

## Interaction of Mannan oligosaccharide with Immune System "Transport of MOS in to the Lamina Propria"

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### Summary

Mannan oligosaccharide (MOS) is a complex that is derived from the cell wall of the yeast *Saccharomyces cerevisiae*. This complex carbohydrate product has been utilized around the world to improve the productivity and wellbeing of poultry, fish and livestock. Questions related to the specific interaction between MOS and the immune cells still remain unclear. The objectives of this study are to investigate if MOS passes through the intestinal epithelium and if it is translocated to the lamina propria of the small intestine. In order to understand the fate of MOS in the gastrointestinal tract and its interaction with the immune related cells, this study compares the translocation of Albumin, the negative control which is known not to be quickly digested and not translocated; that of Dextran, the positive control which is known to be phagocytosed by dendritic cells and that MOS, the experimental group. Pure mannan was obtained from a mannan rich fraction by reacting with 7-methoxycoumarin-3-isocyanate in dimethylsulphoxide. The labeled product was isolated by ethanol precipitation. The MOS was labeled with a fluorescent tag. In this study sixteen one-day old broiler chicks (Cobb x Cobb) were used. They were kept in brooder batteries with four chicks per pen. Each group (n=4) was assigned to a different fluorescent-labeled diet. The control group got the basal diet without fluorescent-tagged molecules in order to determine background levels of fluorescence. The ratio of fluorescent labeled MOS, albumin and dextran to the basic diet was 20 mg/kg. The experiment lasted three weeks. At the end of the study chickens were terminated with carbon dioxide. The removed intestinal segments were preserved in 10% formalin and fixed on the slides using the paraffin method. From each segment, 72 glass slides were prepared. Images captured by fluorescent microscopy were used to determine the extent of translocation of MOS into the lamina propria. The data was analyzed by ANOVA. P value <0.05 was considered to be significant. Foci of fluorescence from albumin were not detectable. The albumin was degraded prior to entrance into the lamina propria as expected in the negative control group. Thus it was not included in the statistical analysis. Comparatively, dextran, the positive control group was transported into the lamina propria, most significantly in the ileum. MOS, the experimental group was transported into the lamina propria. In the duodenum and jejunum, our results indicated that larger amounts of MOS were as transported into lamina propria as compared to dextran. In conclusion MOS does not interact specifically with the epithelial cells but it makes its way to the gut associated lymphoid tissue (GALT) of the lamina propria via an independent method, which appears to be mediated by dendritic cells as an immune surveillance mechanism that is vital in the mucosal immunity. MOS has likely a general adjuvant effect on immune system without causing "danger signals" that are inherent in pathogen. Further studies are needed to identify the mechanism of this interaction especially with M-Cells, which are specialized epithelial cells and play a key role in stimulating the immune system.

