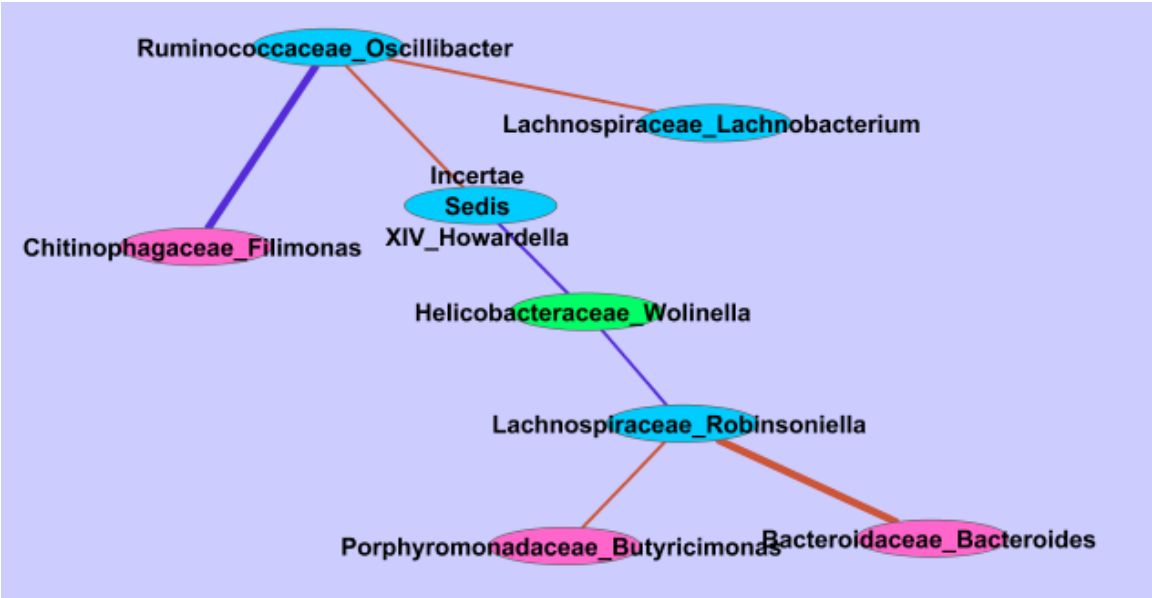

Figure 35: Cecal Mucosa C_WT-P_WT Differential Correlations Network

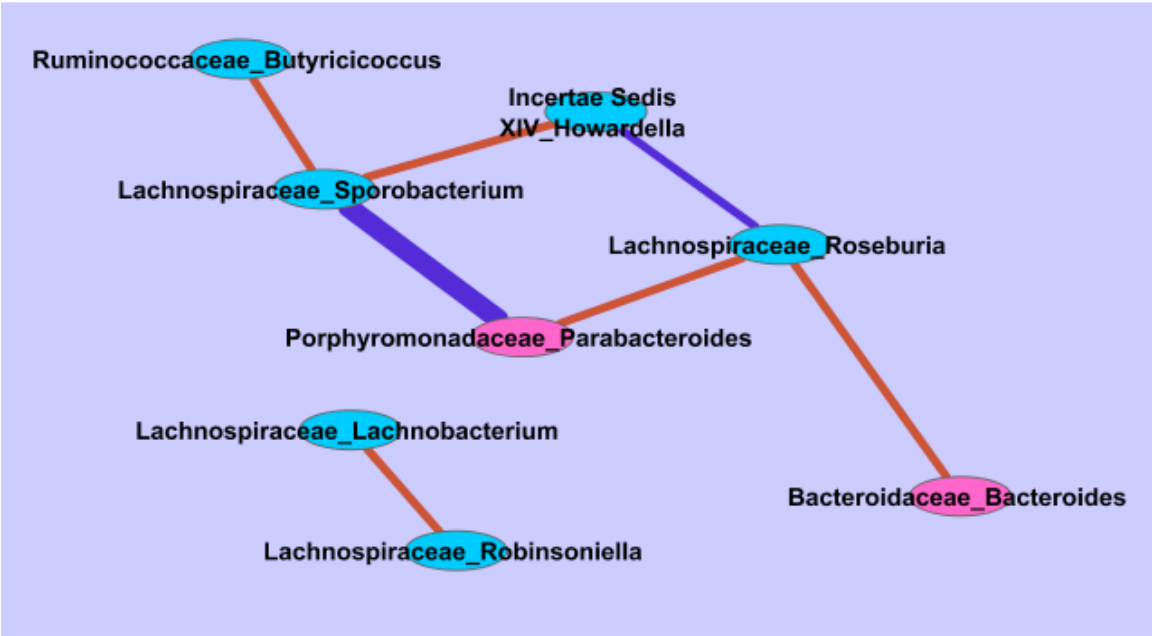

Figure 36: Cecal Mucosa C_IL10-P_IL10 Differential Correlations Network

APPENDIX D

Correlation Networks for Microbiome-Cytokine Data

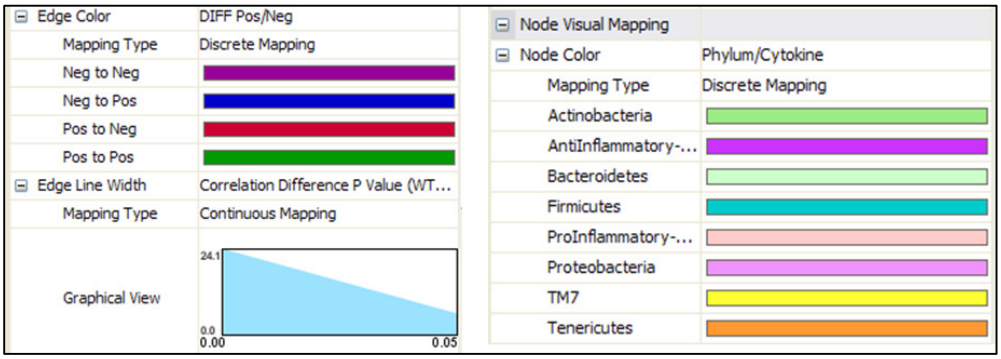

Figure 37: Legend for Microbiome-Cytokine Networks

