**Format for ANSWERING REVIEWERS**

April 14, 2015.

Dear Editor,

We sincerely thank the reviewers for their critique and constructive suggestions on our manuscript. Please find enclosed the edited manuscript in Word format (file name : Revised Review Article.docx). We have also provided a point-to-point response to the reviewers' comments.

**Title:** The role of normal gut microbiota.

**Author:** Sai Manasa Jandhyala, Rupjyoti Talukdar, C Subramanyam, Harish Vuyyuru, Mitnala Sasikala, D Nageshwar Reddy.

**Name of Journal:** *World Journal of Gastroenterology*

**ESPS Manuscript NO:**

The manuscript has been improved according to the suggestions of reviewers:
1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer
First Reviewer

1. The reviewer suggested to change the title more adequately to the content:
   The title of the paper has been changed to "The role of normal gut microbiota".

2. The impact of HGC and LGC on human health, does the occurrence of any of them predispose to the frequency of particular diseases:

   The HGC individuals have a functionally much robust gut microbiome and lower prevalence of metabolic disorders and obesity. On the other hand, LGC individuals harbor a higher proportion of pro-inflammatory bacteria such as *Bacteroides* and *Ruminococcus gnavus,* both of which are known to be associated inflammatory bowel disease[20, 21]Other members of LGC bacteria include *Parabacteroides, Campylobacter, Dialister, Porphyromonas, Staphylococcus* and *Anaerostipes.* In addition, few of the key bacterial metabolites in LGC individuals include modules for β-glucuronide degradation, degradation of aromatic amino acids, and dissimilatory nitrite reduction, all of which are known to have deleterious effects.

   This has now been added to the main text.

3. Enterotypes, what are the chances of possessing a "particular" enterotype on health, how they influence the host metabolism:

   The other way of classifying the gut flora, as proposed by the MetaHIT Consortium[31], is based on species composition which cluster into well-balanced host-microbial symbiotic states that is stable over geography and gender, but can respond differently to diet and drugs. These clusters have been named enterotypes. Interestingly, the abundance of molecular functions however may not correlate with abundance of species within the enterotypes. Furthermore, as shown in a recent study on the association of gut microbiome with atherosclerosis, there may not be significant changes in the enterotype observed in disease conditions [32].There are broadly three enterotypes [29], namely:

Enterotype 1, which has a high abundance of *Bacteroides*; Enterotype 2, which has high abundance of *Prevotella*; and Enterotype 3 which has high abundance of *Ruminococcus*. The bacteria belonging to Enterotype 1 have a wide saccharolytic potential, as evidenced by the presence of genes that code for enzymes such as proteases, hexoaminidases and galactosidases. In view of these set of enzymatic potential, it appears likely that these organisms derive energy from dietary carbohydrates and proteins. Enterotype 2 behave predominantly as a degrader of the mucin glycoproteins that line the gut mucosal layer. Enterotype 3 also is associated with mucin degradation, in addition to membrane transport of sugars. The enterotypes also possess other specific metabolic functions. For instance, biotin, riboflavin, pantothenate and ascorbate synthesis are more abundantly seen in enterotype 1 while thiamine and folate synthesis are more predominant in enterotype 2. However, the concept of enterotyping does not explain the relative distribution of different classes of organisms in different individuals. Since *Bacteroides* and *Prevotella* do not exist in equal proportion in the gut, the concept of enterogradient based upon the dominance of either of these two organisms could be another defining concept. This could explain the inter-individual distribution at the class level in a better way [33].

This has now been added to the main text.

