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β-Glucan successfully stimulated the immune system in different jawed vertebrate species

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ABSTRACT

Several reports have shown the positive effects of β-glucans on the immune. Howeverthese studies have a broad experimental design including β-glucans compounds. Consequently, a study using the same β-glucan molecule, administration route and experimental design is needed to compare the effects of β-glucan across vertebrate species. For this end, during 28 days we fed four different vertebrate species: mice, dogs, piglets and chicks, with two β-glucan molecules (BG01 and BG02). We measured the serum interleukin 2 as an indicator of innate immune response, the neutrophils and monocytes phagocytosis index as a cellular response and antibody formation as an adaptive response. The results clearly showed that the different β-glucan molecules exhibited biologically differently behaviors, but both molecules stimulate the immune system in a similar pattern in these four species. This finding suggests that vertebrates shared similar mechanisms/patterns in recognizing the β-glucans and confirms the benefits of β-glucans across different vertebrate species.

1. Introduction

A variety of natural polysaccharides have shown the ability to stimulate the immune system of invertebrate and vertebrate species. Among these compounds, the glucans stand out and their role as a biologically active immunomodulator has been well documented for more than 40 years. 'Glucans' is the common name given to a group of polysaccharide polymers, classified based on interchain linkages as either α- or β-linked. They are widely distributed in bacteria, algae, fungi and plants, with different structural types [(see Barsanti et al. [1]]. Their structure is comprised of a main chain of β-(1,3)- and/or β-(1,4)-$_D$-glucopyranosyl units in non-repeating but non-random order, with side chains of varying lengths [2].

Specifically, the β-glucans are the most well-known 'glucans' and their benefits have been investigated in a wide range of vertebrates such as humans [3], dogs [4,5], pigs [6], cattle [7], horses [8], sheep [9], chickens [10], frogs [11], fish [12] and invertebrates such as shrimp [13], crab [14], and insects such as bees [15] and drosophila [16]. These benefits include lower stress [17], anti-cancer [18], anti-allergies [19], regulate blood sugar levels [20], prebiotics [21,22], reduce serum cholesterol in hypercholesterolemic animals [23], increase wound healing [24], immune adjuvant [25] and they are extensively used to improve health, growth and general performance in farm animals. In a recent review, Petit and Wiegertjes [26] provided evidence that β-glucans are also able to stimulate a new concept called trained immunity or innate immune memory, which allows macrophages, monocytes, and natural killer cells to show enhanced responsiveness when they reencounter pathogens [27]. In addition, β-glucans have also been successfully used in diverse administration routes such as oral [12], injection or bath [28].

In a review, Soltanian et al. [29] suggested that β-glucan is an immunostimulant that is active across the evolutionary spectrum. Although it is plausible, the studies referred to in the review and also on the current literature did not have the appropriate experimental design to reach this conclusion. The effects of β-glucan are influenced by their molecular weight, degree of branching [2,30], purity, source and extraction process as demonstrated by Pilarski et al. [12]. In addition, the diversity of experimental designs, methods, and administration routes as mentioned before is an aggravating factor for comparing/extrapolating β-glucans' effects across species. Thus, in this study, we fed four different vertebrate species: mice, dog, piglets and chicks, with two β-glucan molecules (BG01 and BG02) and control diet for 28 days. We measured the serum IL-2 production as an indicator of innate immune response, the neutrophils and monocytes phagocytosis index as a cellular immune response and anti-body production against ovoalbumin as an adaptive immune response. This is the first report of this nature on jawed vertebrates.

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2. Material and methods

2.1. Experimental animals and welfare statement

Male and female dogs (average age 2.5 ± 0.5 years, average weight 14.5 ± 2.1 kg, 61% males, and different breeds) were purchased from Marshall Farms, North Rose, NY, USA. Piglets (all white Yorkshire x Landrace; age 3 weeks, average weight 6.3 ± 0.7 kg, 54% males) were obtained from Oak Hill Genetics (Ewing, IL, USA) and leghorn chickens (age 10 days, average weight 65 ± 17.1 g, 50% males) from Hy-Line International, Bryan, TX, USA. All mice used were 8-week-old females of the BALB/c strain from Jackson Laboratory (Bar Harbor, ME, USA). All animals used in our study were grown in conventional conditions with monitored light, temperature and air. At the beginning of the study, all animals were examined for disease and clinically relevant abnormalities, of which none were found.

