Impact of Mannan-Oligosaccharide on Gut Microbial Diversity and Firmicutes/Bacteroidetes Ratio in BALB/c Mice

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ABSTRACT

Gut microbiota is responsible for causing imbalance and metabolic disorder of gut. Mannan-oligosaccharide (MOS) has been used as a potential Prebiotic. A seven day study was performed to detect the impact of MOS on BALB/c mice and to determine changes in gut microbiota and there microbial populations. Mice were administered with MOS (oral, 200 mg/kg b.w.) and vehicle were given to control mice. Animal study identified that MOS significantly decreased populations of faecal microbe colonies of treated mice as compared to control mice. Changes in microbe colonies were also observed in respect of their number, size, shape, colour, opacity, etc. This suggests MOS has capability to improve the gut microbiome by altering Bifidobacterium and Lactobacillus microbes in the colon. These microscopic organisms are known as valuable microorganisms, which are required to improve gut microbiota and gut health. Specifically, the admission of MOS additionally decreased the Firmicutes/Bacteroidetes extent, which are known to cause imbalance of gut microbiota. That is the reason why MOS is useful for controlling gut microbiota and improving health of a host. Along these lines, MOS can be used as a prebiotic agent for regulating the gut microbiota and treating overweight, inflammation and metabolic issue.

Keywords: MOS, Prebiotic, Microbiota, Fimbria, Faecal

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1. Introduction

Gut microbiota is also called gut flora that contains microorganisms that live in the stomach related tracts of animals and humans. The gut microbiota contains many trillions of microorganisms. The gut microbiota get altered because of different environmental factors, for example, pathogenic attack, and so forth. Gut microbiota is liable for keeping up host's

digestion and gut health. Numerous evidences revealed that irregularity of gut microbiota is associated with dysbiosis and metabolic disorders, etc. [1, 2]. Gut microbiota is also responsible for improvement of various ailment including metabolic illness, malignancy and immune responses [3]. Because of these ailments gut microbiota was used as an objective for treating issue related with metabolic disorder, and so on. It was found that gut microbiota of mice get imbalance due to increase in ratio of Firmicutes/ Bacteroidetes bacteria in the gut which causes inflammation or disease in gut [4]. According to research, if a diet is modified with a supplement it can help to improve the gut microbial composition and activity, which affects the host well-being [5, 6]. In many research it was identified that prebiotics are responsible for managing gut microbiota and improving the health of an individual [7, 8].

Prebiotics is "a non absorbable carbohydrates compound that act by using microorganisms in the gut, enhance activity of the gut microbiota, and provide useful physiological effect on the host". Many types of these non-absorbable carbohydrates compounds are available which are known to modulate the gut activity but oligosaccharides compounds has most beneficial effects on regulating gut microbiota. It has been recognized that oligosaccharides and dietary fiber can improve the health of the gut [9, 10]. Similarly, with the ability to tie and limit the colonization of gut pathogens, prebiotics has been shown to be an effective option of antibiotics free diets [11].

Mannan oligosaccharide derived from cell wall of yeast, Saccharomyces cerevisiae. MOS was found to inhibit colonization of enteric pathogens, for instance, Salmonella, E.coli, Campylobacter, etc. which can modify the composition and function of the gut microbiota [12-14]. MOS work by attaching pathogenic microscopic microbes to type1 fimbriae and prevent their adherence to the intestinal mucosa and decreased pathogen causing inflammation [15]. Due to these pathogenic decolonization activity of MOS it can use in animal products as a supplementation to improve well-being of gut [16, 17].

In the intestinal tract MOS enhances the growth of helpful microorganisms, for example, Lactobacillus spp., Bifidobacterium spp. which improves gut health and decreases the harmful microorganisms likes Firmicutes, Bacteriodetes which cause inflammation or disease in gut [18, 19]. As a result of these points of interest, MOS used in animal feed to improve gastrointestinal health and well-being [20]. It was found in many research that gut microbiota is modulated by prebiotics which helps to prevent dysbiosis, body weight changes of an individual [21, 22].