**Keywords:** Immune system, Lamina propria, Mannan oligosaccharide

## Mannan oligosakkaridin İmmün Sistemle Etkileşimi "MOS'un Lamina Propria'ya Geçiş Mekanizması"

### Özet

Mannan oligosakkarid (MOS), *Saccharomyces cerevisiae* mayasının hücre duvarından elde edilen kompleks bir karbonhidrattır. Bu karbonhidrat Dünyada kümes ve besi hayvanları ile balıklarda verimlilik artışı ve sağlığın korunması amacıyla kullanılmaktadır. MOS'un bağışıklık sistemiyle olan etkileşimi ve mekanizması henüz tam olarak anlaşılmamıştır. Bu çalışmamızda MOS'un intestinal epitelyumdan geçiş geçmediğini ve ince bağırsakta lamina propria'ya girip girmediğini incelemek amaçlanmıştır. MOS'un gastrointestinal kanalda akıbetini araştırmak ve bağışıklık sistemi hücreleriyle olan etkileşimini anlayabilmek için albumin, dekstran ve MOS kullanılmıştır. Albumin negatif kontrol grubu olup, kolayca sindirilemez ve lamina propria'ya nakledilmez. Dekstran pozitif kontrol grubudur. Dendritik hücreler tarafından fagozitoya uğramaktadır. MOS ise deneysel grup için kullanılmıştır. Saf mannan, mannanca zengin bir çözültiden 7-methoxycoumarin-3-isocyanate in dimethylsulphoxide ile reaksiyona girerek ve etanol çözültisi ile çöktürülmesi sonucu elde edilmiştir. Elde edilen MOS çözültisi floresans ile işaretlenmiştir. Çalışmada 16 adet 1 günlük piliçler kullanılmıştır. Her kafeste 4 piliç olmak üzere toplam 4 grup oluşturulmuştur. Her grup farklı diyetlerle beslenmiştir. Kontrol grubuna floresans işaretleme olmayan bazal diyet verilmiştir. Floresans işaretli MOS, albumin ve dekstranın bazal diyete göre rasyonu 20 mg/kg'dır. Araştırma üç hafta sürmüştür. Üçüncü haftanın sonunda piliçler karbondioksit ile sonlandırılmış; çıkarılan intestinal doku örnekleri %10'luk formalin çözültisinde korunmuştur. Örneklerden parafin metoduyla tespit edilmiştir. Her örnekten 72 adet kesit hazırlanmıştır. MOS'un lamina propriaya geçişini tespit edebilmek amacıyla floresans mikroskopisi kullanılmıştır. Elde edilen veriler ANOVA ile analiz edilmiştir. P<0.05 değeri anlamlı kabul edilmiştir. Negatif kontrol grubu olarak albumin lamina propriada tespit edilmiştir. Albuminin lamina propria girişinden önce degrede olduğu düşünülmektedir. Bu nedenele istatistiksel hesaplamaya dahil edilmemiştir. Pozitif kontrol grubu olan dekstranın lamina propriaya özellikle ileuma geçiş yaptığı görülmüştür. Deney grubu olan MOS'a lamina propriada rastlanmıştır. Duodenum ve jejenumda dekstran ile karşılaştırıldığında MOS'un daha fazla miktarda nakledildiği bulunmuştur. Sonuç olarak, MOS epitel hücreleriyle bir şekilde etkileşime girmektedir. Ancak, lamina proprianın GALT'ına (gut associated lymphoid tissue) henüz anlaşılmayan bir mekanizmayla geçiş yaptığı görülmüştür. MOS, muhtemelen bir adjuvan gibi çalışarak bağışıklık sistemi tarafından patojen olarak algılanmamış ve bu sonuçtan hareketle bağışıklık sistemini harekete geçirmede olumlu yönde etki gösterdiğini söylenebilir. Ancak, çalışmalar daha ileri götürülerek M-cells ile etkileşimi araştırılmalıdır.