All the animals were monitored throughout the experiments. None of them developed any disease including lethargy, or GI problems. Similarly, none of these animals either died or were euthanized during this study. The use of animals was approved by the University of Louisville IACUC committee (#12029 and 10080).

2.2. Experimental design

After acclimation, animals were fed with three experimental diets (see diet preparation below): a control diet without β-glucan, a diet supplemented with 15 mg $kg^{-1}day^{-1}$ of BG01 or 25 mg $kg^{-1}day^{-1}$ of BG02. The diets' supplementations were designed as iso-glucan and the difference in the concentration is due to the purity of both β-glucans. Additional information and the compositions of both β-glucan samples were previously described by Pilarski et al. [12] and are shown in Table 1. After feeding for 28 days, the blood was drawn from five animals per group by puncture of the jugular vein. We measured IL-2 on serum, the index of monocytes and neutrophils phagocytosis and antibody formation.

Table 1
Composition of β-glucans.

Parameters	BG01	BG02	Reference
pH	6.40	2.88	AOAC 981.12 [31]
Moisture (%)	7.00	7.98	AOAC 934.01 [31]
Protein (%)	3.50	12.70	AOAC 990.03 [31]
Fat (%)	4.10	3.08	AOAC 922.06 [31]
Ash (%)	2.40	0.62	AOAC 942.05 [31]
Glucan (%)	77.30	55.70	[32]
Mannan (%)	1.47	2.20	[32]
Others carbohydrates (%)	5.32	16.88	[33]
Ca (%)	0.08	0.04	
P (%)	0.09	0.22	
K (%)	0.07	0.02	
Na (%)	0.93	0.03	
Mg (%)	0.07	0.01	
Cu (mg/kg)	1.45	7.40	
Fe (mg/kg)	82.99	67.60	
Mn (mg/kg)	5.58	0.50	
Zn (mg/kg)	365.74	6.00	
Co (mg/kg)	< 0.50	< 0.50	
Mo (mg/kg)	< 0.50	< 0.50	
Ni (mg/kg)	< 0.50	< 0.50	
Pb (mg/kg)	< 0.50	< 0.50	
Cr (mg/kg)	0.60	< 0.50	
As (mg/kg)	< 0.10	< 0.20	
Ba (mg/kg)	2.90	0.60	
Al (mg/kg)	57.81	3.10	
Cd (mg/kg)	< 0.50	< 0.50	
S (%)	0.03	0.06	

2.3. Diet

Mice were given Standard Rodent Diet 50001 (Purina). Dogs were given regular food (standard Purina Dog Chow; Purina, USA). Piglets were given Laboratory Porcine Diet Grower #5084 (Purina Mills, Inc., Richmond, IN). Chicks were given regular food (high protein DuMORR Chick Starter 24%; Purina, Harrisburg, PA). The amount of food used in this study was based on the manufacturer's instructions. Feed was supplemented orally for 28 days with either BG01 or BG02. The 4 weeks duration of feeding was based on the immunization protocol. Details about individual glucans are represented in Table 1.

2.4. Innate immune response – serum production of interleukin 2

Whole blood was allowed to clot at an ambient temperature for 30 min, followed by centrifugation at 1700 ×*g* for 10 min. at room temperature. The serum layer was collected, divided into aliquots, and frozen at −80 °C until use. Sera isolated from animals fed with glucans or control feed were collected, filtered through 0.45 μm filters and stored at −80 °C until experiment. The presence of IL-2 was evaluated using a dog IL-2 ELISA kit (Bethyl Laboratories, Montgomery, TX, USA), pig IL-2 ELISA kit (R&D Systems, Minneapolis, MN, USA), chicken ELISA kit (Genorise Scientific, Paoli, PA, USA) and mouse IL-2 ELISA kit (R&D Systems), resp. The assay procedure was similar for each species. The optical density was determined by using a STL ELISA reader (Tecan U.S., Research Triangle Park, NC) at 492 nm, and the amount of IL-2 was calculated from the standard curves included in each kit.

2.5. Cellular immune response - index of monocytes and neutrophils phagocytosis

The technique employing phagocytosis of synthetic polymeric microspheres was described earlier [34,35]. Briefly: peripheral blood cells or isolated peritoneal cells were incubated *in vitro* with 0.05 ml of 2-hydroxyethyl methacrylate particles (HEMA; 5×10^8 ml^{-1}). The test tubes were incubated at 37 °C for 60 min., with intermittent shaking. Smears were stained with Wright stain. The cells with three or more HEMA particles were considered positive, and cell types were distinguished based on their morphology. All experiments were performed in triplicate. At least 300 cells in 60 high-power fields were examined in each experiment.