These information’s are not fully complete to use MOS as a prebiotic. That why more investigations are need to be done to confirm the therapeutic effect of MOS. Consequently, we inspected the effects of MOS on gut microbiota utilizing BALB/c mice. The events of faecal weight changes, changes in faecal microorganism’s populations with and without MOS treatment were observed. These investigations were means to decide the medicating effect of MOS, and to use MOS as a prebiotic to treat gut microbiota and metabolic related disorders.

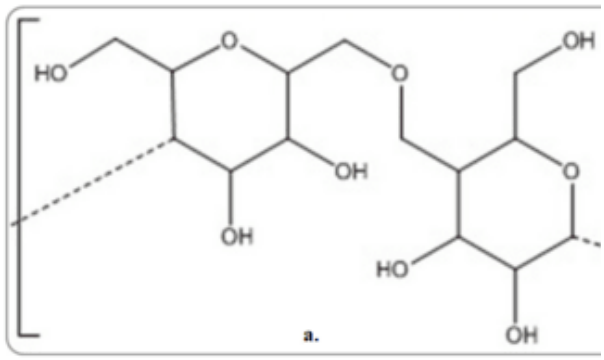


Fig. 1 (a) Chemical structure of Mannan-oligosaccharides **(b)** Mechanism of action of MOS [23]

2. Materials and Methods

2.1 Materials used and Experimental Design

MOS Standard (molecular weight 666.6 g/mol) was provided from Sigma-Aldrich and MOS API (Active Pharmaceutical Ingredient) was synthesized in INMAS-DRDO, Delhi, India. All other required reagents was obtained and prepared using deionised water in research lab. Mice were house in the animal facility department under standard conditions in INMAS-DRDO. Mice were randomly divided into two treatment group's one male group and one female group (Three mice per group). In our experimental study, from both group total four mice was fed with MOS API orally (200 mg/ kg body weight) for 2 days and there effects was observed for 7 days. The remaining mice were used as control. For all groups, the administration volume of the gavage was 0.08 ml per 48 gm body weight.

During the examination changes in weight of faecal material, body weight and faecal microbe's colonies was observed and compared with control. All animals procedures and care protocols were followed according to the rules of Institutional Animal Ethics Committee.

2.2 In Vitro Study

Carbohydrates test

a. Benedict test

This in-vitro test was used for examination of carbohydrates in Mannan oligosaccharide API powder. In Benedict test a blend of copper (II) sulfate, sodium citrate, and sodium carbonate was utilized for detection of diminishing sugars. In this test Mannan oligosaccharide API powder (1mg) was blended with (1ml) of Milli Q and afterward include (2 ml) of benedict reagent in a test tube and warmth it for 5 minutes in heating mantle. After that observe the colour change in solution of test tube the colour will change from blue to red precipitate [24].

b. Barfoed's test

In this test drug Mannan oligosaccharides API powder was used to recognize monosaccharaides from diminishing disaccharides. Barfoed's test reaction involves drug sample (1 mg) was dissolved with (1 ml) of Barfoed reagent in a sample tube and warmth it for 5 minutes in heating mantle. Loom for the development of colour change to brick red precipitate [24].

c. Iodine test

This test was utilized to identify Polysaccharides in drug molecule. A test sample of MOS API powder (1mg) was blended with 2-3 droplets of iodine reagent

in sample tube and observes any shading changes [24].

Fat and Fixed oils test

a. Ethanol emulsion test

This process was utilized to identify fats and fixed oils in MOS API. In this test (1 ml) sample solution was prepared and mixed with ethanol. Then Shake the solution slowly and add deionized water in small amount. If the colour of solution changes and suspension occur it confirms the positive test.

2.3 In Vivo Study

a. Zone of Inhibition

Zone of Inhibition is a test which is used to detect an area around which there is no growth of microorganisms. Zone of Inhibition was determined by streaking faecal microbes on Nutrient agar plates and then inducing drugs by making wells. Another strategy for detection of Zone of Inhibition is by spreading medications through sterile spreader on N. agar plates and afterward streak faecal organisms. Both methods show its clinical advantage to decide antibiotics action by inhibiting growth of microorganism and to determine antibiotic resistance. This Zone of Inhibition can be observe and estimated easily outwardly in N. agar plates [25].

