DIETARY SUPPLEMENTATION WITH MANNANOLIGOSACCHARIDES AND β GLUCANS IN GROWING RABBITS. 2 GUT BARRIER AND INTESTINAL MICROBIOTA AFTER WEANING

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ABSTRACT

The aim of this work was to determine the effect of yeast mannanoligosaccharides (MOS; Actigen, Alltech) and yeast β-glucans on gut barrier and intestinal microbiota in rabbits. A control diet (C) was formulated to meet rabbit growth requirements. G1 and G2 diets were obtained by supplementing C diet with β-glucans from *Saccharomyces cerevisiae* (100 and 200 mg/kg respectively), M1 and M2 diets were supplemented with mannanoligosaccharides (Actigen, Alltech) (400 and 800 mg /kg respectively), and GM diet was supplemented with 100 mg/kg of β-glucans + 400 mg/kg of MOS. Ninety six rabbits weaned at 32 days of age were randomly assigned to the six experimental diets and slaughtered 15 days after weaning. The type of diet did not affect villus length and goblet cell counts, but crypt depth was reduced at the highest levels of MOS and β-glucans supplementation. Both MOS and β-glucans increased specific sucrose activity ($P = 0.001$) and soluble protein concentration ($P < 0.05$) in jejunal mucosa; they also modified ileal and caecal microbiota and modulated the immune response. Compared with the control diet, the supplementation with 200 mg/kg of β-glucans (G2) and 800 mg/kg of MOS (M2) reduced the expression of iNOS in ileum. Rabbits fed the M2 diet showed also the lowest mRNA abundance of IL6. In appendix, rabbits fed G2 showed increased IL6 expression and those fed the M2 reduced the expression of INFγ. In conclusion, MOS and β-glucans changed intestinal microbiota and modulated the immune response by reducing the expression of some pro-inflammatory cytokines in ileum and caecum of weaned rabbits.

Key words: Yeast mannanoligosaccharides, yeast β-glucans, weaning rabbit, immunity, microbiota, jejunum morphology

INTRODUCTION

Mannanoligosaccharides (MOS) and β-glucans extracted from *Saccharomyces cerevisiae* are promising alternatives to antibiotic growth promoters (Yang et *al*., 2008; Bovera et *al*., 2012). They have shown beneficial effects on mucosal integrity and are capable of modulating the immune response and intestinal microbiota in chicken and pigs (Castillo et *al*., 2007; Yang et *al*., 2008). In rabbits, recent publications suggest that these prebiotics increase villus length (Mourao et *al*., 2006) and reduce coliforms count in the caecum (Bovera et *al*., 2012). Wu et *al*. (2011) observed that β-glucans improved maternal humoral immunity at late lactation by elevating serum Ig concentrations in does. However, information about the effect of MOS and β-glucans on gut barrier and intestinal microbiota in weaned rabbits is scarce. The aim of this work was to determine the effect of yeast MOS and β-glucans on gut barrier, intestinal microorganism and immune response in rabbits around weaning.

MATERIALS AND METHODS

Animals and experimental diets

Ninety six Hyplus rabbits (Grimaud Frères, France) were weaned and randomly assigned to 6 experimental diets (16 animals per treatment). From weaning (32 days of age) to 46 days old (1634 ± 137 g LW), rabbits were fed *ad libitum* as follows: C (control diet without additives), G1 and G2 (control diet supplemented with 100 mg/kg or 200 mg/kg of β-glucans, respectively, extracted from *Saccharomyces* cerevisiae by the Instituto de Estudios Biofuncionales, Madrid), M1 and M2 (control diet supplemented with 400 mg/kg or 800 mg/kg of MOS, respectively, Antigen, Alltech, Spain) and GM (control diet supplemented with 100 mg/kg of β-glucans + 400 mg/kg of MOS). Ingredients and chemical composition of diets have been described by Tazzoli et *al*. (2012). All rabbits were housed individually and kept under controlled environmental condition.