2.6. Adaptative immune response - antibody formation

The formation of antibodies was evaluated using ovalbumin as an antigen and ELISA assay [36]. Animals were s.c. injected twice (two weeks apart) with albumin (100 μg for mice, 200 μg for chicken, and 400 μg for dogs and pigs) and the serum was collected 7 days after the last injection. The Freund adjuvant was used together with the antigen in a positive control group. The optical density was determined using a STL ELISA reader (Tecan U.S., Research Triangle Park, NC) at 492 nm.

2.7. Statistical analyses

The experiment was conducted according to a completely randomized design. All data were evaluated for normality (Cramer-von Mises) and homoscedasticity (Brown-Forsythe). The data were analyzed using one-way ANOVA followed by Tukey's studentized range test to examine the effects of both β-glucans on the immune response. $P < 0.05$ was used as the level of statistical significance in all analyses. Values in the figures are presented as the means ± 1 standard deviation (S.D.) of the mean.

3. Results

As shown in Table 1, purity of BG01 is 77%, whereas purity of BG02

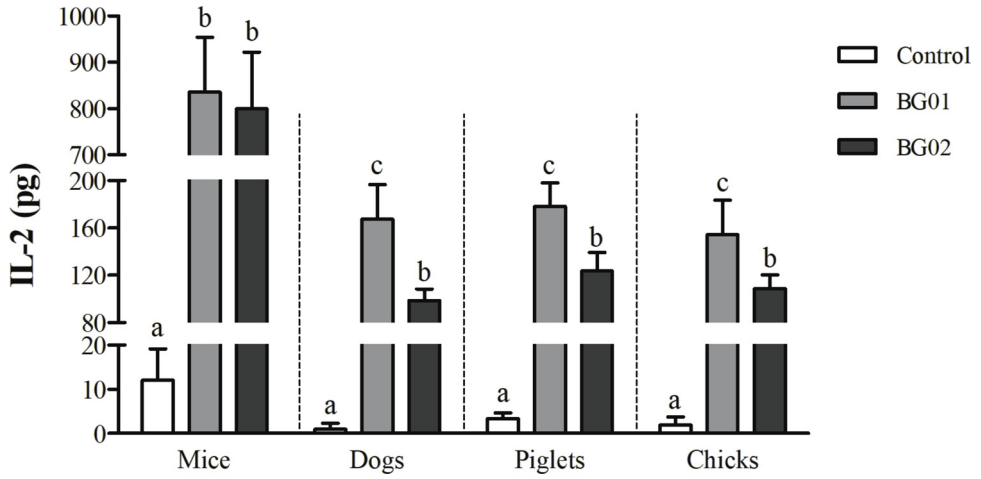

Fig. 1. Serum interleukin 2 of mice, dogs, piglets and chicks fed a control diet and one supplemented with 15 mg kg^{-1} day^{-1} of BG01 or 25 mg kg^{-1} day^{-1} of BG02. Dissimilar lower case letters indicate a difference among treatments within the same animal group. The values are means ± 1 standard deviation (S.D.), N = 5.

is 55%. In order to use similar doses of glucan, we used different dose (15 mg/kg or 25 mg/kg) to make the treatments more comparable regard glucan level, since that it is the major active compound. Although it not to be the exactly same glucan level, the small difference between glucan levels in the diet (11.5 mg for BG01 and 13.7 mg for BG02) are really biologically irrelevant and not responsible to trigger the quite different biological responses between BG01 and BG02 found in our study.

Both β-glucan molecules significantly increase IL-2 production in all species. However, dogs, piglets and chicks fed with BG01 had the highest value for IL-2, while animals fed with BG02 had higher values than the control group ($P < 0.05$, Fig. 1). Both β-glucan molecules had similar results for mice in particular.

The two β-glucan molecules also significantly increase the phagocytosis index of neutrophils and monocytes in all species (Fig. 2A and B), except BG01 for the neutrophils phagocytosis index in dogs, which showed no significant difference (Fig. 2A). BG01 and BG02 showed similar positive effects in mice and piglets for the phagocytosis index of neutrophils, while BG02 alone had a positive effect for dogs and the highest value for chicks ($P < 0.05$, Fig. 2A). Animals fed with BG02 had the highest phagocytosis index for monocytes in all species ($P < 0.05$, Fig. 2B), while BG01 had a higher index than the control.