b. Gut microbiota analyses

Gut microbiota analyses was done by administrating MOS API drug oral to selected mice as per groups. During the 7 days study, MOS was given to four mice for two days in a gap of three days and two mice used as control. Before and after administration of drug mice faecal samples was collected every day of study. There faecal microbes was streak on Nutrient agar plates through streak plate method on same day. Identification and comparison was done by observing microbes colonies changes on N. agar plates from day 1 of study to 7 day of study. The gut microbiota investigation and analyses was finished by streaking faecal microbes of treated and untreated mice with MOS on Nutrient agar plates.

c. Gram Staining

Gram Staining is use to recognize the specific microbes and their overall structure of cells (e.g., cocci, rods, spirals, filaments, etc.). Gram staining test was done by taking 300 ul of PBS (Phosphate buffer solution) in eppandorf tubes. After that add bacterial colonies with nichrome loop in these eppandorf tubes and vortex them totally. After forming of solution, take a loopful of microscopic microbes in clean slide and make a smear of it. This smear is then stains first with crystal violet and allows it to stand for 2 minutes. After that make the slide tilt and flush the slide with distilled water using a wash bottle. Then tenderly flood the slide with Gram's iodine and let it stand at that point for 2 minutes then flush the smear using wash bottle, the smear will show up as a purple shading circle on slide. Decolorize the slide by decolourizing agent and tilt the slide to apply liquor drop by drop for up to 10 seconds so as not to decolorize and promptly flush with water. Rinse the slide with safranin to counterstain and let stand for 1 minute. Tilt the slide somewhat and delicately flush using wash bottle. Then airs dry the slide and view under microscope 100x for best result [26].

d. Motility test

The motility test of microscopic organisms was used for differentiation and classification of bacteria. Bacteria are characterized into two types motile and non-motile. A soft agar stabbing (Tube Method) was performed to distinguish motility of microbes which was isolated from specific faecal microbe colonies of treated and untreated mice with MOS. Arranged 6 test tubes of N. agar media by melting agar in microwave oven for 1-2 minutes. Sterilised nichrome wire was used for stabbing, a loop of microbes was used to stab 2/3 way down to bottom of test tube and then incubate these tubes in incubator shaker for growth. Analyse these test tubes in next day for result. The line of inoculation show a well dispersed growth if the microbes are motile and no well dispersed growth if the microbes are non-motile [27].

2.4 Ultraviolet-visible spectroscopy

The UV Spectrophotometry of two drugs MOS (Standard) and MOS API (Active pharmaceutical ingredient) was performed using NanoPhotometer. To perform UV Spectrophotometry, we make MOS Standard (1mg/ml) solution and take 4 samples from it which are 25ul, 50ul, 100ul, 200ul and dilute it to make 1 ml. Then check the absorbance of the entire 4 sample using a cuvette. Follow the same procedure for the absorbance of another drug [28].

2.5 Fourier transform infrared spectroscopy

FTIR of two drug MOS (Standard) and MOS API (Active pharmaceutical ingredient) was done by taking 1 mg of powder. FTIR was performed on Perkin Elmer Spectrum Two Spectrophotometer to determine molecular composition and structure of a test compound and standard [29].

2.6 Statistical analyses

The statistical comparisons and differences considered to be statistically significant at p 0.05. Mean values and standard deviation for each parameter were evaluated by using Microsoft excel software 2019 version.

3. Result

3.1 In vitro test

These in vitro tests were done to investigate sugars, fats in MOS API powder. In Benedict test the colour changes was seen from blue to green-red precipitate shows the nearness of 0.1-0.5% sugars. In Barfoed test the nonattendance of red colour or no colour change was seen which implies monosaccharaides are absent. In Ethanol Emulsion test the absence of no colour change confirms the nonappearance of fats and oils. In Iodine test no change in colour of solution shows that polysaccharides are absent.

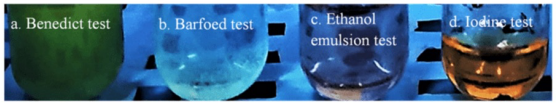


Fig. 1 (a) Positive Benedict test **(b)** Negative Barfoed test **(c)** Negative Ethanol Emulsion test **(d)** Negative Iodine test

3.2 Determination of Zone of Inhibition

The Zone of Inhibition was observed by streak faecal microbes of mice on Nutrient

agar plates. The Spots around wells confirming antimicrobial action in which MOS drugs (Standard which is denoted by S, API denoted by I, Pure denoted by P) were induced in wells. Through pour plate technique Zone of Inhibition was also determined, by spreading these three drugs through sterile spreader and afterward streak faecal microbes on N. agar plates. These treated plates showing less microbial colonies when compared with control. It was observed that the both techniques restraining development of microorganism but MOS API drug show better inhibiting result as compare to Standard and Pure.