**Anahtar sözcükler:** Bağışıklık sistemi, Lamina propria, Mannan oligosakkarid



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## INTRODUCTION

Nutrition has a significant role in the human and animal health by impacting the immune system. Today prebiotics have been widely used to enhance the immune system and health in the human life. Prebiotics are nondigestible food ingredients that beneficially affect the animal host by selectively stimulating the growth of certain bacteria which are advantageous to the host by serving as selective substrates for so-called probiotic bacteria<sup>1</sup>. Physical and chemical aspects of the diet can modify the populations of microorganisms in the gastrointestinal tract, the capacity of pathogens to attach to enterocytes, and the integrity of the intestinal epithelium<sup>2</sup>. In recent years, there has been increasing biotechnological and commercial interest in yeast cell wall components, including their use as biological response modifiers, anti-cancer agents, bioadsorbents, ingredients in food processing and cosmetic formulations, and as systems for immobilizing oral vaccines, antibodies and enzymes of industrial significance<sup>3</sup>. Mannoproteins are a functionally heterogeneous, heavily mannosylated groups of proteins found in many fungal species including the yeast *Saccharomyces cerevisiae*. The mannan and mannoproteins represent 25-50% of the yeast cell wall and determine the cell wall properties<sup>4</sup>, which are believed to be the basis of the three primary modes of action of MOS: (1) adsorption of pathogenic bacteria containing Type 1 fimbriae; (2) modulation of the host immune response; and (3) enhancement of intestinal integrity<sup>5</sup>. The cell wall comprises of yeast 15-30% of the dry weight of the cell with the major components being  $\beta(1,3)$ -glucan,  $\beta(1,6)$ -glucan, mannoproteins and chitin<sup>6</sup>. Mannan oligosaccharides (MOS) are complex mannose sugars derived from the cell wall of the yeast *Saccharomyces cerevisiae*. This complex carbohydrate product has been utilized around the world to improve the productivity and wellbeing of poultry, fish and livestock. MOS has been one of the key interest areas for the researchers. Experiments using a variety of species demonstrate that the positive effects of MOS on performance can be attributed to an improvement in health. A portion of this activity is due to the ability of MOS to block the attachment of bacteria via Type 1 fimbriae to intestinal villi<sup>7</sup>. Many studies have been reported on the improved performance benefits thanks to feeding yeast cultures to growing poultry<sup>8-10</sup>. MOS alter faecal microbial populations and certain indices of the immune system of senior dogs<sup>11</sup>. Supplementation of MOS beneficially altered indices of gut health by improving ileal and fecal microbial ecology and also altered immune function by causing a shift in blood immune cells<sup>12</sup>. Yeast mannan directly inhibit *in vitro* antigen-driven T-cell proliferation from millimolar to nanomolar concentrations acting to block early events required for normal antigen processing/presentation in regulating the human immune response<sup>13</sup>. MOS and possibly other oligosaccharides, serve as alternate attachment sites for Gram-negative pathogens, thereby preventing attachment onto enterocytes and subsequent

enteric infection. MOS optimizes several parameters of immune competence within the intestines, including secretory IgA secretion and enhanced levels of antigen-specific and natural antibodies<sup>14-18</sup>. MOS stimulates gut associated and system immunity by acting as a non-pathogenic microbial antigen<sup>19</sup>. The effect of MOS was also examined on the phenotypic and functional competence of immune cells in cecal tonsil (CT), which is a major GALT<sup>20</sup>. Mannose residues exposed on glycoproteins present at the gut epithelial cell surface form important attachment sites for several unfavorable organisms<sup>21</sup>. A study was also carried out to investigate the effects of MOS and probiotic supplementation on hematological and immunological parameters in turkeys. The results showed that MOS or probiotic may elevate IgG and IgM levels in turkey. The MOS and probiotic that enhance immunoglobulin levels will have a more positive effect on growth performance, production and the ability to resist any disease<sup>22</sup>. In addition, previous reports suggest that MOS supplementation resulted in significant improvement in antibody responses in broiler and layers<sup>23</sup>. This results show that MOS may bind to pattern-recognition receptor on a variety of defense cells of the GALT and in turn activate immune defenses such as phagocytosis, the alternative complement pathway and the lectin pathway<sup>24</sup>. Some experiments to determine the effects of dietary supplementation of yeast culture at different levels of dose on the growth performance, intestinal microflora, and immune response in weanling pigs. The results indicated that dietary yeast culture supplementation had a positive effect on growth performance of nursery pigs by improving jejunal villus height and villus height/crypt depth ratio and by modulating gut immune response<sup>25</sup>. On the other hand some studies have indicated that MOS does not have any positive effect on the health and immune system or it has still been unclear. MOS conferred intestinal health benefits to chickens by improving its morphological development and microbial ecology. But, there were no additional benefits of the higher MOS dosage<sup>26</sup>. A study showed that 0.05% MOS did not affect plasma immunoglobulins in broilers, but the heterophil/lymphocyte ratio, basophil level, and microbial population in the ileum were significantly affected<sup>27</sup>. A study showed that MOS regulated the expression of nonimmune and immune genes in pig leukocytes, perhaps providing benefits by enhancing the immune responses of pigs to an infection, while preventing over-stimulation of the immune system<sup>27</sup>. Plasma immunoglobulins are not affected by MOS prebiotics but the heterophil : lymphocyte ratio, basophil level, and microbial population in the ileum are significantly affected. A dose-response evaluation of spray-dried yeast cell wall supplementation of diets fed to adult dogs on nutrient digestibility, immune indices, and fecal microbial populations was also investigated. The results showed that the effects on immunological indices appear limited<sup>28</sup>. A study was conducted to determine the effects of yeast culture and modified yeast culture (cell