For antibody production, the groups injected with adjuvant showed the highest values in all species ($P < 0.05$, Fig. 3). Further, animals fed with BG01 and BG02 also had a significant increase in antibody production compared to the control. For dogs in particular, the group fed with BG02 had higher values than the group fed with BG01 ($P < 0.05$, Fig. 3).

4. Discussion

So far, no published study has investigated the effects of β-glucan molecules on different vertebrate species using the same experimental protocol. Here, we show that both β-glucan molecules stimulate four individual immune responses in similar patterns in mice, dogs, piglets and chicks, but with different magnitudes of responses between individual β-glucan samples. Pilarski et al. [12] using the same two β-glucan molecules from this study also showed that the β-glucan samples exhibited biologically different behaviors, but both increased resistance against bacterial infection in fish. The results support two main findings, (1) the vertebrate species share the same mechanism of action for the β-glucan and, consequently, (2) this allows us to interpolate the glucan effects which are well described in human beings and mice to other species.

The IL-2 plays a central role in the growth, proliferation, and differentiation of T cells. In addition to its effects, IL-2 also stimulates the natural killer cells and cytotoxicity of monocytes [37,38]. Our results showed that both dietary β-glucan molecules stimulate the production of IL-2 in mice, dogs, piglets and chicks. Vetvicka and Oliveira [4,5], using a similar protocol observed an increase in IL-2 in pigs after the dietary administration of β-glucan. Mao et al. [39] also noticed an increase in IL-2 in pigs fed with β-glucan after an immune-challenge with LPS. In a recent study, Mo et al. [40] showed that β-glucan stimulated IL-2 production in mice. Orally administered β-glucan also increases IL-2 production in chicken [41]. To the best of our knowledge, this is the first study showing an increase in IL-2 production in dogs after the oral administration of β-glucan. It is generally accepted that glucan treatment results in signaling processes leading to the modulation of various cytokines [42].

For the most part, phagocytosis is an efficient process that eliminates invading pathogens and helps maintain homeostasis. Some specialized cells such as neutrophils and monocytes/macrophages perform this very efficiently and have therefore been named professional phagocytes [43]. In our study, with the exception of BG01 for dogs, both β-glucan molecules significantly stimulated the phagocytosis index of monocytes and neutrophils in mice, dogs, piglets and chicks. Similar results were also obtained in pigs [4,5], mice [44] and chickens [45,46]. The increase in IL-2 and the phagocytosis index triggered by the β-glucan corroborates a wide range of studies showing that β-glucan promotes bacterial and viral resistance in vertebrates [12,47,48].

Several studies have shown that β-glucan supplementation increases antibody response and consequently the β-glucan can be broadly used as vaccine adjuvant [49–52]. These effects were observed in mice [53], dogs ([54]; Vetvicka and Oliveira [4,5]) and in clinical trials involving humans [55]. Thus, both β-glucan molecules increase antibody production against ovoalbumin in mice, dogs, piglets and chicks, which corroborate the findings discussed in the literature. As described previously, the increase in IL-2 production in all four species by β-glucan may also assist in the increase of antibody formation.

During the course of their evolution, vertebrates encountered the harmful effects of fungi. Therefore, vertebrates have developed mechanisms to protect themselves against these fungal invaders [29,56]. Glucans are highly conserved structural components of cell walls of fungi and β-glucans have been considered major fungal pathogen-associated molecular patterns (PAMPs). Thus, throughout the course of their evolution, the vertebrates created mechanisms/receptors to recognize fungi, especially β-glucan. As the innate immune system is fairly conserved among vertebrates due to its ancient roots in evolutionary history and as, in general, the two β-glucan molecules showed a similar pattern response among the species, we hypothesized that these