Slaughter and samples collection

During two consecutive days (at 45 and 46 days of age), 96 rabbits (16/diet) were slaughtered by cervical dislocation. Immediately, 10 cm from middle jejunum were collected. The first 5 cm were washed with saline solution (0.9 % NaCl), frozen in dry ice and stored at -80ºC until determination of sucrase activity. The other 5 cm were stored in 10% buffered formalin solution (pH: 7.2-7.4) for analysis of mucosa morphology and goblet cell count. One gram of caecal and ileal content was collected and placed in sterile tubes containing 3 ml of ethanol solution (98%) and stored at 4ºC for microbial analysis. For cytokine analysis, only rabbits from diets C, G2 and M2 were used (6 rabbits/diet). In these animals, 3 cm of the ileum (between ileo-caecal junction and Payer patch) and the *vermiform appendix* were taken. All samples were washed with saline solution (0.9 % NaCl), longitudinally opened and mucosal scrapings placed in vials containing 2 ml of RNA-later (Ambion) and stored at -80ºC until immunological analyses.

Analytical methods

Sucrase activity (EC 3.2 1.48) was determined using sucrose as substrate and following the procedure described by Gómez-Conde et *al*. (2007). Glucose liberated was measured by using a commercial kit (Randox, Gluc-PAP). The soluble protein concentration was determined on jejunum homogenates using a commercial kit (Thermo scientific, Pierce® BCA Protein Assay Kit). To evaluate the jejunal mucosa morphology, goblet cell count, and gut microbiota samples were processed according to Gómez-Conde et *al*. (2007). To study the immune response, the expression of selected cytokines was assessed by real time RT-PCR with the ABI Prism 7300 Sequence Detector using the SYBR green dye (Applied Biosystems,). Total RNA was isolated from mucosal scrapings using Trizol and the GeneElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich,) with an additional on-column DNase I digestion step. To synthesize cDNA, the High-Capacity cDNA Archive Kit was used (Applied Biosystems) according to manufacturer's specifications. The relative abundance of INOS, INFγ, TNFα, IL-2, IL-6, IL-8 and IL-10 mRNA for appendix and of iNOS, IL-6 and IL-10 for ileum was studied. The specific primers for rabbit housekeeping (GADPH), IFN-γ, IL-10 and TNF-α were selected according to Menoyo et *al*. (2010).

Statistics analysis

Data of mucosa morphology and enzymatic activity was analyzed as a completely randomized block design with a diet as the main source of variation by using a Mixed procedure of SAS (1999). Contrasts were used to compare means. For each contrast, the changes in gene expression resulted from the comparison of the first treatment group relative to the second, following the model described by Steibel *et al*. (2009) using the SAS Mixed procedure. For genes displaying efficiencies different from 2 (E≠2), Ct values were adjusted according to the model described by Steibel et *al*. (2009).

RESULTS AND DISCUSSION

The type of diet did not affect villus length and the number of goblet cells per villus (Table 1), showing normal values for 45-d-old rabbits. However, both additives included at the highest levels reduced the crypt depth ($P < 0.05$). These results agree with those reported in pigs and broiler (Castillo et *al*., 2007, Yang et *al*., 2008), but contrast with those of Mourao et *al*. (2006) in rabbits. These latter reported that MOS supplementation increased the length of villus in ileum compared to a no supplemented diet. This difference between studies might be due to differences of health status (3.5 vs. 8.5% mortality during fattening period). The good health of the animals in the present study is compatible with a good gut morphology and lower crypt cell turnover. The β-glucans and MOS supplementation increased the specific sucrase activity and soluble protein concentration compared to the control diet ($P < 0.05$), which suggests an improvement of enterocyte maturity and/or functionality as in other species. Chicken fed diet supplemented with MOS showed higher specific activity of maltase and alkaline phosphate, which is used as indicator of intestinal maturity (Iji et *al*., 2001; Yang et *al*., 2008). This effect can also be explained by changes of intestinal microbiota, as suggested by Yang et *al*. (2008) who reported an increase of *Lactobacillus* in broilers fed MOS. This increase seems to improve the intestine digestive enzyme activity (Sissons, 1989). Dietary treatments did not affect the gene expression of cytokines in appendix, except for IL6, which increased ($P < 0.05$) in animals fed G2, and INFγ, which displayed a lower expression pattern ($P < 0.05$) in rabbits fed M2 (Figure 1). Compared to the control, rabbits fed G2 and M2 diets reduced the expression of INOS in ileum and M2 also reduced the expression of IL6. Since rabbits affected by ERE showed a higher mRNA abundance of IL6 and INOS in ileum relative to healthy animals (Menoyo et *al*., 2010), these additives could be useful to reduce the pro-inflammatory response of infected animals. However, this point needs to be deeply studied. In this trial we did not characterize or count the intestinal microbiota but the supplementation with MOS and β-glucans led to a modification of both ileal and caecal microbiota (Figure 2). In conclusion, MOS and β-glucans changed intestinal microbiota and modulated the immune response by reducing the expression of some pro-inflammatory cytokines in ileum and caecum of rabbits around weaning.