4. Effect of microbiota on metabolism and biological activity of polyphenols (Nutrient Metabolism):

Recent studies have shown that human gut microbiota is also involved in breakdown of various polyphenols (phenolic compounds) that are consumed in the diet. Polyphenolic secondary metabolites found in a variety of plants, fruits and plant derived products (tea, cocoa, wine), for example, flavanols, flavanones, flavan-3-Ols, anthocyanidins, isoflavones, flavones, tannins, lignans and chlorogenic acids. Of these, flavanoids and flavanoid sub-families are most commonly absorbed by the intestine. Polyphenols exist as glycosylated derivatives bounded with sugars such as glucose, galactose, rhamnose, ribulose, arabinopyrinose and arabinofuranose. Polyphenols, which usually remain inactive in diet are bio transformed to active compounds removal of the sugar moiety by the gut microbiota, among other factors. Structural specificity of polyphenol and individual richness of microbiota determines the level of biotransformation that occur in the intestine. The final active products are absorbed by the portal vein and travel to other tissues and organs, thereby providing antimicrobial and other metabolic action. This can be exemplified by the conversion of inactive isoflavones to the aglycon equol, which has anti-androgenic and hypolipidemic effects. [50]Table 2 shows an elaborate list of the dietary polyphenols and the gut microbiota involved in its transformation. (51-69)

This has now been added to the main text.

5. Inhibitory impact of polyphenols:

Dietary polyphenols, besides their systemic antimicrobial and metabolic functions, also play a role in the inhibition of gut bacteria. While the polyphenolic compound querectin are degraded by *Bacteroides distasonis*, *Bacteroides uniformis*, *Bacteroides ovatus*, *Enterococcus casseliflavus*, and *Eubacterium ramulus*are the bacteria that degrade this flavanol, hesperetine (a rutinoside containing aglycon) is poorly degraded by the colonic microbiota. This aglycon has an inhibitory activity against vancomycin- intermediate *Staphylococcus aureus* and *H. pylori*. [142]

This has now been added to the main text.

6. Impact of Sea weeds on food consumption and gut microbiota:

Seaweeds are the active resources with bioactive compounds and various biological activities such

as antibacterial, anti-oxidant, anti-inflammatory, anti-coagulant, anti-viral and apoptotic activity. They are rich source of fiber with nearly 50-60% of water soluble fibers, and are also rich in sulfated polysaccharides such as porphyrans, and agarases. Few species of red sea weeds like *Palmaria decipiens* and *Pterocladiella capillacea* contains sulfated polysaccharides and uronic acids (i.e., xylans and xylogalactans) respectively.[143] Several human and rat studies have demonstrated a significant shift in the gut microbiota upon the use of seaweeds as a food supplement. In humans, supplementation of *Gelidium* seaweed has significantly increased the expression of *Bifidobacterium* genera, without any change in the others. There was also an increase in the production of the SCFA's. [144] Another study conducted on Japanese populations explained the transfer of porphyranases and agarases to the gut bacteria *Bacteroides plebius* through carbohydrate active enzymes (CAZymes). [145] These studies points towards the feasibility the use of sea weeds as a potential prebiotic.

This has now been added to the main text.

7. The 'micron' unit which was represented as 'um' was corrected to "μm" in the anti-microbial section of the article.
8. All the Latin words has been corrected to italics.
9. Grammatical corrections has been corrected all over the article with the suggested changes such as:
    a. Removal of colons near the subheadings.
    b. Removal of a), b) etc.
    c. The spelling of $p^H$ has been corrected in the figure 2. *Streptococci* has been changed to *Streptococcus*
    d. The coloration of the figure 1 has been corrected.

Second Reviewer:

1. The recent advances on culture-based studies:
   Isolation, identification and enumeration of the vast majority of gastrointestinal microorganisms using conventional culture based techniques is an arduous task. Earlier, using culture based techniques, scientists were able to isolate only 10-25% of the microbiota, and this was because most of the microorganisms in the gut are anaerobic. Later, with the improvements in the anaerobic culturing techniques, dominant genera were identified as, *Bacteroides, Clostridium, Bifidobacterium* etc. The major drawback in using these techniques was difficulty in studying the culture characteristics of various colonies on a petri plate. Secondly, it is time consuming. [8, 9, 10]
   This has now been added to the main text.
2. The contradictory results of metagenomics between labs, the poor reproducibility of these studies:

   Even though NGS could provide voluminous data with fair to good accuracy, they are not free from problems. A recent study have shown that sequencing could be prone to errors that most likely results from the library preparation methods and choice of primers.[13]The other issue of concern in 16S rRNA based sequencing is the variability of results across different sequencing centers, both for predominant and minor taxa. This variation again could be result of differences in primers used to generate the amplicon libraries. [14]

   This has now been added to the main text.
3. Strong evidence for the alterations of gut microbiota to various disorders:

Significant interest have evolved on the gut microbiota in the recent years within the scientific community; and the gut microbiota have been associated with a large array of human diseases ranging from luminal diseases such as inflammatory bowel diseases (IBD) [2] and irritable bowel syndrome (IBS)[3], metabolic diseases such obesity and diabetes [4], allergic disease [5]to neurodevelopmental illnesses, though the strength of evidence is not robust with many of them.