wall product containing mannan oligosaccharides) in pig diets on the blood cell composition of weanling pigs and to determine whether these dietary supplements could replace antimicrobial growth promoters in pig diets. It was obtained that adding MOS to diets would not improve the performance or health of weanling pigs above that of yeast culture alone and more insight into the mode of action of MOS was recommended<sup>29</sup>. A number of isolates of *Saccharomyces cerevisiae* have been associated with disease in immunocompromised individuals<sup>30</sup>. The gut epithelium behaves as a primary lymphoid organ responsible for the differentiation of a major local T cell set<sup>31</sup>. These observations imply that MOS is taken up into the intestinal epithelium where it stimulates regulatory and/or effectors cells of the gut associated lymphoid tissue (GALT)<sup>32</sup>. Questions relating to the fate of mannan in the gastrointestinal tract and the specific interaction of mannoproteins and the host immune cells still remain unclear. MOS-immune system also cross talk would be expected and this cross talk can be affect improvement in health. In this study it is investigated that if MOS passes through the intestinal epithelium and if it is translocated to the lamina propria of the small intestine

## MATERIAL and METHODS

This experiment was conducted in the Department of Animal Science at UC Davis Animal Unit at Meyer Hall and was approved by the UC Davis Committee on Animal Care and Use. Addition of a fluorescent label to the mannan structure would allow researchers the ability to identify and localize the key cells involved in its uptake and immune recognition in the gastrointestinal tract.

### Preparation of MOS from Yeast Cell

A method was also described for the synthesis the florescent reagent 7-methoxycoumarin-3-isocyanate and

its attachment to mannan-OH group via a urethane bond. The synthesis of the reagent has not been previously described. The first step was to isolate pure mannan from mannan rich fraction. This material was prepared by reacting mannan rich fraction with 7-methoxycoumarin-3-isocyanate in dimethylsulphoxide (DMSO). The labeled product was isolated by ethanol precipitation and purified from fluorescent residue by-products by an extensive ethanol wash. The chemical composition of the product was: mannan 73.1%, glucan 10.9%, protein 13.6% and label 2.4% (all weight %). The labeling ratio is ~1 molecule of the label per 52 mannopyranose and 7.8 glucopyranose monomers. The distribution of the molecular weights for the product is under investigation. Solubility: ~5mg/ml water, 50 mg/ml DMSO. Maximum wave-lengths are: absorption 345nm and emission 417 nm, for the solution in water at 100 µg/ml<sup>33</sup>. FITC dextran (FD 70S Fluorescein isothiocyanate) and oval albumin were both purchased from the Firm "Sigma". Ovalbumin was labeled via FITC method. 10 mg of FITC was mixed with DMSO. 100 mg ovalbumin was stirred in 10 ml of NaHCO buffer for 4 h at ambient temperature. It was transferred to the Mini dialysis Unit. The solution was collected from the tube and freeze dried it.

### Labeling Macromolecules

In this study four groups of one-day old broiler chicks were arranged. Each group (n=4) was assigned to a different fluorescent labeled diet. The first group is the control group fed with the basal diet without any fluorescent tagged molecules as shown in the *Table 1*. The second group is the experimental group fed with MOS labeled with a fluorescent tag as shown in the *Fig. 1*. The third group is the albumin fed group as the negative control which is known not to be quickly digested and not translocated. The fourth group is the dextran as the positive control which is known to be phagocytosed by dendritic cells. The ratio of fluorescent labeled MOS, albumin and dextran to the basic diet was 20 mg/kg. The