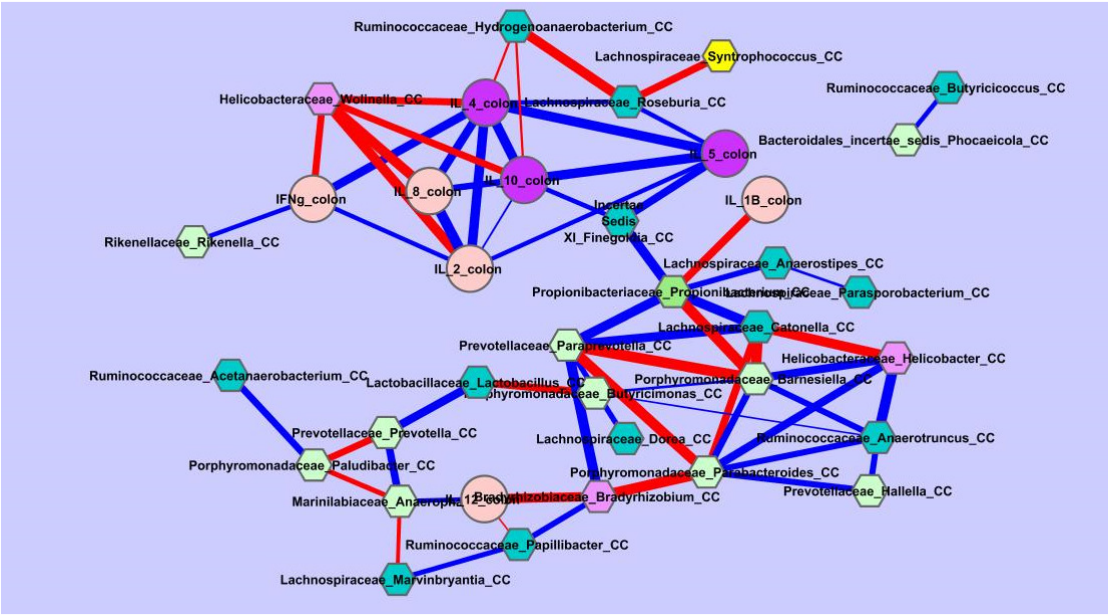

Figure 38: C_WT Cecal Content -Colon Cytokine Correlations Networks

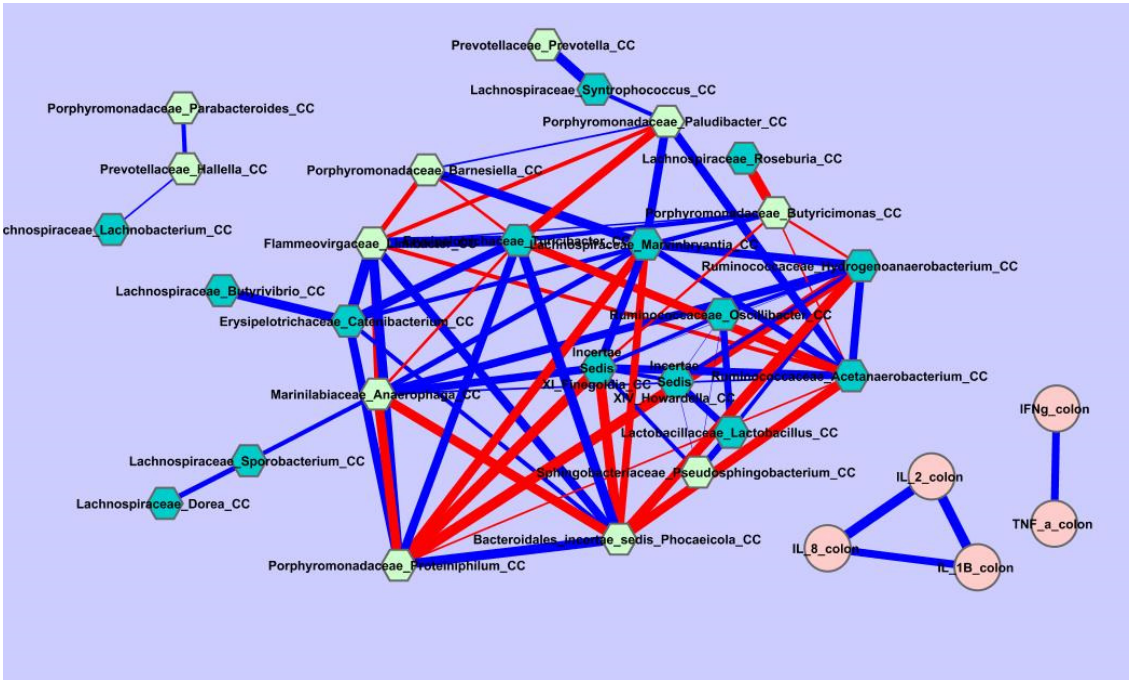

Figure 39:C_IL10 Cecal Content -Colon Cytokine Correlations Networks

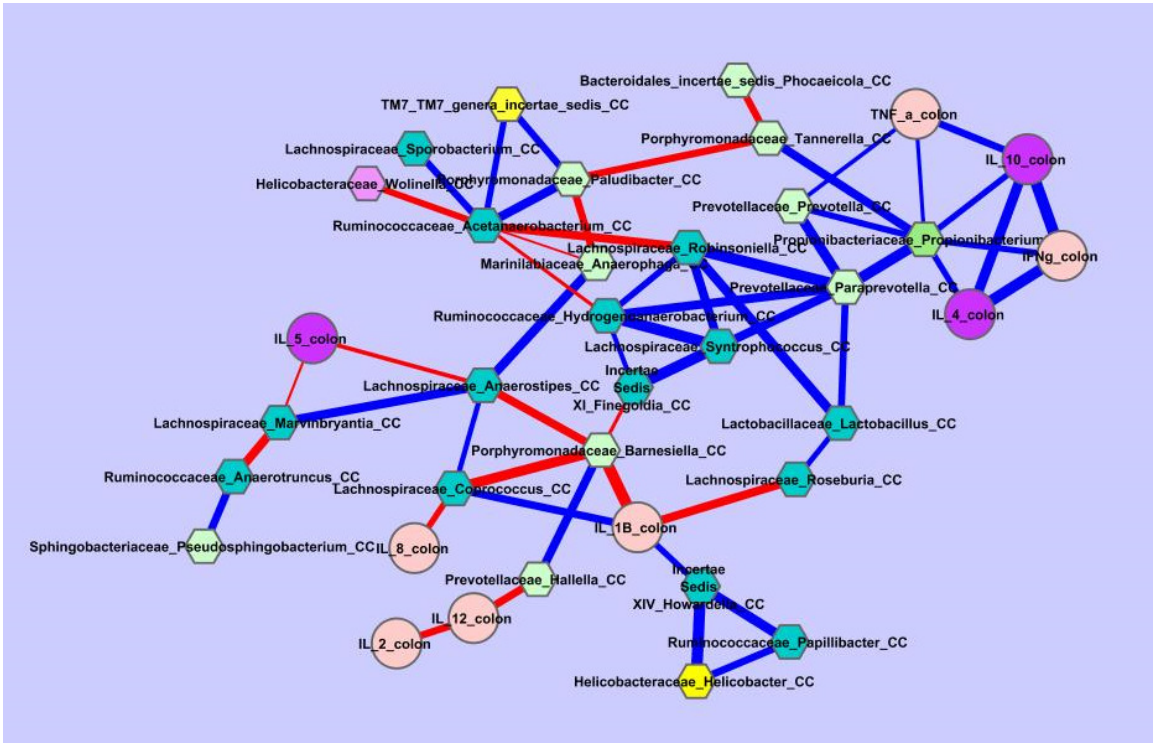

Figure 40: P_WT Cecal Content Colon Cytokine Correlations Networks

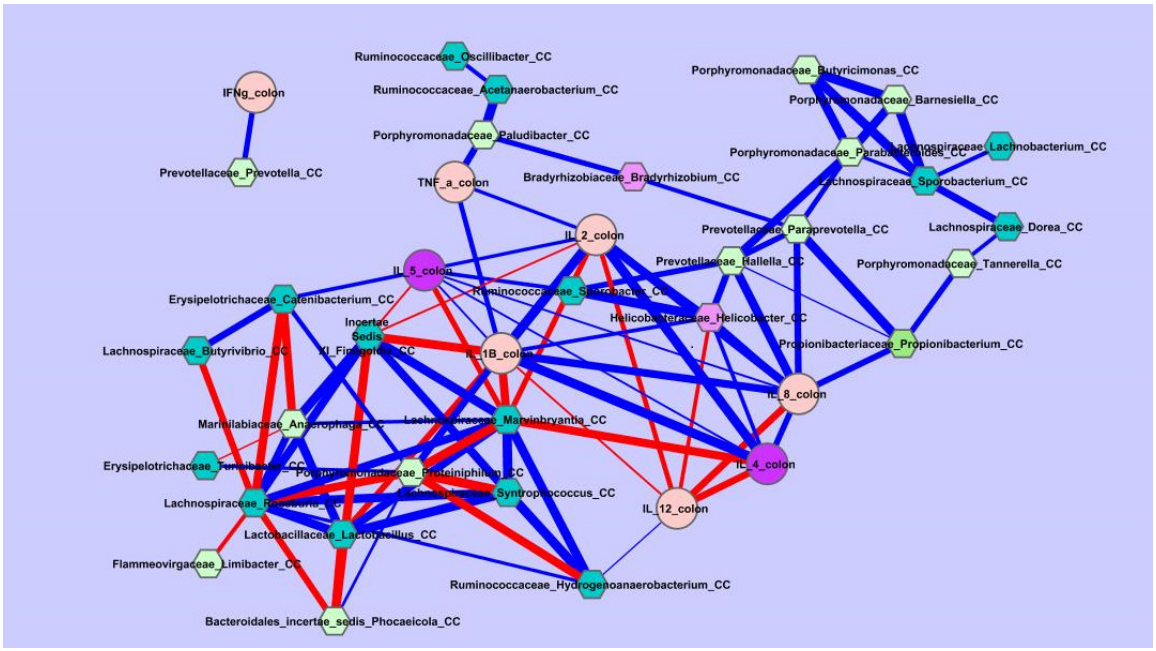

Figure 41:P_IL10 Cecal Content -Colon Cytokine Correlations Networks