Table 1: Effect of diet on morphology, goblet cell counts and sucrose activity of jejunal mucosa in 45 days old rabbits.

	Diet[1]						*RSD*	Contrast P-value[2]						
	C	G1	G2	M1	M2	GM		C *vs*. Others	C *vs*. G1+G2	C *vs*. M1+M2	G1+G2 *vs*. M1+M2	G1 *vs*. G2	M1 *vs*. M2	G1+M1 *vs*. MG
Rabbits, n	16	15	15	15	16	15								
Villus length, µm	650	670	669	666	669	658	*65.5*	NS	NS	NS	NS	NS	NS	NS
Crypts depth, µm	110	114	107	114	107	112	*8.71*	NS	NS	NS	NS	0.02	0.03	NS
Villus length/crypts depth	6.04	6.08	6.40	5.98	6.36	5.96	*0.62*	NS	NS	NS	NS	NS	0.09	NS
Specific sucrose activity[3]	7352	8669	8821	10778	11654	9702	*1066*	0.001	0.001	0.001	0.001	NS	0.02	NS
Soluble protein, µg/100 mg tissue	8.30	8.75	8.53	8.54	8.72	8.39	*0.51*	0.04	0.03	0.04	NS	NS	NS	0.038
Goblet cell count/villus	16.2	15.6	16.0	16.3	16.8	16.2	*1.60*	NS	NS	NS	0.07	NS	NS	NS

[1] C control diet without additive. G1: diet C with 100 mg of β-glucans/kg. G2:diet C with 200 mg of β-glucans/kg. M1: diet C with 400 mg of MOS/kg. M2: diet C with 800 mg of MOS/kg. MG: diet C with 100 mg of β-glucans+ 400 mg of MOS/kg. [2] NS: $P > 0.10$. [3] measured at 37ºC during 30 min, µmol of glucose/g of protein.