This has now been added to the main text.

4. *Bacteroides* a genus and Firmicutes as a phylum have been corrected all over the review paper.
5. Changes have been made with the taxonomic classification: phylum *Proteobacteria (Campylobacter jejuni, Salmonella enterica, Vibrio cholera and Escherichia coli), and* phylum *Bacteroidetes (Bacteroides fragilis)*
6. *Eschereichia* has been changed to *Escherichia.* Similarly, Rosuburia has been changed to *Roseburia*
7. Scheme of the Gut microbiota has been included in the form of a figure.
8. For the accuracy of various NGS platforms in the Table 1, we have included the reference. Table 1 presents the accuracy, advantages and disadvantages of the currently available sequencing techniques [15, 16]

Third Reviewer:

1. Section on the immune system should be expanded to include the roles of the different cell:

The gut microbiota contribute to gut immunomodulation in tandem with both the innate and adaptive immune systems. The components and the cell types from the immune system that participate in the immunomodulatory process includes the gut associated lymphoid tissues (GALT), effector and regulatory T cells, IgA producing B (plasma) cells, Group 3 innate lymphoid cells, and, resident macrophages and dendritic cells in the lamina propria.

The role of gut microbiota in shaping a normal GALT is implied by the impaired development of the Peyer's patches and isolated lymphoid follicles that are marked by the abundance of $IgE^+$ B cells instead of the normally seen $IgA^+$ B cells [91].The effector T cell responses in the intestine have also been shown to be primarily controlled by Th2 responses as opposed to the Th1 responses [92]. The latter is primarily mediated by Th1 and Th17 cells under a physiological milieu; and gut commensals are believed to result in TLR-MyD88 signaling mediated activation of IL1β which in turn promote development of IL17[93].

Intestinal microbiota is also essential for the normal development and function of $Foxp3^+$ T regulatory (Treg) cells. However, the mechanism by which this is mediated is still not clear. For example, in the case of certain *Clostridium* clusters it could be either independent of PRRs or dependent on My-D88 dependent mechanisms [94]. In the case of *Bacillus fragilis,* induction of Tregs appear to be mediated by TLR2 signaling by polysaccharide A [95].SCFAs, especially butyrate have also been implicated in the development and function of Tregs. SCFAs are shown to activate G-protein coupled receptors expressed by the IECs and regulate Treg by epigenetic regulation (increased acetylation) of the *Foxp3*locus [96, 97, 98].

As mentioned in the previous section, mucosal plasma cells produce secretory IgA upon induction by DCs. Though the mechanisms are not clear, it is speculated that this function is mediated by My-D88 signaling in lamina propria and follicular DCs. My-D88 signaling can be activated by the gut microbiota. Furthermore, in addition to class switching of sIgA by APRIL mediated stimulation, the gut

microbiota also stimulate DCs in the payer's patches to secrete TGF-β, CXCL13, and B-cell activating protein (BAFF), which leads to IgA production and class switching[99].

Another set of innate immune cells, namely the innate lymphoid cells (ILCs) are capable of responding rapidly to epithelium-derived cytokine signals [100]. ILCs arise from common lymphoid precursors and have a cytokine expression pattern that is similar to that of T helper subsets (particularly Th17 cells); but the differentiation is more dependent on microbial composition rather than somatic recombination [101]. Based on the functional properties, ILCs can be divided into three groups, namely, group 1 (T box expressed in T cells [T-bet] $^{+}$), Group 2 (Gata binding protein 3 [GATA-3] $^{+}$], and group 3 (retinoid-related orphan receptor gamma t [RORγt] $^{+}$). Of these, RORγt$^{+}$ ILCs appear to be most closely associated with regulation of gut immunity [102].Even though the precise mechanisms are unclear, it is speculated that gut microbes could regulate ILCs both directly and indirectly. Evidence in favor of the former is provided by the observation that the bacterial metabolite indole-3-aldehyde stimulates ILC via the aryl hydrocarbon receptor to induce synthesis of IL22 [103].Indirect mechanism of ILC regulation, on the other hand, is via the recruitment of other immune cells such as the $CX_3CR1^{+}$ intestinal macrophages[104].