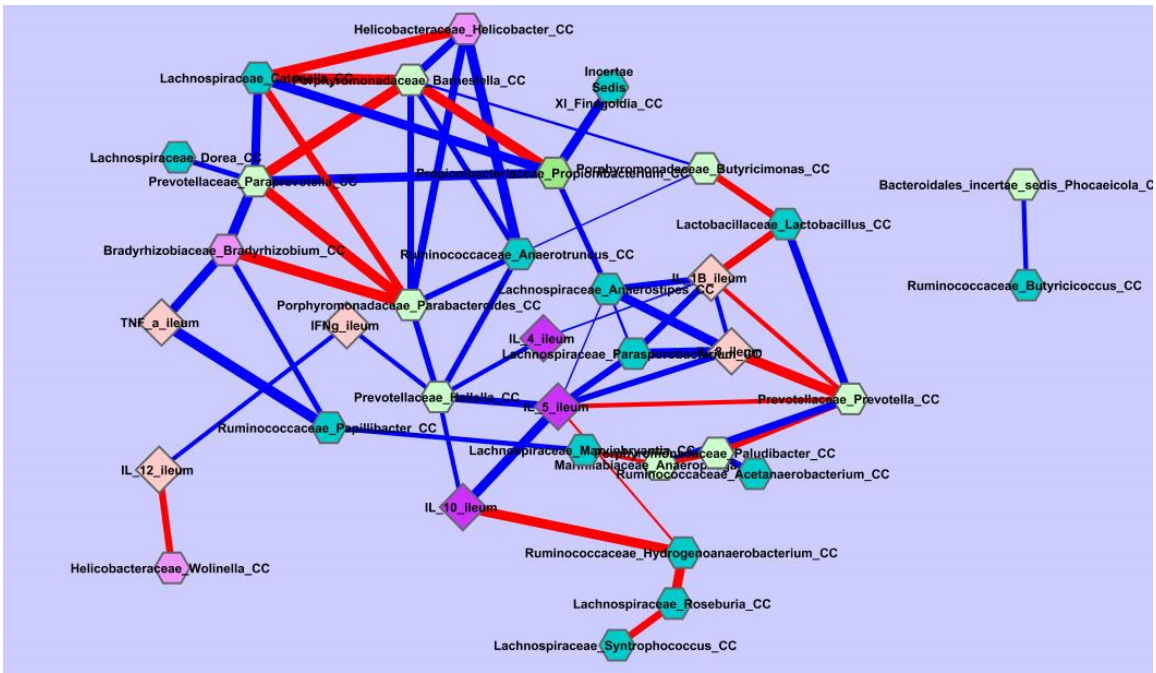

Figure 42:C_WT Cecal Content -Ileum Cytokine Correlations Networks

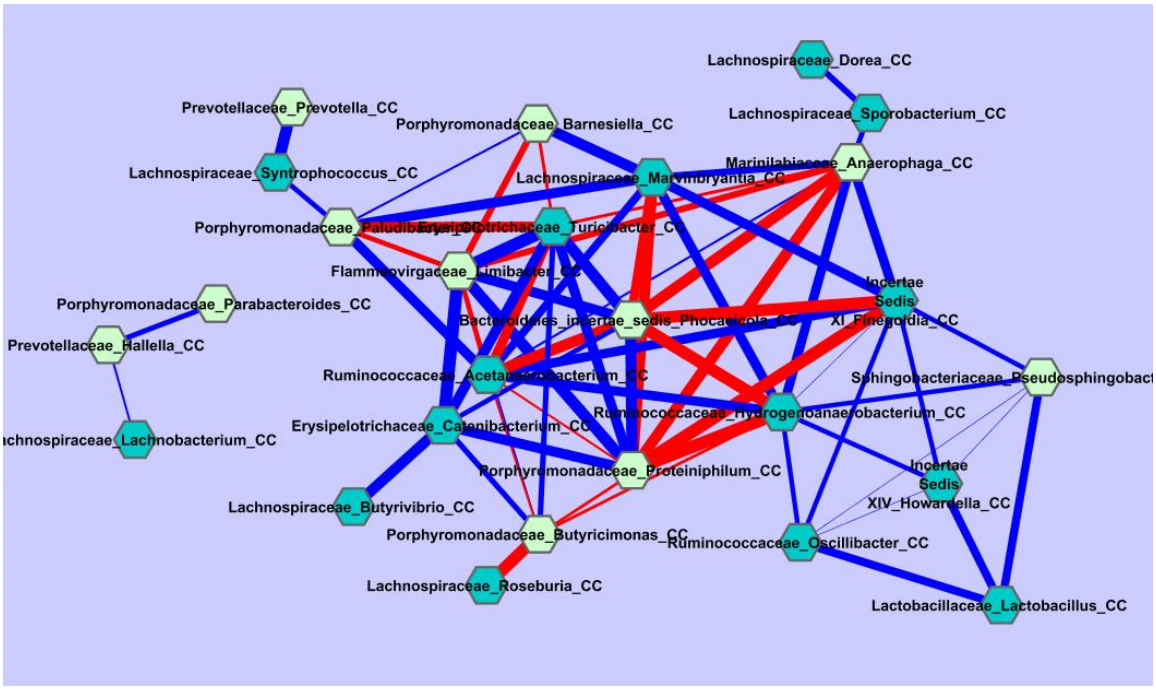

Figure 43: C_IL10 Cecal Content -Ileum Cytokine Correlations Networks

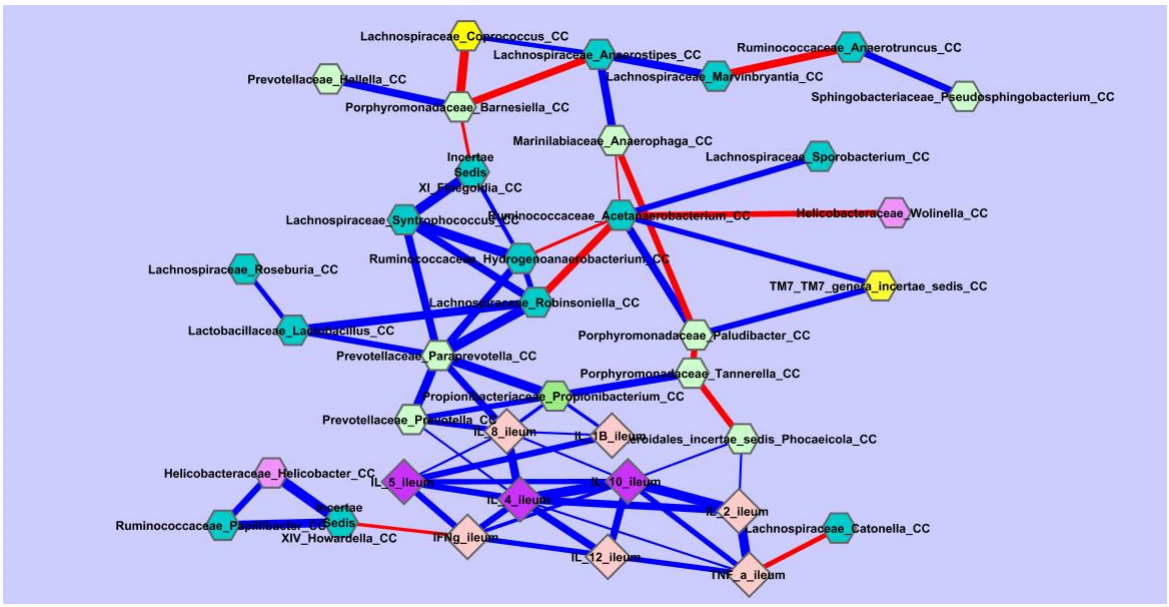

Figure 44: P_WT Cecal Content -Ileum Cytokine Correlations Networks

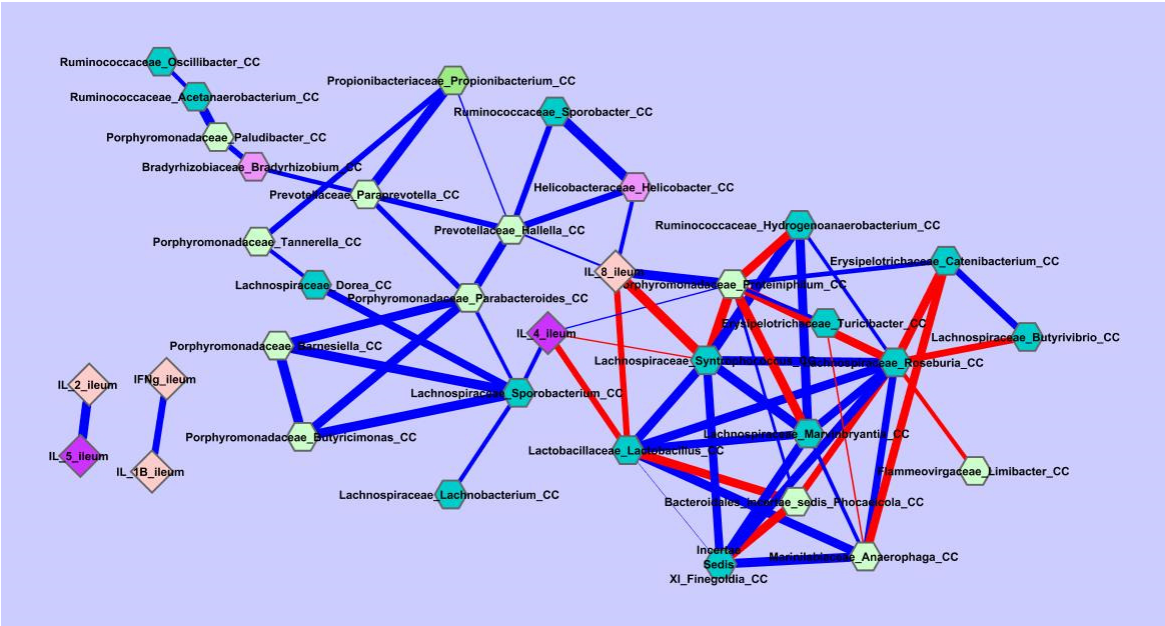