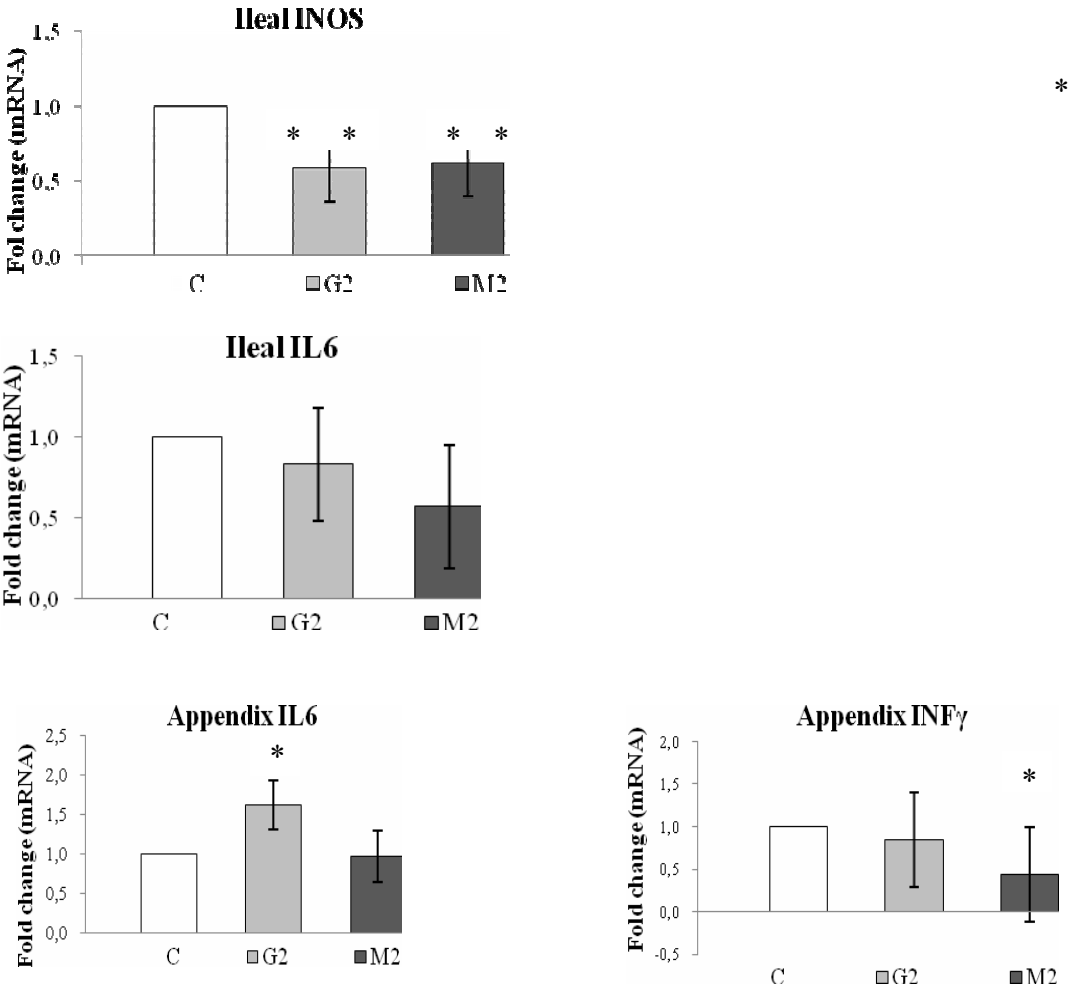

Figure 1: Relative gene expression of selected cytokines in animals fed G2 (200 mg of β-glucans/kg) and M2 (800 mg of MOS/kg) diets compared with rabbits fed C diet