Fig. 2 Arrows showing Zone of Inhibition of MOS API which is bigger than others **(a)** Streak plate method **(b)** Pour-plate method **(c)** Streak plate and Pour-plate method

3.3 Effect of MOS on gut microbiota of mice

It was identified that health of mice was improved after treatment to MOS. By examination of 7 day study of N. agar plates streak faecal microbes of treated and control mice. It was seen that significant changes in faecal microbes colonies concerning size, number, shape, opacity, colour was observed and also reduction in populations of pathogenic microbial colonies, weight of faecal material and weight of mice was seen with MOS treatment as compared to control in **figure 4.**

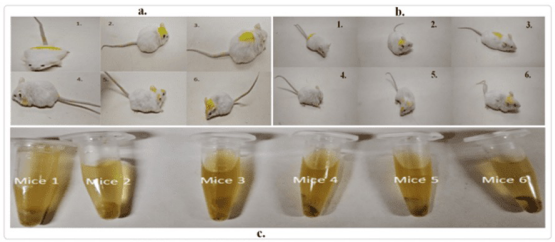


Fig. 3 (a) BALB/c mice male & female before drug treatment **(b)** Mice after drug treatment

(c) Faecal microbes for streaking

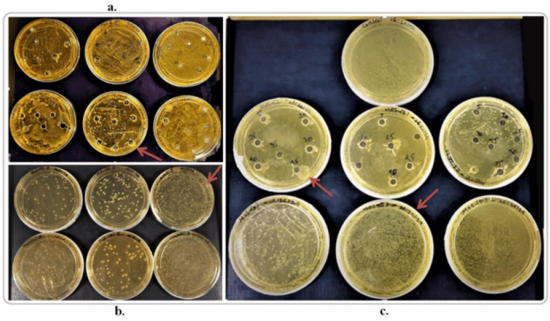
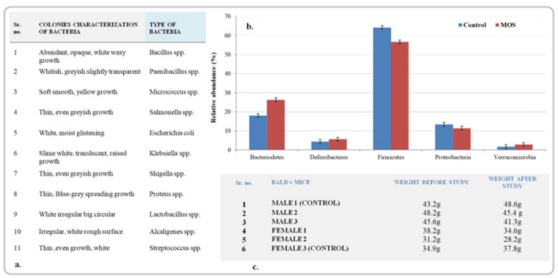


Fig. 4 Animal Study **(d, f)** Streak plate without drug

(e, g) Streak plate with drug **(h), (i), (j)** Streak plate without drug

3.4 Gut microbiota modulated by MOS



The relative abundance of gut microbiota of mice was seen in the beneath graphical record. It was identified that MOS decreased the plenitude of bad bacteria like Firmicutes, Proteobacteria and increment the abundance of good bacteria like Bacteriodetes, Deferribacteres, and Verrucomicrobia for keeping up healthy gut. These beneficial effects were observed on gut microbiota of mice after treatment to MOS orally.