Figure 45: P_IL10 Cecal Content -Ileum Cytokine Correlations Networks

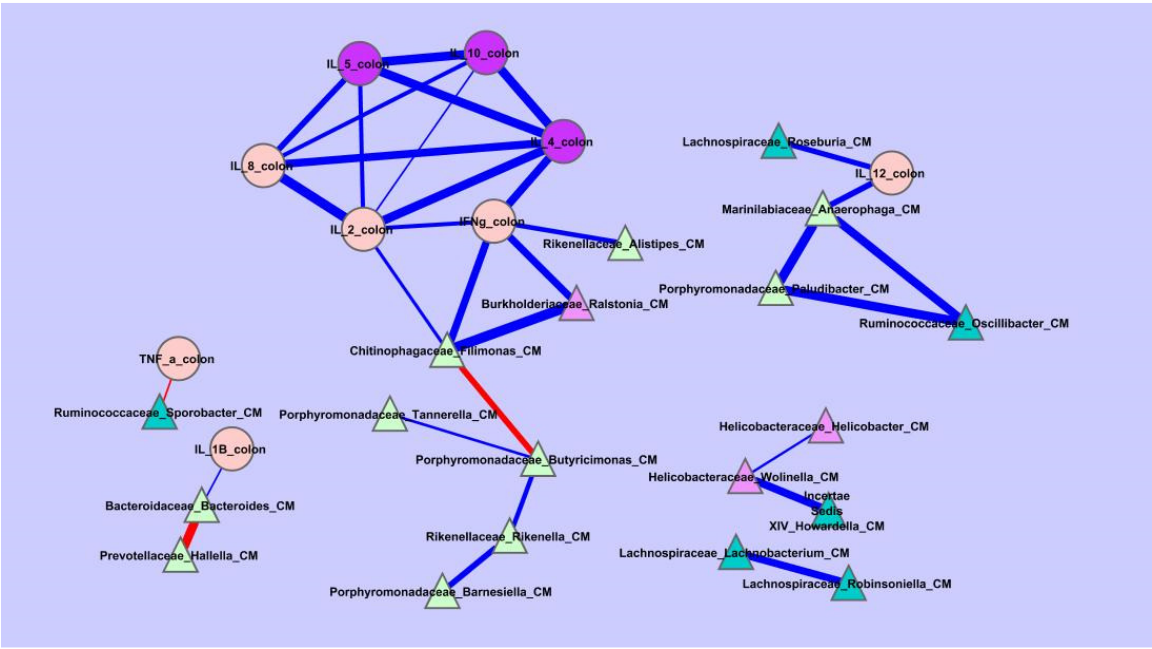

Figure 46: C_WT Cecal Mucosa -Colon Cytokine Correlations Networks

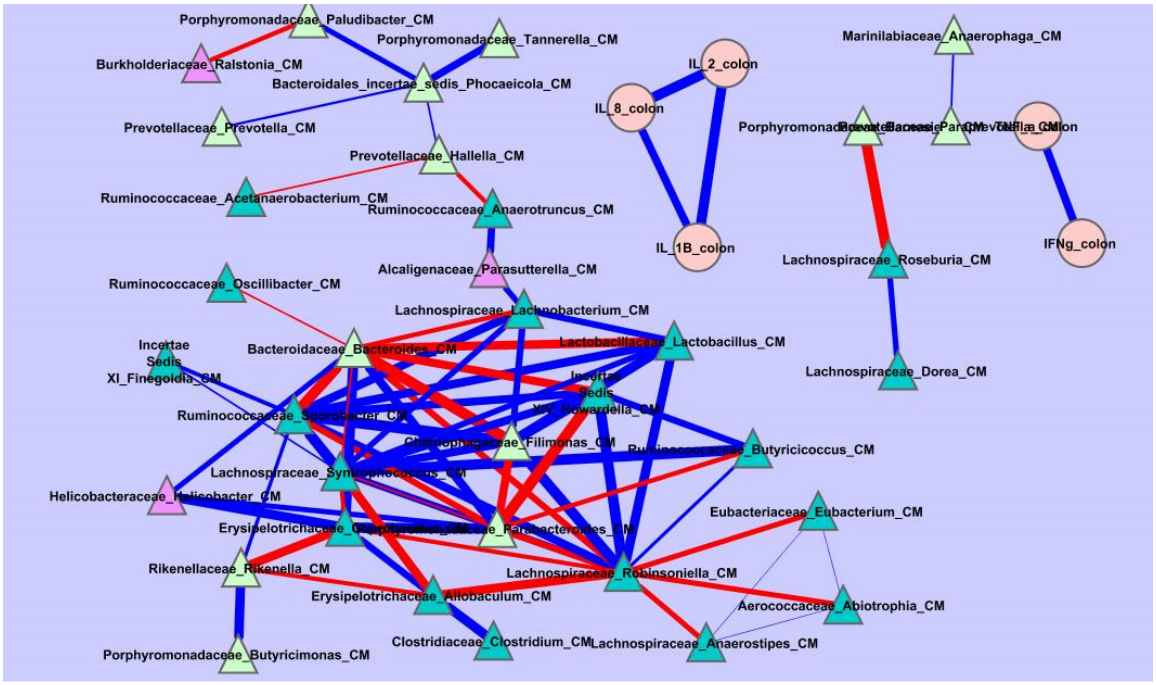

Figure 47: C_IL10 Cecal Mucosa -Colon Cytokine Correlations Networks

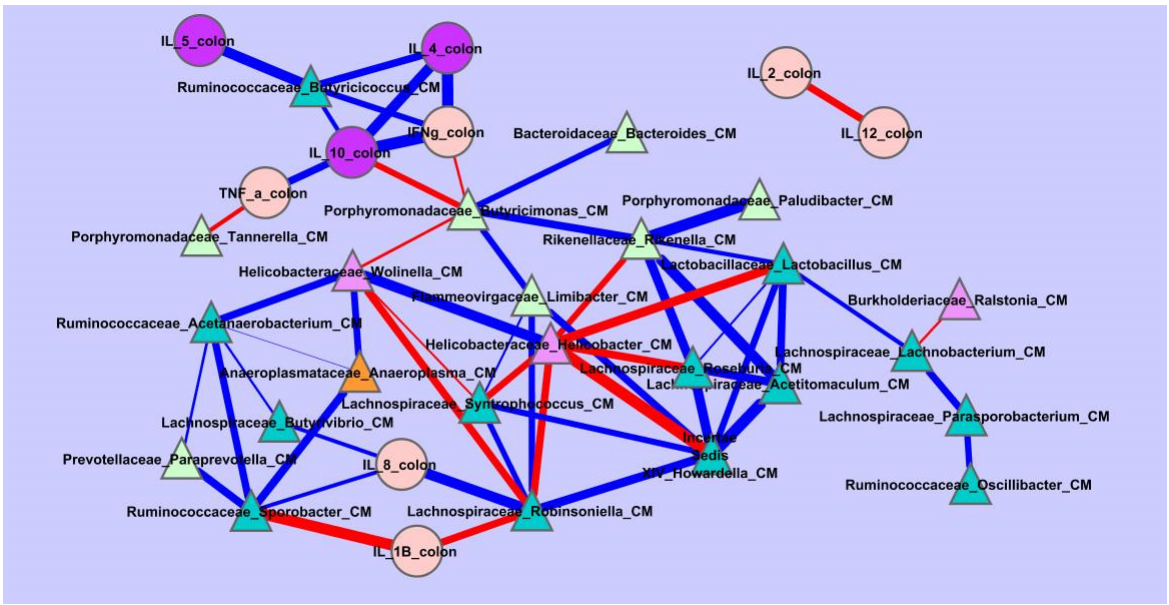

Figure 48: P_WT Cecal Mucosa -Colon Cytokine Correlations Networks