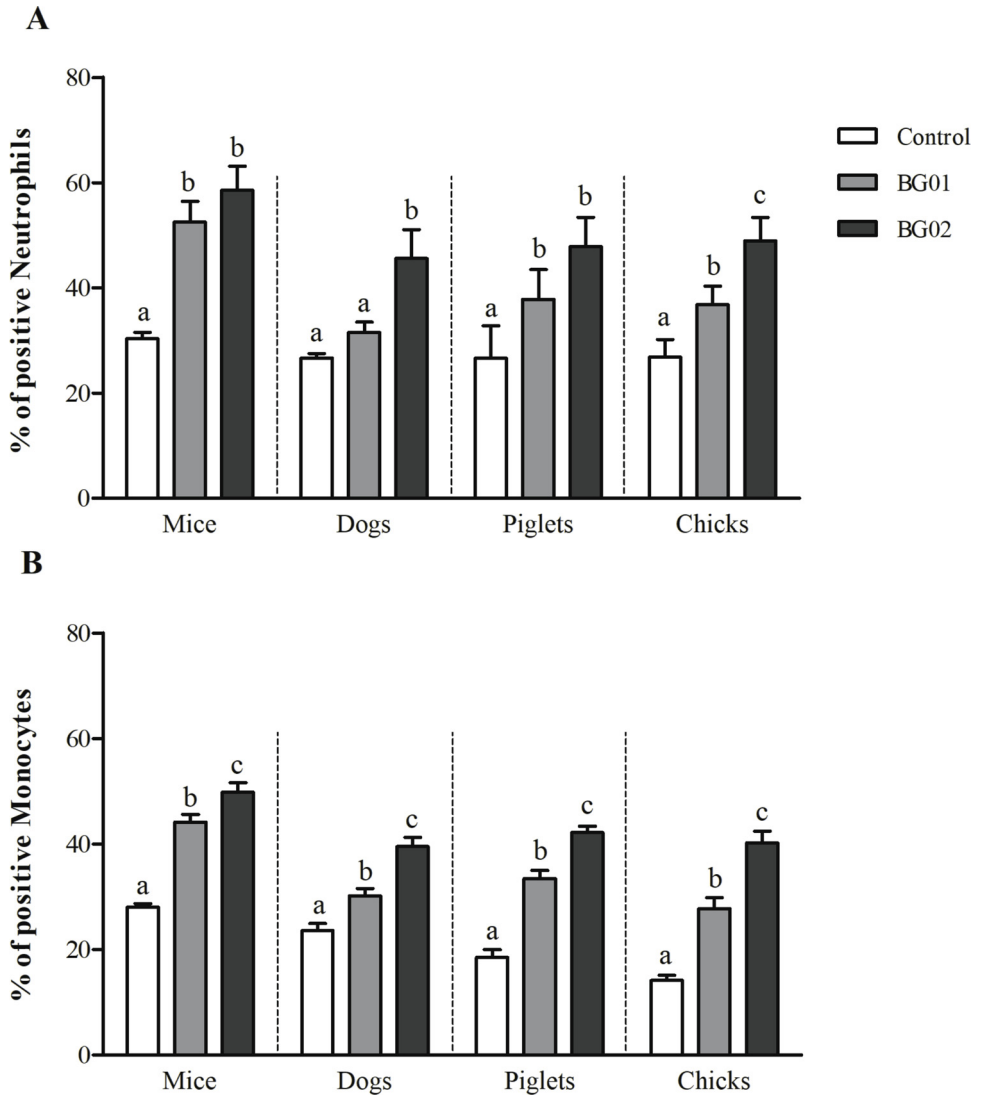

Fig. 2. Percentage of positive cells for phagocytosis of neutrophils (A) and monocytes (B) of mice, dogs, piglets and chicks fed a control diet and one supplemented with 15 mg kg^{-1} day^{-1} of BG01 or 25 mg kg^{-1} day^{-1} of BG02. Dissimilar lower case letters indicate a difference among treatments within the same animal group. The values are means ± 1 standard deviation (S.D.), N = 5.

species shared the same mechanism behind the β-glucan effects found here.

In general, our results showed that both β-glucan molecules had a similar pattern response among the species, but with a different magnitude response between the β-glucan samples. This finding allows us to extrapolate that the same mechanism of β-glucans action is shared among these species as mentioned above. However, considering all the information available about β-glucans actions in different species, we