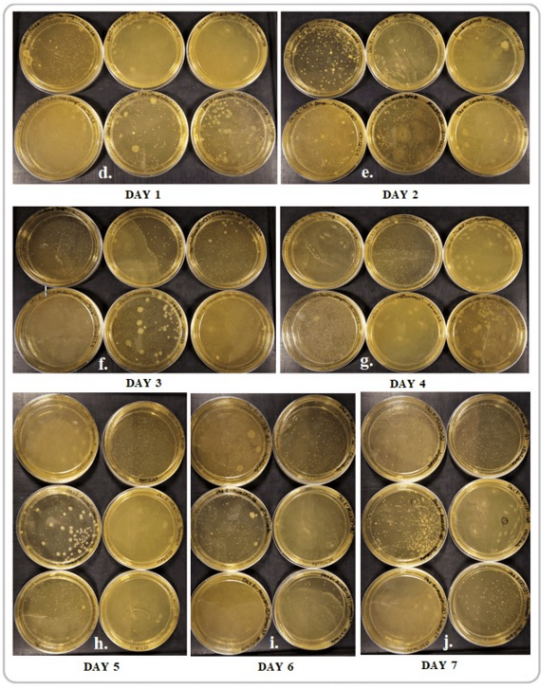


Fig. 5 (a) Bacteria identification by observing colonies **(b)** Graphical representation of relative abundance of bacteria in gut microbiota of mice with MOS treated and control **(c)** Mice weight before and after study

3.5 Gram Staining

The Gram Staining was done to distinguish different types of bacteria. The isolated microbes of faecal material of mice which was used for gram staining showing both gram positive and gram negative bacteria. The Purplish shading appeared on slide **(a), (c)** Gram positive microorganisms and pink shading on slide **(b)** Gram negative microorganisms. The colour on slide **(d)** showing both Gram positive and Gram negative microorganisms. Subsequently, Gram staining help to understand the well-being status of gut microbiota through identification of different types of bacteria.

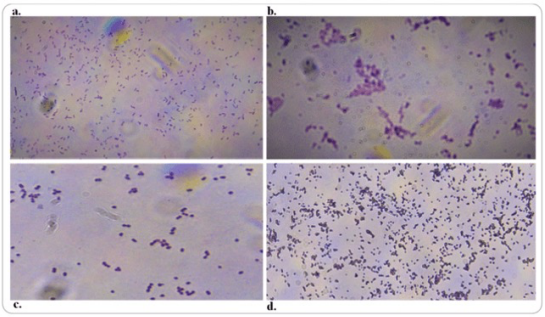


Fig. 6 (a) Gram positive bacteria **(b)** Gram negative bacteria **(c)** Gram positive bacteria **(d)** Gram positive and negative bacteria

3.6 Motility test

In motility test it was identified that all the faecal microorganisms of treated mice are non-motile. This test was use for examination of motile and non-motile microbes and distinguishes the various microscopic microbes by their motility. In this manner, it was found that all the non motile microscopic microbes present are valuable for healthy gut [26].

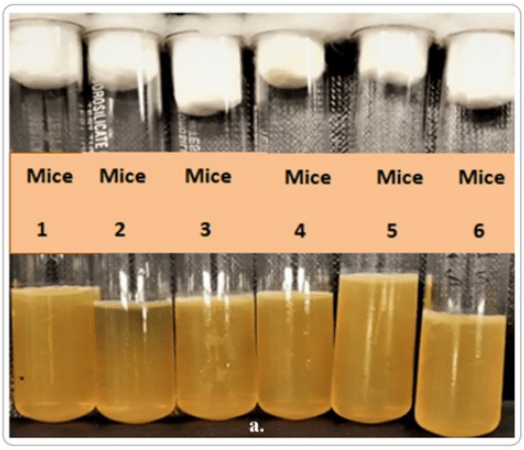


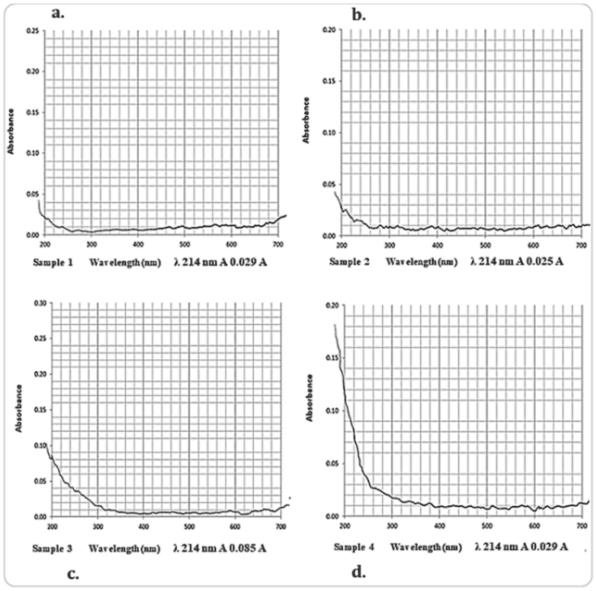
Fig. 7 Gut microbiota treated by MOS show no growth and line of inoculation identifying that bacteria are non-motile **(a)** Motility test