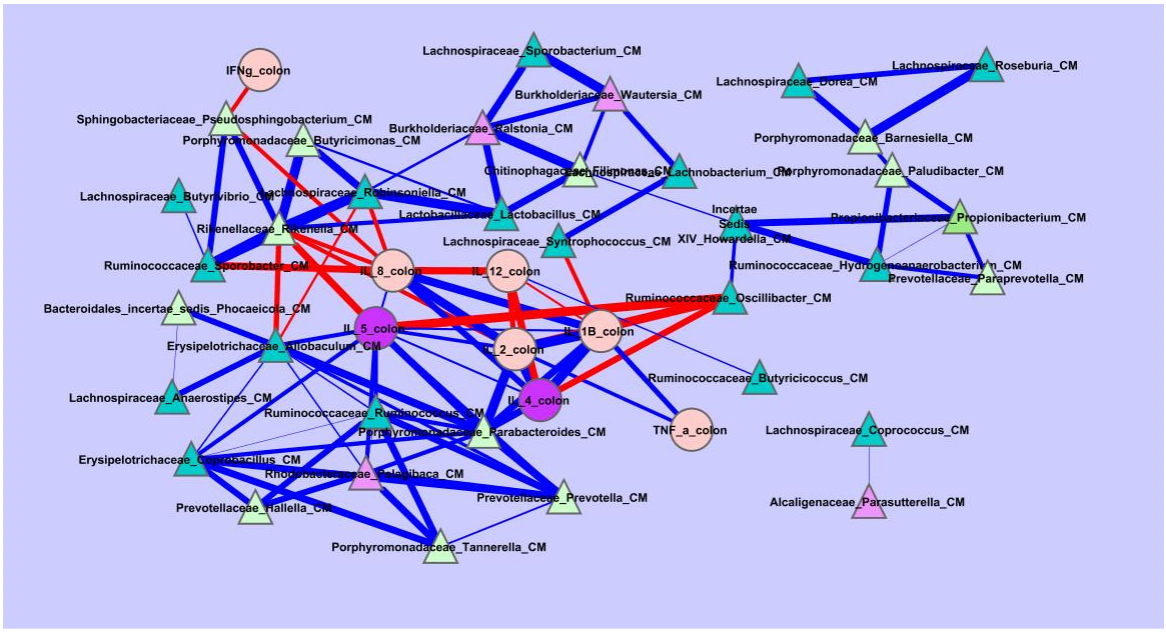

Figure 49:P_IL10 Cecal Mucosa -Colon Cytokine Correlations Networks

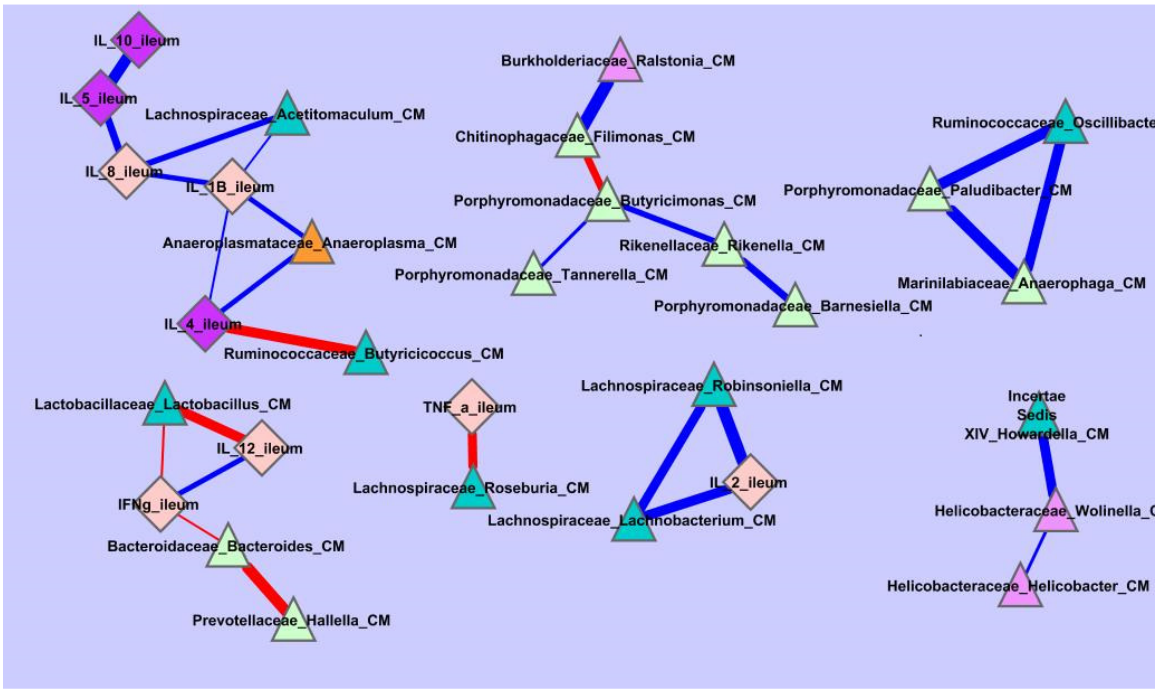

Figure 50: C_WT Cecal Mucosa-Ileum Cytokine Correlations Networks

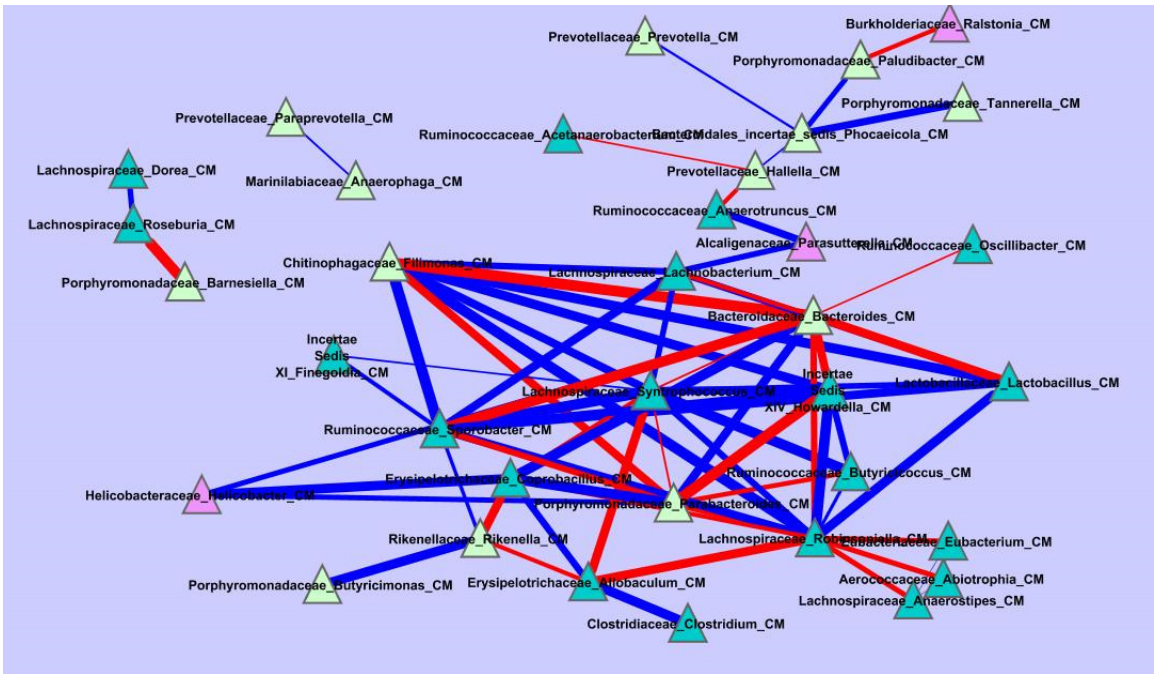

Figure 51: C_IL10 Cecal Mucosa -Ileum Cytokine Correlations Networks

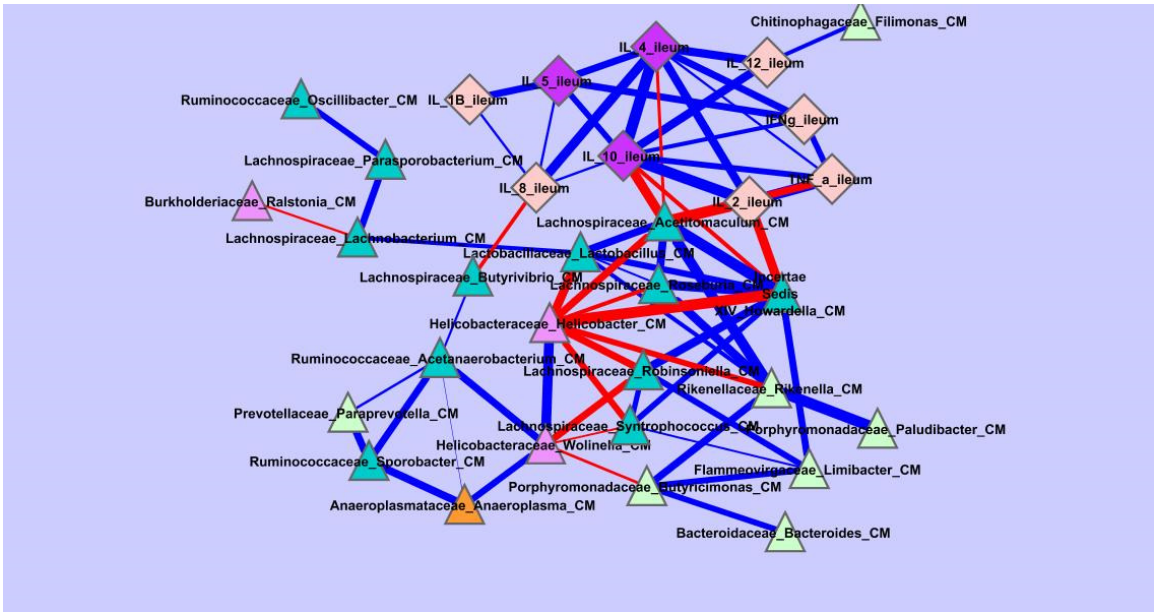