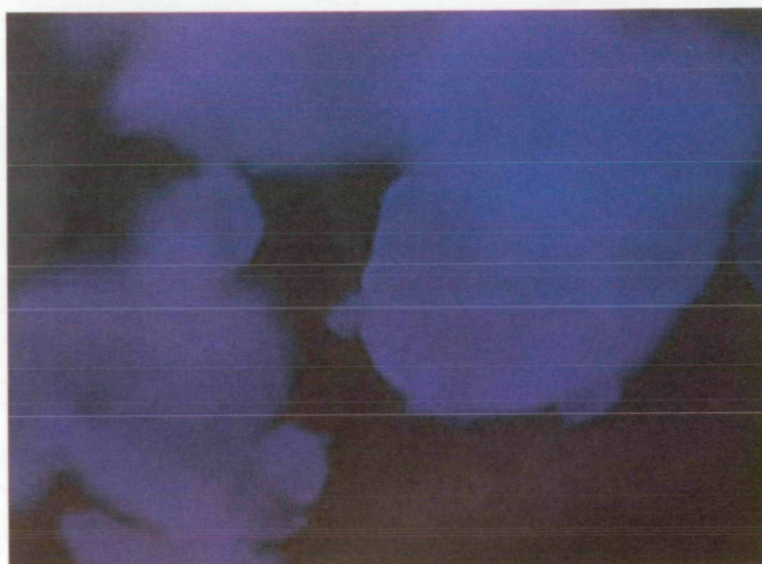


Fig 1. Fluorescent labeled feed

Şekil 1. Floresans işaretli diyet

**Table 1.** Composition of basal diet (0%, as-fed basis)**Tablo 1.** Bazal Diyet Kompozisyonu (%0, KM)

Composition	(g/kg)
Ingredient	Amount
Corn	577.79
Soybean seeds	323.82
Vegetable oil	50.67
Calcium phosphate	17.97
Limestone, ground	13.27
Salt	4.51
DL-Methionine 99%	3.49
Mineral premix – NRC	2.50
Vitamin premix – NRC	2.50
L-Lysine 95%	1.16
Threonine	1.07
Choline chloride	0.75
Ferrous sulfate	0.50
<b>Total</b>	<b>1.000.00</b>

next step after labeling macromolecules was to check the labeled MOS in the feed -mix with fluorescent microscopy to see fluorescent signals as shown in the Fig. 1.

The basal diet composition without fluorescent tagged molecules was prepared to feed the broiler chicks as given in the Table 1.

### Chicken Management and Experimental Design

In this study 16 of one-day old broiler chicks (Cobb x Cobb) were used. Control chicks were examined in order to distinguish and correct for auto fluorescence inherent in feed ingredients. They were kept in brooder batteries with four chicks per pen<sup>34</sup>. Each group (n=4) was assigned to a different fluorescent labeled diet as seen in the Table 1.

### Data Collection

The experiment lasted three weeks. After three weeks,

chickens were terminated with carbon dioxide. The intestinal segments were removed and preserved in 10% formalin and were fixed on the slides using the paraffin method. From each segment, 72 glass slides were prepared. Fluorescent microscopy was used to determine the extent of translocation into the lamina propria and images were captured. Slides were evaluated quantitatively by interrogation of color intensity of foci of translocated macromolecules (foci) using a commercial image analysis program that converts color intensity at specific wavelengths into numerical values of intensity.

## RESULTS

Introduction of fluorescent labeled MOS into segments utilized samples of intestine from three sections: segments from the distal duodenum that lack Peyer's patches and dendritic cells were used to examine the uptake by epithelial cells; segments from the proximal ileum that contain dendritic cells was used to clarify uptake by phagocytes. Slides were evaluated quantitatively by interrogation of color intensity of foci of translocated macromolecules (foci) using a commercial image analysis program that converts color intensity at specific wavelengths into numerical values of intensity.

This data was analyzed by ANOVA. P value < 0.05 was considered to be significant. One-way ANOVA: Lumen, epithelium and lamina Propria versus Treatment; Units are % fluorescence of treatment foci over background<sup>35,36</sup>. Foci of fluorescence from albumin were not detectable and albumin was apparently degraded prior to entrance into the intestines. Thus, it was not included in the statistical analysis. Dextran the positive control group was transported into the lamina propria, especially in the ileum as seen in the Fig. 2.