The immunomodulatory action of resident macrophages in the lamina propria is to express pro-IL1β in the steady state, which aids in the rapid production of mature IL1β in response to pathogen invasion. MyD-88 dependent mechanisms induced by commensal flora is essential for this action; while the microbiota regulated IL-10 production by the macrophages entail MyD-88 independent mechanisms[105, 106].

Apart from the gut microbiota, other factors also play a role in modulation of the gut immune system. For example, the IECs secrete an isoform of alkaline phosphatase (intestinal alkaline phosphatase) that dephosphorylates the LPS endotoxin [107]. Another example is the reduced neutrophil recruitment into the intestinal lumen in response to tumour necrosis factor-α (TNF-α). This action is mediated by the intestinal alkaline phosphatase [107]. Furthermore, an immunoprotective mechanism that is acquired at birth and is seen predominantly with vaginal delivery is the down regulation of IL-1 receptor-associated kinase (IRAK-1), which acts through TLR4 [108].

This has now been added to the main text. We have also added a new figure depicting the immune mechanisms.

2. How taking probiotics helps regulate the gut flora:

The World Health Organization defines probiotics as live microorganisms that can provide benefits to human health when administered in adequate amounts, which confer a beneficial health effect on the host. Several species such as *Lactobacillus casei, Lactobacillus planatarum, Lactobacillus bulgaricus, Lactobacillus acidophilus, Bifidobacterium longum, Bifidobacterium infantis, Streptococcus thermophilus, Escherichia coli* strain Nissle 1917, to name a few have been shown to impart immunomodulatory and gut barrier functions. These and several others have been used commercially in the management of human illnesses for e.g. IBD, antibiotic associated diarrhea. The fundamental concept of using these organisms in the treatment armamentarium is mimicking the physiological health promoting functions of the 'good' bacteria. Addition of a prebiotic could possibly augment the effect of the probiotics. Prebiotics are defined as food ingredients that contain non-digestible oligosaccharides (e.g. Galacto oligosaccharides and inulin); and a probiotic and prebiotic are together called a symbiotic. The gut bacteria selectively ferment these fibers resulting in the synthesis of SCFAs, which in turn imparts the pro-health effects (*vide supra)*. A detailed discussion on pro- and prebiotics is out of the scope of this review since it deals predominantly on a normal gut microbiota.

Nevertheless, we believe that even though dietary fibers and healthy gut microbiota are known to promote health, use of synbiotics for maintenance of health needs to be studied with much robustness before using them commercially as health promoters. [157, 158]

This has now been added to the main text.

Fourth Reviewer:

1. The impact of preterm birth on the microbiota:

   In pre-term infants, bacteria that colonize the gut include *Bifidobacterium* and *Lactobacillus* and basically, these differ depending on the type of feeding habits. In formula-fed infants, *Enterococci* and *Enterobacteria*, *Bacteroides*, *Clostridia*, and other anaerobic *Streptococci* dominates the gut niche. Whereas, in breast-fed infants, *Bifidobacterium* and *Lactobacillus* dominates. Human milk oligosaccharides (HMO's) in breast milk are easily broken down by these bacteria. Pre-term microbiota are said to maintain the Gut associated lymphoid tissue (GALT), and is involved in generating the innate immunity during development. Therefore, abnormal colonization of the gut microbiota may result in pediatric diseases because of poor immunity. [129, 130]

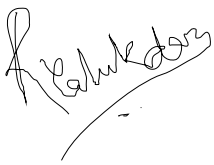
   This has now been added to the main text.

3. The temporal alteration is affected by dietary patterns, lifestyle, life events, and environmental factors including antibiotic use.' would be better referenced: Respective reference has been added in the text.

4. References and typesetting were corrected

We are indeed, thankful to the *World Journal of Gastroenterology* for providing us value reviewers.

Sincerely yours,



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