Figure 52: P_WT Cecal Mucosa -Ileum Cytokine Correlations Networks

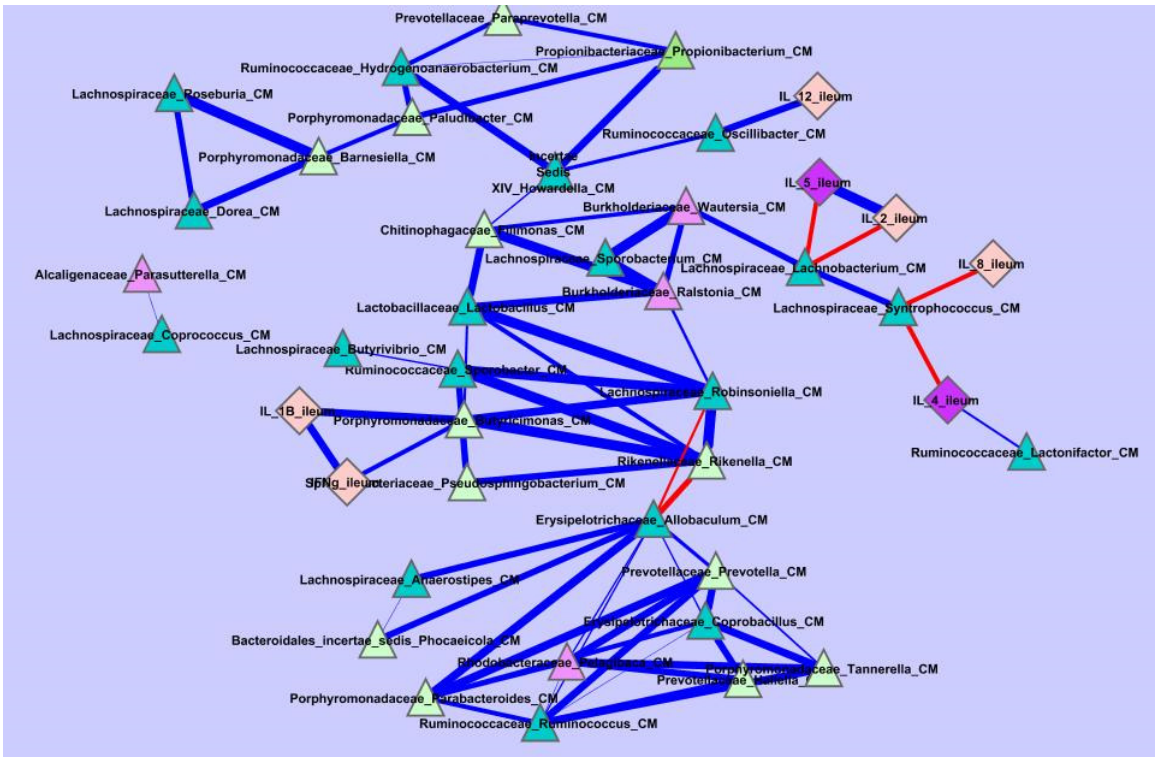

Figure 53: P_IL10 Cecal Mucosa -Ileum Cytokine Correlations Networks

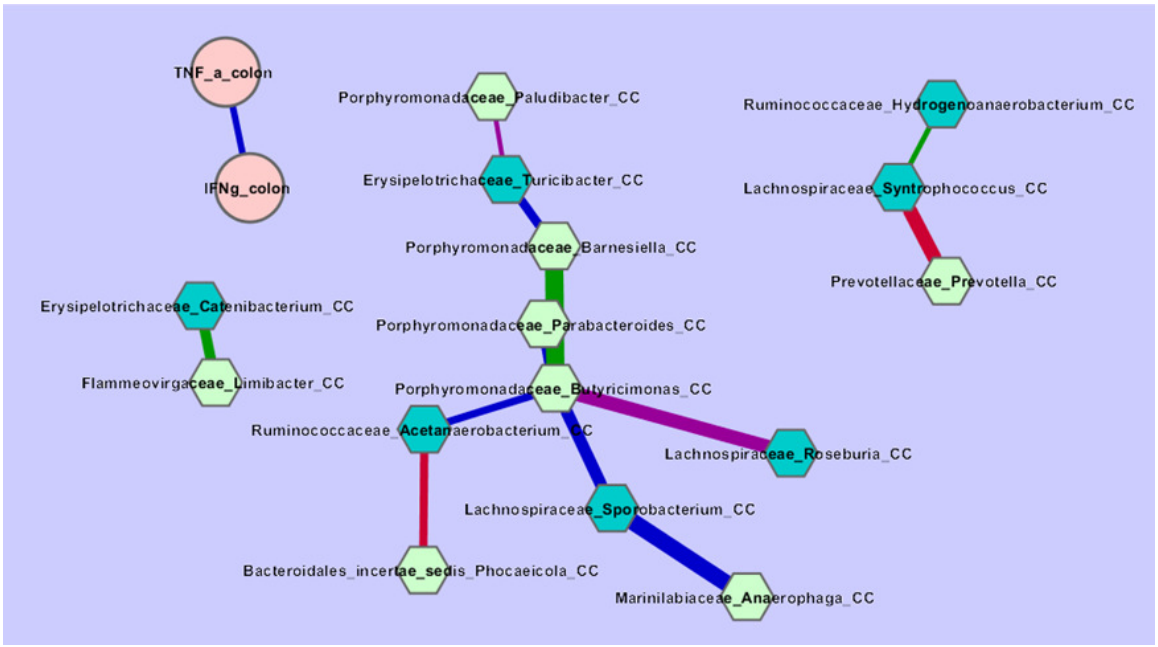

Figure 54: C_IL10- P_IL10 Cecal Contents -Colon Cytokine Networks

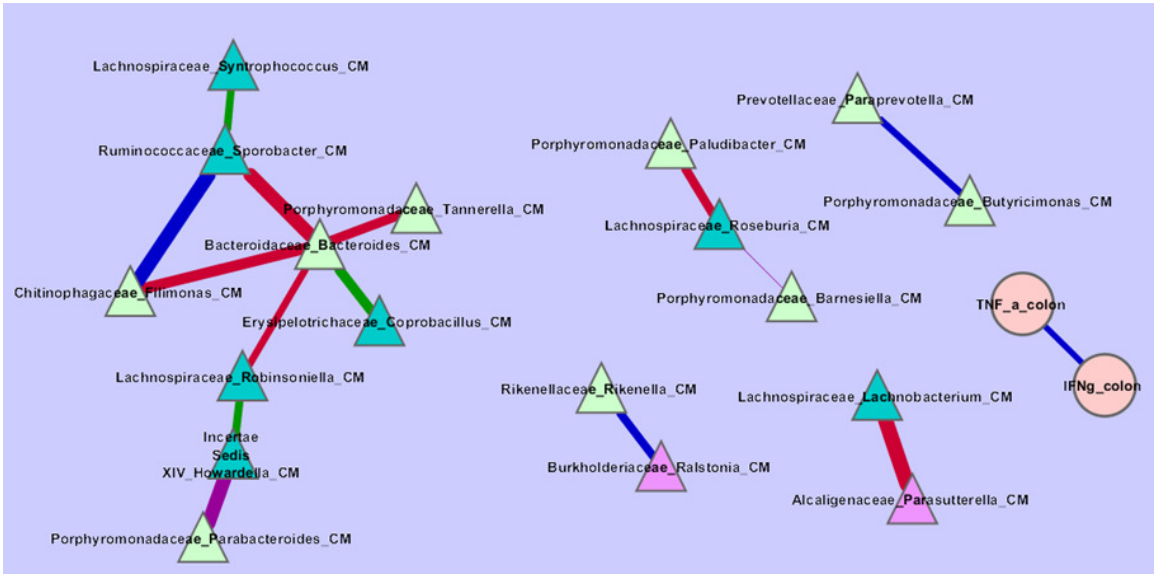

Figure 55: C_IL10- P_IL10 Cecal Mucosa-Colon Cytokine Differential Correlations Networks