Mannan oligosaccharide (MOS), the experimental group, was transported into lamina propria, duodenum and jejunum as seen in the Fig. 3 in larger amounts especially in to the lamina propria than dextran. The supplied mannan

**Table 2.** Lumen, epithelium and lamina propria versus treatment; units are % fluorescence of treatment foci over background (One-way ANOVA)**Tablo 2.** Dekstran, MOS ve Kontrol Grubu Lumen, epitelyum ve lamina propria floresans ışıma değerleri

Treatment	Lumen			Epithelium			Lamina Propria		
	n	mean	(±)SD	n	mean	(±)SD	n	mean	(±)SD
Dextran (Duodenum)	5	-3.75	11.25	5	1.400	7.285	5	137 <sup>b</sup>	10.05
Dextran (Distal. Ileum)	5	-6.39	34.56	5	0.579	6.706	5	213 <sup>c</sup>	17.68
Dextran (Jejunum)	5	2.27	12.38	5	-1.149	3.867	5	132 <sup>b</sup>	13.45
MOS (Duodenum)	5	-10.1	10.54	5	-2.915	7.483	5	338 <sup>d</sup>	89.32
MOS ( Distal Ileum)	5	0.06	37.08	5	2.936	12.815	5	223 <sup>c</sup>	13.99
MOS ( Jejunum)	5	-9.51	24.83	5	-0.196	5.769	5	213 <sup>c</sup>	17.78
Control (no treatments; background)	5	-9.22	10.29	5	-0.158	6.456	5	5 <sup>a</sup>	10.73
P value	0.953			0.970			< 0.0001		

from The Firm "Sigma" as the experimental group was also transported into the lamina propria. Albumin in

the negative group was not transported into the lamina propria.