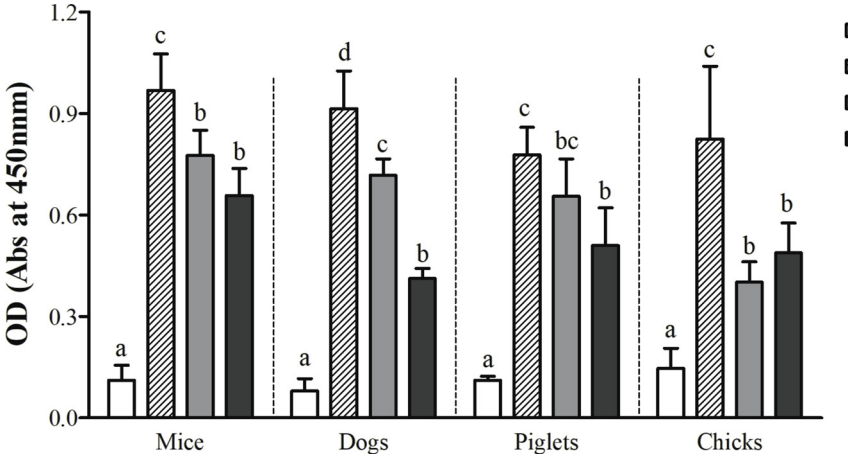

Fig. 3. Antibody production after stimulation with ovalbumin of mice, dogs, piglets and chicks fed a control diet and one supplemented with 15 mg kg^{-1} day^{-1} of BG01 or 25 mg kg^{-1} day^{-1} of BG02. ADJ means adjuvant control. Dissimilar lower case letters indicate a difference among treatments within the same animal group. The values are means ± 1 standard deviation (S.D.), N = 5.

discussed below the plausible mechanism shared among these species. When orally administered, β-glucans are non-digestible by enzymes of vertebrates and the main absorption and immune-stimulation begin in the small intestine. Specifically, β-glucans can modulate the mucosal immune response by cells of Peyer's patches as well as intestinal intraepithelial lymphocytes [57,58]. The M cells (in Peyer's patches) are a unique subset of specialized epithelial cells that transport macromolecules and particulate antigens through the epithelial layer [59]. Thus, one path for β-glucans uptake in the lumen could be a gate through M cells in the Peyer's patches of the small intestinal lumen. Second, the dendritic cells (DCs) of the follicle-associated epithelium extend projections into the lumen to recognize antigens for presentation to intraepithelial lymphocytes [60,61]. The DCs capture β-glucans by binding to several receptors, such as dectin-1, toll like receptors 2 (TLR) and 6, CR3, scavenger receptors, or lactosylceramide [62]. Third, the innate immune cells in the intestine recognize pathogens through pathogen recognition receptors (PRRs). In response to invading fungi, the innate immune cells of Peyer's patches recognize the fungi membrane components such as mannan and β-glucan [56]. The β-glucans are recognized by dectin-1, TLR2, and TLR6, whereas mannans are recognized by dectin-2, mannan receptors (MRs), TLR4, DC-SIGN, galectin 3, and FcRγ [48,63,64]. Through phagocytosis process, macrophages and DCs fragmentize the β-glucans that are bound to dectin-1 and TLR2. Adaptive immune cells such as B and T cells can also be activated by TNF-α, IL-2, IL-10, and IL-12 secreted by macrophages and DCs [65] (For review see Brown and Gordon [66], Volman et al. [48], Soltanian et al. [29], Batbayar et al. [56] and De Smet et al. [49]).

In this study we used two different β-glucan molecules. The β-glucan molecules showed a similar pattern response in all species, but with difference magnitudes between the β-glucan samples. Rice et al. [67] investigated the absorption of three soluble glucans after oral administration and showed a difference in oral pharmacokinetics among the glucans. Brown and Gordon [66] have suggested that high molecular weight (MW) and/or particulate β-glucans from fungi directly activate leukocytes, while low MW β-glucans from fungi only modulate the response of cells when they are stimulated with, for example, cytokines. Pilarski et al. [12] using the same two β-glucan molecules from this study, also observed different magnitudes in growth and immunity in fish. Specifically, BG01 increased immunostimulation, while BG02 improved growth performance. These effects can be related to absorption, where BG01 absorbed more and consequently caused a higher immune-stimulation and BG02 absorbed less and caused a higher prebiotic effect on the intestine and consequently higher growth performance. *In vivo* effects of β-glucan may depend on their molecular weight, which might be caused by differences in uptake from the intestinal lumen. As we discussed before, the mechanism of β-glucan absorption is complex and even minor changes in the glucan structure can influence their effects. Thus, these statements can explain the different magnitude responses between the β-glucan samples found here.

5. Conclusions

The current literature recognizes more than 31,000 papers that discuss the immunological and other physiological activities of glucans. However, scientific reports directly comparing individual glucans are limited [12,67–69], and a direct comparison of the effects of glucan in several species is completely absent. Therefore, this report is not important because it describes the biological activity of two samples of glucan, but because it is the first report demonstrating that two different β-glucan samples showed a similar pattern response in four vertebrate species. This fact allows us to interpolate with more reliability the glucan effects obtained in one vertebrate species to another species.

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