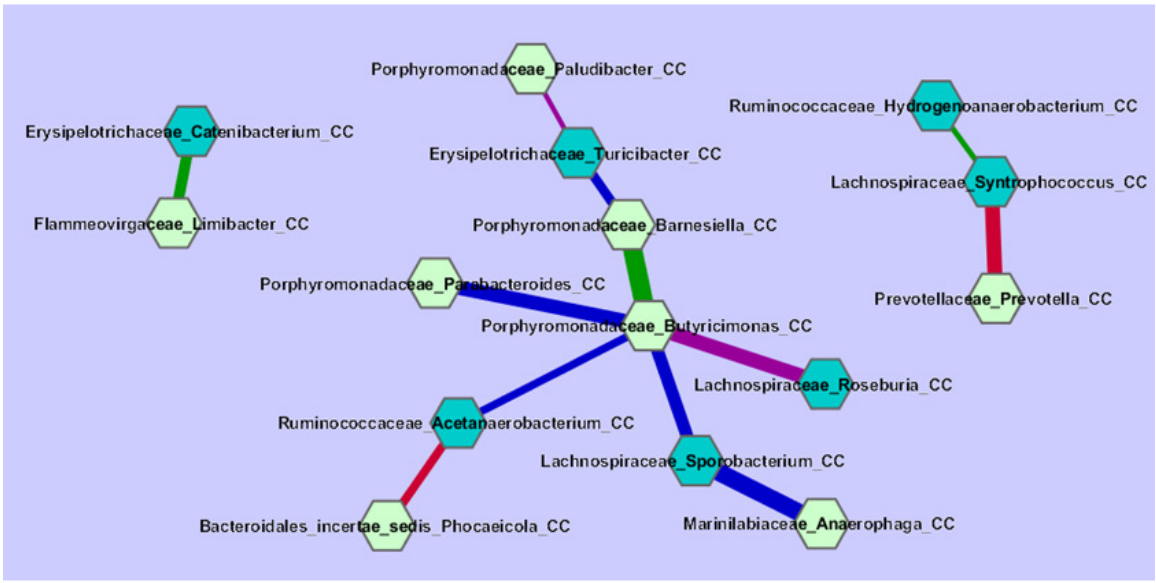

Figure 56: C_IL10- P_IL10 Cecal Contents -Ileum Cytokine Differential Correlations Networks

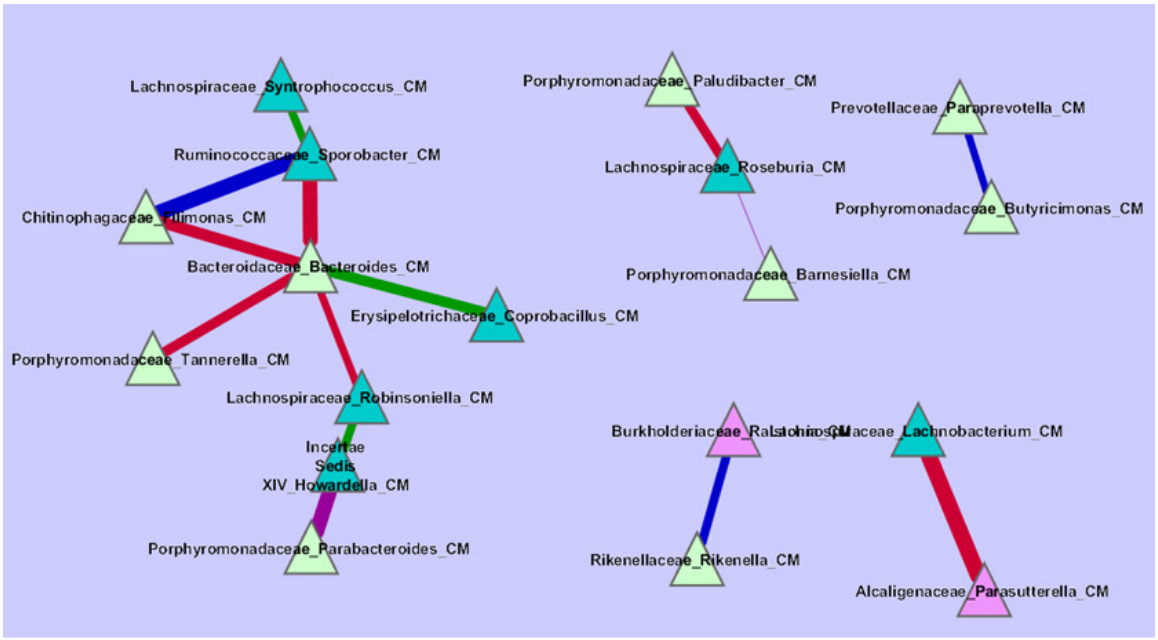

Figure 57: C_IL10- P_IL10 Cecal Mucosa -Ileum Cytokine Differential Correlations Networks

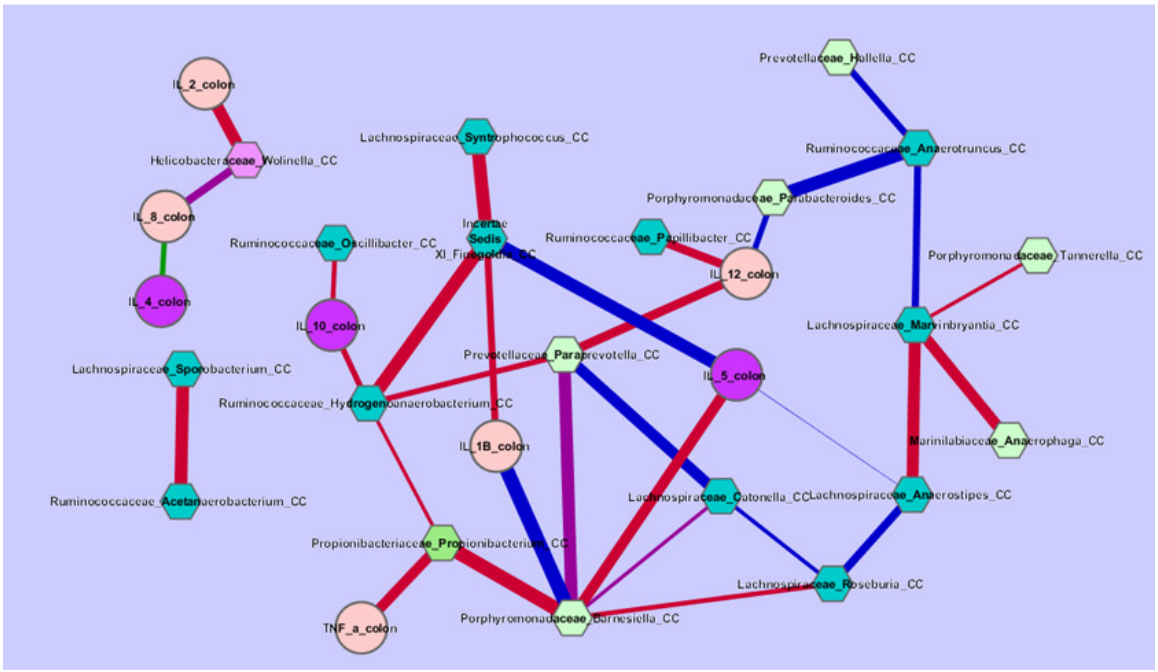

Figure 58: C_WT- P_WT Cecal Contents -Colon Cytokine Differential Correlations Networks