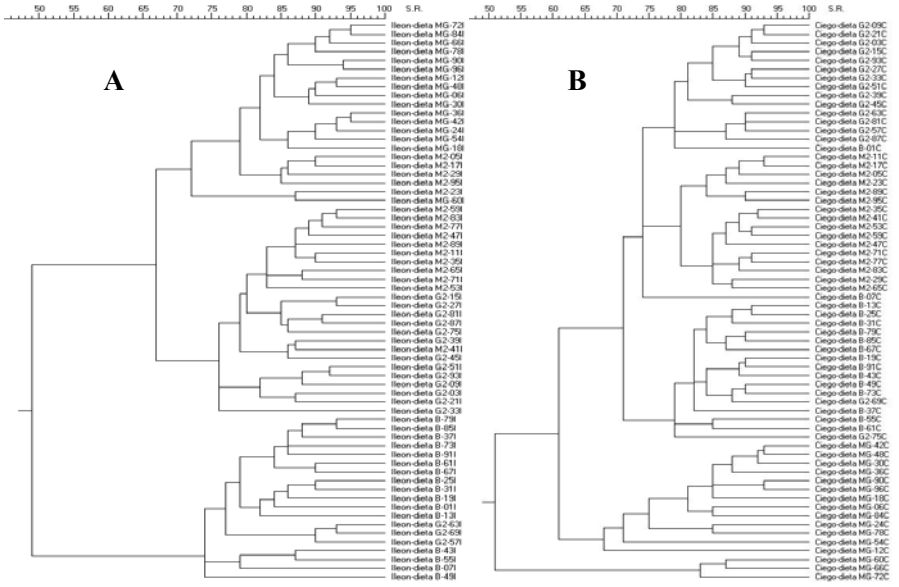

Figure 2: Effect of diet on similarity rate of PCR-RFLP banding pattern of the ileal (A) and caecal (B) microbiota (SR, expressed in percentage) in 45-d-old rabbits. (B: control diet, G2:diet C with 200 mg of β-glucans/kg, M2: diet C with 800 mg of MOS/kg; MG: diet C with 100 mg of β-glucans+ 400 mg of MOS/kg). The number of each animal is its identification.

ACKNOWLEDGEMENTS

This study was funded by the Ministerio de Ciencia y Tecnología, Spain (Project AGL 2008 – 00627).

REFERENCES

Bovera F., Lestingi A., Marono S., Iannaccone F., Nizza S., Mallardo K., de Martino L., Tateo A. 2012.Effect of dietary mannan-oligosaccharides on in vivo performance, nutrient digestibility and caecal content characteristics of growing rabbits. *J. Anim. Phys. Anim. Nut.,96, 130-136.*

Castillo M., Martín-Orúe S.M., Taylor-Pickard J.A., Pérez J.F., Gasa J. 2008. Use of mannanoligosaccharides and zinc chelate as growth promoters and diarrhea preventative in weaning pigs: Effects on microbiota and gut function. *J. Anim. Sci., 86, 94-101.*

Gómez-Conde M.S., García J., Chamorro S., Eiras P., García-Rebollar P., Pérez De Rozas A., Badiola I., De Blas J.C., Carabaño R. 2007. Neutral detergent-soluble fiber improves gut barrier function in twenty-five-day-old weaned rabbits. *J. Anim. Sci., 85, 3313-3321.*

Iji P.A., Saki A.A., Tivey D.R. 2001. Intestinal structure and function of broiler chickens on diets supplemented with a mannan oligosaccharide. *J. Sci. Food Agric., 81, 1138-1192.*

Menoyo D., Gutierrez M., Ghanavi H.R., García J., Carabaño R. 2010. Immune response to Epizootic Rabbit Enteropathy. *In Proc: 4° Congreso de las Americas. Córdoba, Argentina Sept. 2010.*

Mourao J.L., Pinheiro V., Alves A., Guedes C.M., Pinto L., Saavedra M.J., Spring P., Kocher A. 2006. Effect of mannan oligosaccharides on the performance, intestinal morphology and cecal fermentation of fattening rabbits. *Anim., Feed Sci. Technol., 126, 107-120.*

Sissons J.W. 1989. Potential of probiotic organisms to prevent diarrhea and promote digestion in farm animals: a review. *J. Sci. Food Agric., 49,1-13.*

Steibel J.P., Poletto R., Coussens P.M., Rosa G. 2009. A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. *Genomics 94, 146-152.*

Tazzoli M., Xiccato G., Trocino A., Majolini D., El Abed N., García J., Eras M.A., Carabaño R. 2012. Dietary supplementation with mannanoligosaccharides and β-glucans in growing rabbits. 1. growth performance, health status and carcass traits. *In Proc: 10th World Rabbit Congress, Sharm El Sheik, Egypt, September 2012 (in press)*

Wu H.H., Weng B.B.C., Chen K.L., Chiou P.W.S., Yu B. 2011. Effect of dietary supplementation of β-1,3–1,6-glucan on reproductive performance and immunity of New Zealand White does and their pups. *Livestock Sci., 135, 70-75.*

Yang Y., Iji PA., Kocher A., Mikkelsen L.L., Choct M. 2008. Effects of dietary mannanoligosaccharide on growth performance, nutrient digestibility and gut development of broilers given different cereal-based diets *J. Anim. Phys. Anim. Nut., 92, 650-659.*