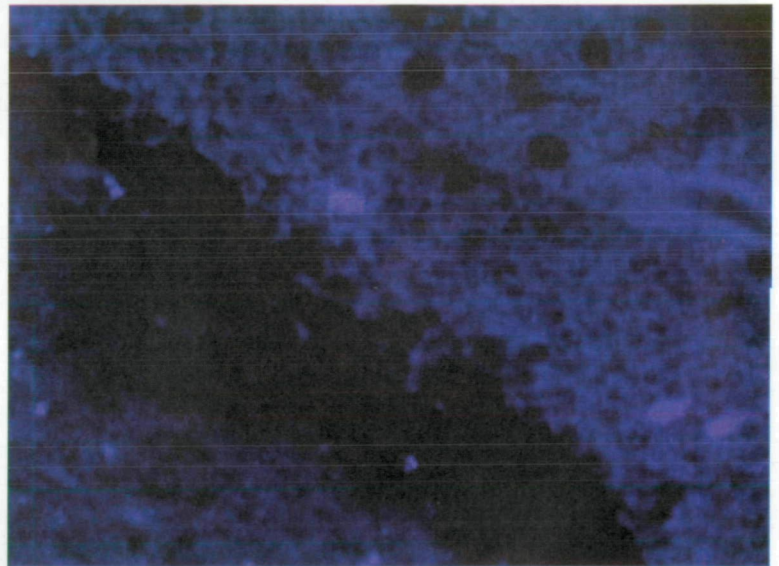


**Fig 2.** Dextran in Lamina propria

**Şekil 2.** Lamina propriada dekstran

**Fig 3.** MOS in Lamina propria

**Şekil 3.** Lamina propriada MOS



**Fig 4.** Albumin in lumen

**Şekil 4.** Lumende albumin



## DISCUSSION

Oligosaccharides composed of monosaccharide molecules come together to form a larger molecule. Mannose is a monosaccharide forming the main building block of MOS. The small intestine does not contain the enzymes required to break down MOS bonds, that's why they reach the large intestine intact after ingestion and passage through the small intestine<sup>37</sup>. Mannose which is present on the surface of intestinal epithelial cells act as receptor binding sites for certain pathogens with type-1 fimbriae that contain mannose-specific lectins<sup>38</sup>. This adherence to the intestinal cell wall causes the initiation of colonization by pathogenic organisms in the gastro-intestinal tract<sup>39</sup>. As a binding occurs, translocation across the intestinal wall can occur<sup>40</sup>. The lamina propria contains capillaries and a central lacteal (lymph vessel) in the small intestine, as well as lymphoid tissue. Lamina propria also contains glands with the ducts opening on to the mucosal epithelium that secrete mucus and serous secretions. But despite many researches done in this field of interest there has not been clearly obtained data about the translocation of MOS in to the lamina propria and its metabolism. Our experimental results showed that MOS was transported into the intestinal mucosa consist of epithelium and lamina propria. A portion of this activity could be due to the ability of MOS to block the attachment of bacteria via Type 1 fimbriae to intestinal villi. The surface of the mucosal sites, the intestinal tract that is covered by epithelial cells is protected from invading pathogens by an acquired immune system, referred to as the mucosal immune system, in which epithelial cells and lymphocytes function cooperatively<sup>41</sup>. The intestinal immune system must elicit robust immunity against harmful pathogens but must also restrain immune responses directed against commensal microbes and dietary antigens. The mechanisms that maintain this dichotomy are poorly understood<sup>42</sup>. Maintenance of this critical balance is attributed to mucosal dendritic cells residing in organized lymphoid tissue and dispersed in the subepithelial lamina propria<sup>43</sup> because dendritic cells in the intestinal lamina propria play a key role in mucosal immunity<sup>44</sup>. Oligosaccharides have been shown to have variety of effects on the immune system, such as inhibition of cancer metastasis<sup>45</sup>. MOS also optimizes several parameters of immuno-competence within the intestines, including secretory IgA secretion and enhanced levels of antigen-specific and natural antibodies. There are also many studies available in the scientific portals related to the effects of MOS on the immune system. Supplementation of Broiler chicks with MOS beneficially influenced the bacterial populations in the digestive system. The results have mostly indicated that MOS has enhanced performance, improved immune function and inhibited colonization of the gastro intestinal tract by unfavorable microorganisms in a number of livestock species. MOS has the ability to

influence the microbial population in the intestinal tract. Recent studies demonstrated that pathogenic bacteria were inactivated in the animals fed diets supplemented with MOS. Supplementation of broiler chicks with MOS provide in control of pathogenic *Clostridium perfringens* and *Escherichia coli*<sup>46-48</sup>. These experiments were conducted to find out if MOS crosses the intestinal epithelium. This modification is accomplished by the ability of MOS to attach to mannose binding proteins on the cell surface of some strains of bacteria, thereby preventing these bacteria from colonizing the intestinal tract by interfering with the binding of carbohydrate residues on epithelial cell surfaces<sup>47</sup>. These observations imply that MOS is taken up into the intestinal epithelium where it stimulates regulatory and/or effectors cells of the gut associated lymphoid tissue (GALT). The GALT is also the site where immune system and components of the diet interact<sup>48</sup>. These data imply that MOS is taken up into the intestinal epithelium where it stimulates regulatory and/or effector cells of the gut associated lymphoid tissue (GALT). It has been seen that there have been many studies in the literature investigating the MOS and its effects on the performance of the animals fed with MOS. Among these studies to determine the interaction of MOS with immune system, its transportation in to lamina propria and its relation with M Cells have not been determined yet. Both M cells and dendritic cells possibly to promote tolerance against pathogens are involved by the uptake of IgA mechanism that is a challenging opportunity to understand the mechanism of the transportation across the cell membrane and immune-related studies<sup>48</sup>.

This study has shown that MOS does not interact specifically with the epithelial cells but it makes its way to the GALT of the lamina propria via an independent method, which appears to be mediated by dendritic cells, as an immune surveillance mechanism that is vital in the mucosal immunity. It is likely that MOS has a general adjuvant effect on the immune system without causing "danger signals" that are inherent in pathogens. Thus it alerts but does not alarm. In conclusion further studies are needed to identify which mechanism makes MOS transport to lamina propria and especially if it interacts with M-Cells, which are specialized epithelial cells and play a key role in stimulating the immune system.

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