3.7 Ultraviolet Visible Spectrophotometry

The UV spectrophotometry of MOS (Standard) powder & (API) powder was

recorded on NanoPhotometer. 1 mg ml^{-1} powder of both drugs was used to make 4 dilutions of each test sample for absorbance. From the figure below the absorbance of sample 1 MOS (Standard) was found to be 0.029 at 214 nm and MOS (API) 0.037 at 218nm. Absorbance of sample 2 (Standard) 0.025 and 0.046 (API) at 214 nm. Absorbance of sample 3 (Standard) 0.085 and 0.080 (API) at 214 nm. Absorbance of sample 4 (Standard) 0.029 and 0.097 (API) at 214nm. Out of all the UV spectra the sample 1 and sample 3 spectra of MOS Standard and API are indicating same outcome. It was determined that MOS API has the same property as that compared to MOS Standard.

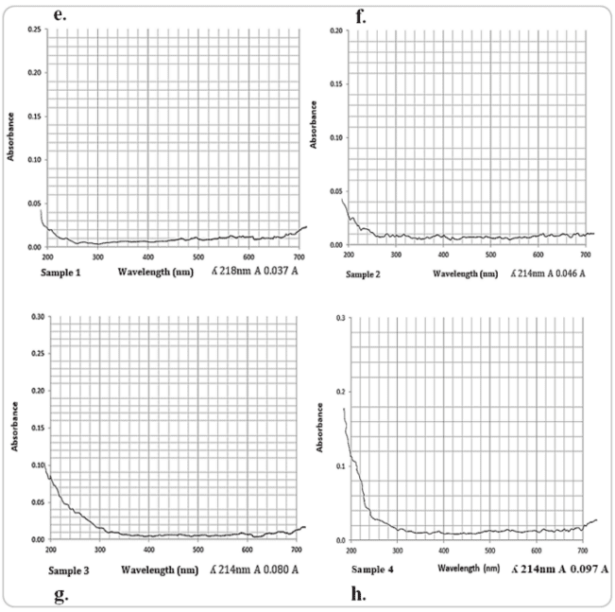
Fig. 8 UV Spectrophotometry of Mannan



oligosaccharides (Standard) and (API) drug matched with each other but API somewhat shows better absorbance than Standard **(a-d)** UV Spectrophotometry of **MOS Standard (e-h)** UV Spectrophotometry of **MOS API**

3.8 Fourier Transform Spectroscopy

Attenuated total reflectance – Fourier transform infrared spectroscopy of MOS (Standard and API) was recorded on Perkin Elmer spectrum two spectrophotometer at 4000-500cm^{-1}. **From figure 9** it was determined that all the major peaks of Standard powder sample of MOS matched with API powder. The characteristic peaks of Standard MOS **(a)** was found at 3318.18cm^{-1} while in API **(b)** it was 3317.29cm^{-1} that indicated the presence of Weak N-H (2°- amines) stretching vibration. Medium CH_2-CH_3 deformation bending vibration was observed in Standard sample of MOS as well as in API at 1363.41cm^{-1} and 1372.96cm^{-1}. Medium C-N stretching vibration of MOS API at 1018.06 cm^{-1} also matched with Standard



sample at a range of 1021.17 cm^{-1} respectively.