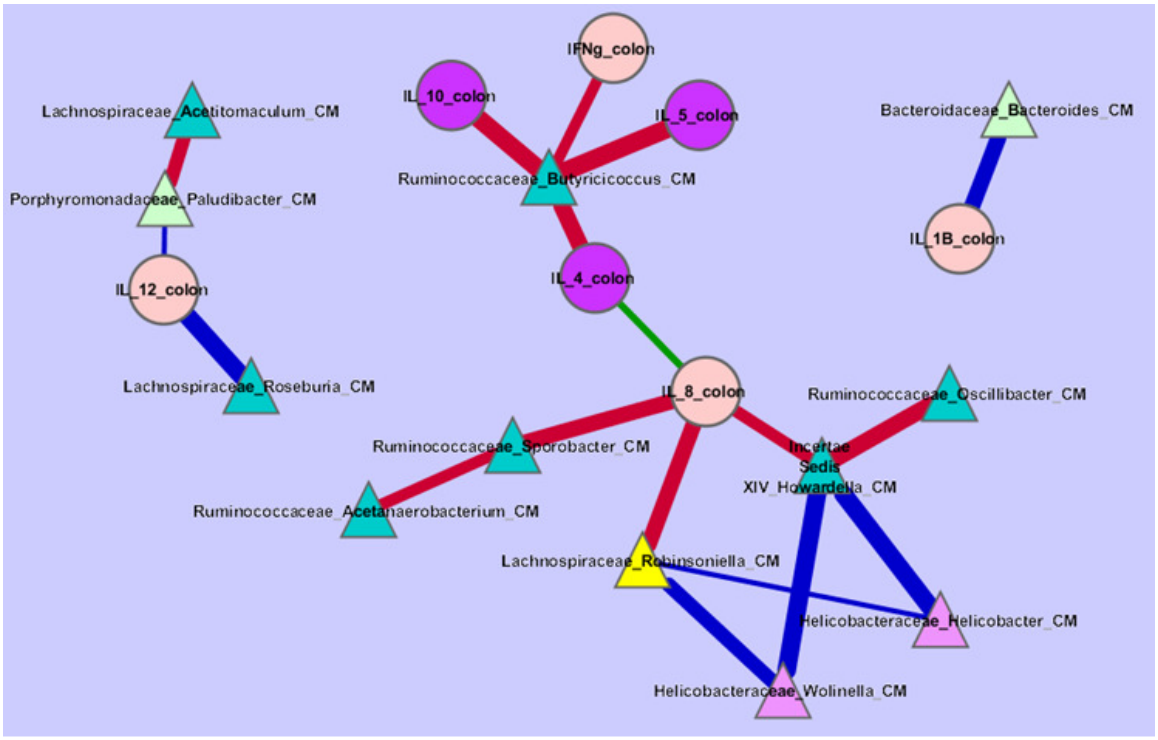

Figure 59: C_WT- P_WT Cecal Mucosa -Colon Cytokine Differential Correlations Networks

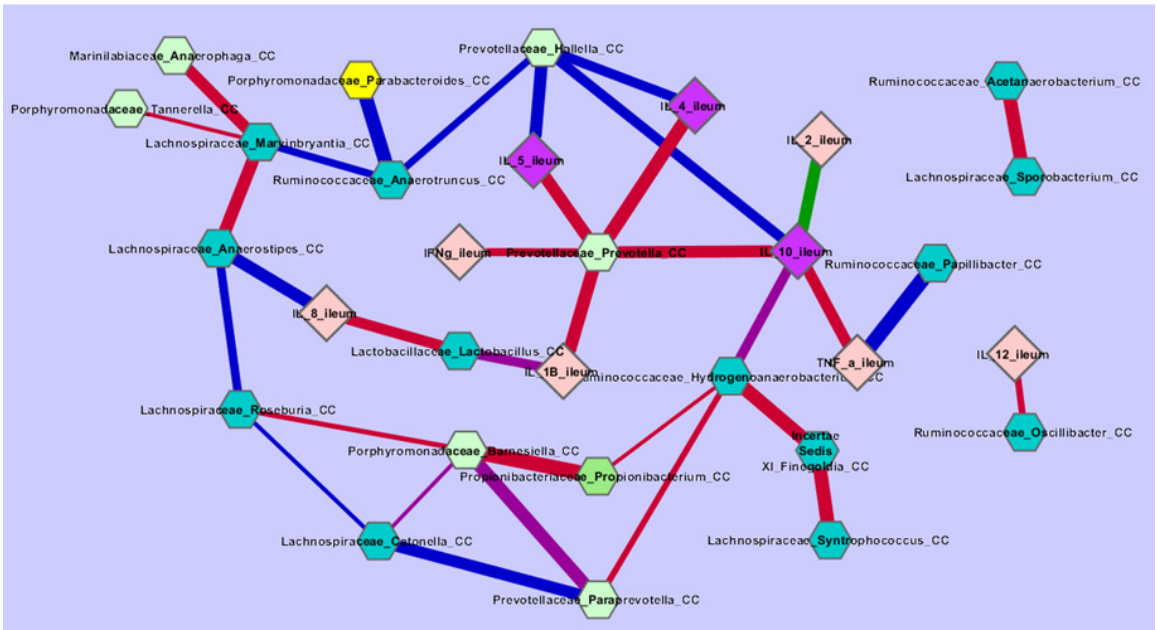

Figure 60: C_WT- P_WT Cecal Contents -Ileum Cytokine Differential Correlations Networks

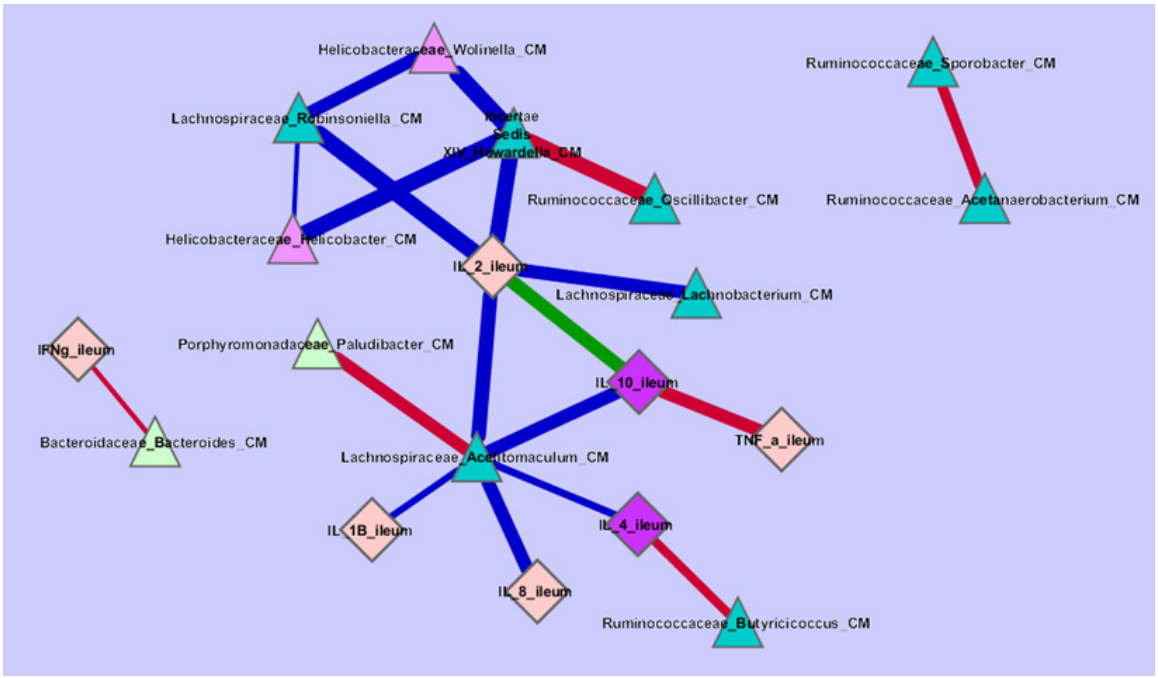

Figure 61: C_WT- P_WT Cecal Mucosa-Ileum Cytokine Differential Correlations Network

7: LIMITATIONS AND FUTURE WORK

7.1. Limitations

In the method used in chapter 3, the strong correlations (r >= 0.9) were selected/filtered first, followed by visualization in Cytoscape. When comparing different tissue types, correlations can change not only slightly, but even change their direction of correlation, from sign. This information is lost when filtering by correlation values is done first, and can result in losing significantly different metabolite-metabolite interactions if their correlations do not lie in the applied threshold. This can be overcome by visualizing all the correlations in Cytoscape, and then using the filters set in Cytoscape to select sub-networks based on their node and edge attributes.

7.2. Future work

A significant correlation or significant differential correlation results, from a set of treatment conditions, can be investigated further by experimentation or by validating from published literature. A correlation between variables does not determine causality. In a cross-sectional study where data is collected at one time point, the results can be best used for generating hypotheses to investigate the relationship. However, longitudinal studies will allow us to understand the changes in microbiome over time. The limitations for longitudinal studies include longer times, and a decreasing sample size over time.

Although, the microbiome composition is diverse at the species level, and the microbial pathways in the gut microbes is stable, the microbiome metabolic profile can

be reconfigured under different experimental conditons (Candela et al., 2012). This variable microbiome metabolic network, is a result of the functions of the microbiome genes expressed under that condition. Metatranscriptome data (of IL10 knock-out mice fed with control and probiotic diet) can help elucidate the differential metabolic networks functions under varying conditions.

An immediate area of future work is to look closely at the gut-liver-heart axis that is now emerging in current literature. Our data shows striking reversal of negative correlations in Control IL10 knock-out mice to positive correlations in Probiotic fed IL10 knock-out mice. Such a severe change in the correlation pattern indicates a marked change in the regulation of the liver system. Hence, the need to carry out multifaceted -omics approach involving liver metabolomic and transcriptomic data to understand the system level changes in the liver. If done in conjunction with gut microbiome, metabolome and transcriptome data, we can begin to gain insight into the functional correlations between systems.

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