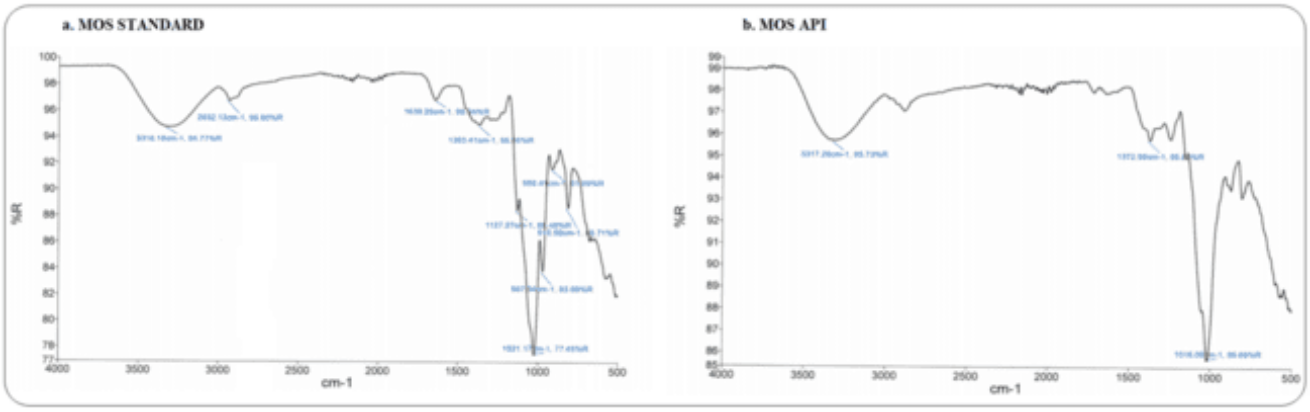


Fig. 9 Fourier transform infrared spectra of Mannan oligosaccharides **(a) Standard and (b) API**

4. Discussion

The present investigations demonstrated that dietary supplementation of MOS balanced the gut microbiota of mice. Considerably, as a prebiotic, MOS keep up the strength of gut by modifying microbial metabolites. Unpalatable sugars including inulin, galactooligosaccharide, xylooligosaccharide, and soybean oligosaccharides, have been found to altered gut microbiome and microbial metabolites [30, 31]. Past examinations have also shown that dietary MOS regulated gut microbiota when administered in other species such as chickens, [32, 33] juvenile rainbow trout, [34] or turkeys [35]. It was shown by various examinations that the gut microbiota imbalance play an important role in creating dysbiosis, stoutness and metabolic disorders [36].

This is the reason, to understand the effect of MOS on gut microbiota by in vitro, in vivo examination and further analysing there beneficial effect. It is known that MOS is one of the incredible prebiotics, has been commonly utilized as an anti-oxidant, anti-inflammatory, etc. MOS works by restricting the string like fimbriae on pathogenic microbes, getting them away from joining to the intestinal mucosa and promoting there inhibition of colonization in the gut. MOS redesigned gut microbiota by upgrading SCFAs (Short chain fatty acids) generation and increase the production of Bifidobacterium and Lactobacillus in gut microbiota which are helpful in maintaining gut health. In our investigation, MOS was supplemented orally to selected mice to survey the gut microbiota changes. The investigation of gut microbiota was done by observing outwardly on faecal streak Nutrient agar plates and understanding the changes in plates dependent on faecal microbes colonies populations.

A Significant changes in gut and faecal microbes colonies of mice after treatment to MOS were observed which may help in preventing obesity, dysbiosis and metabolic related disorder. Bacteroidetes and Firmicutes are the two important bacterial divisions in the gut, and numerous investigations exhibited that accumulation of Firmicutes expanded while Bacteroidetes diminished in obesity. Our investigation demonstrated that there were

generally less Firmicutes and more Bacteroidetes, Actinobacteria, Verrucomicrobia and Deferribacteres ratio in the gut of treated mice contrasted with that of control mice as per **figure 5 (b).**

In addition, mice treated with MOS show a reduction in faecal pathogenic microbes colonies populations, faecal material and body weight as compared to control mice. These outcomes demonstrated that intake of MOS act by selectively enhancing the populations of several beneficial species and diminishing species related to dysbiosis, obesity and metabolic disorder, which is helpful in contributing the health promoting effects of MOS. These results also indicated that MOS can be used as Prebiotic to regulate the gut microbiota and to maintain gut health.

5. Summary

In summary, the admission of MOS improve the faecal populations of valuable bacteria and diminished the faecal populations of pathogenic bacteria, also diminishes colonization of pathogenic faecal microbes, weight of faecal material, body weight, in treated BALB/c mice. Considering that MOS could forestall the gut dysbiosis which is a key to prevent obesity and metabolic syndrome. Our examination provides vital information regarding the therapeutic efficacy of MOS and confirms that dietary MOS can be applied as an option to antibiotics to treat gut health and metabolic related issues.

Conflict of Interest

There are no conflicts of interest to declare.

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