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A review of the immune activity of chitooligosaccharides

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Abstract

Under the influence of the COVID-19, people's awareness of physical health and immunity has increased significantly. Chitooligosaccharide is an oligomer of β-(1, 4)-linked D -glucosamine, furthermore, is one of the most widely studied immunomodulators. Chitooligosaccharide can be prepared from the chitin or chitosan polymers through enzymatically, chemically or physically processes. Chitooligosaccharide and its derivatives have been proven to have a wide range of biological activities including intestinal flora regulation, immunostimulant, anti-tumor, anti-obesity and anti-oxidation effects. This review summarizes the latest research of the preparation methods, biological activities in immunity and safety profiles of Chitooligosaccharide and its derivatives. We recapped the effect mechanisms of Chitooligosaccharide basing on overall immunity. Comparing the effects of Chitooligosaccharide with different molecular weights and degree of aggregation, a reference range for usage has been provided. This may provide a support for the application of Chitooligosaccharide in immune supplements and food. In addition, future research directions are also discussed.

Keywords: Chitooligosaccharide; intestinal flora; immunity; anti-tumor; safety.

Practical Application: In the USA, COS has been applied as a supplements ingredient. It has been added to daily foods and beverages in China, Japan and Canada. In Europe, COS appeared in meal capsules.

1 Introduction

The world experienced the outbreak of coronavirus disease 2019 (COVID-19) recently, posing an enormous threat to global public health and economies (Ugur & Buruklar, 2022). Consumers are looking for natural immune ingredients from daily supplements and foods.

Chitooligosaccharide (COS), an oligomer of β-(1, 4)-linked D -glucosamine, can be prepared from the chitin or chitosan polymers, inside, the chitin is rarer and must be extracted from the exoskeletons of arthropods, such as shrimp and crab, and insects and the cell walls of fungi. The molecular weights (MW) of COS is highly related to its biological activity, and its polymerization degree (DP) is 2~10. Therefore, COS can be dissolved in water, is non-cytotoxic, can be readily absorbed through the intestines and excreted in urine (Muanprasat & Chatsudthipong, 2017). The reduced MW couples with different degree of deacetylation (DD) and DP, makes COS a strongly viable option for a variety of applications in daily supplements, food, cosmetics, animal nutrition and agriculture industries (Tabassum et al., 2021; Ngoc et al., 2022; Okutan & Boran, 2022). It has been applied to the food industry around the world as a prebiotic: a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017). In USA, COS has been applied as a supplement's ingredient. It has been added into daily foods and beverages in Japan and Canada. In Europe, COS appeared in meal capsules.

Modern biomedical research largely focuses on significant immunoregulation effects of COS. The present review briefly summarizes the key roles of COS in the treatment of immunoregulation, with a focus on the functions of COS in intestinal and tumor immunity regulation, and describes recent clinical and pre-clinical trials investigating the potential of COS in immunoregulation therapy.

2 Preparation process of COS

Since the chitin from marine raw materials is a complex of calcium carbonate, protein, and small amounts of lipids, it must be further processed for industrial applications. After shredding and cleaning the crustacean shell waste, minerals and proteins need to be removed (Arnold et al., 2020). Due to the high MW and degree of polymerization (DP), the water solubility of these polymers is limited. Furthermore, the industrial applicability of chitin and Chitosan is also restricted. However, there are converted into soluble and more biologically active forms, COS

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and GlcNAc, and these problems are effectively solved (Qin & Zhao, 2019). COS can be prepared by enzymatic hydrolysis methods using specific enzymes or non-specific enzymes, physical methods such as ultrasonic, ultraviolet or microwave treatment, and chemical methods such as acid or alkali hydrolysis. Specific enzymes is chitosanases,meanwhile, non specific enzymes include cellulose, protease, amylase, etc. Preparation methods of COS are shown as Table 1.

The COS obtained by enzymatic hydrolysis also has three types of reactive functional groups. In addition to the amino/acetamido group at C-2, namely the primary and secondary hydroxyl groups at the C-3 and C-6 positions. The existence of these functional groups makes COS possess various biological activities.

3 Intestinal flora regulation of COS

The intestinal flora is divided into probiotics, pathogenic bacteria and conditional pathogens. The occurrence of many diseases is related to the imbalance of intestinal flora (Yan et al., 2021; Zhang et al., 2021). COS have been extensively researched for their prebiotic activities. which can promote the proliferation of probiotics while inhibiting the growth of pathogenic bacteria (Ying et al., 2002; Koppová et al., 2012). The effect methods have been summarized:

1)COS directly acts on microorganisms

0.1% COS can directly destroy the morphology of Actinomycetes (Choi et al., 2001). It has been reported that COS can rupture the cells of Gram-positive pathogenic, *Bacillus cereus,* and dissolve solute. Furthermore, it can inhibit the growth of Gram-negative bacteria, *Escherichia coli,* by blocking nutrient absorption (Kim et al., 2003).

At present, researchers thought that the main mechanisms of COS against pathogenic bacteria are as follows:

The high concentration of amino groups of COS enables them to bind and adsorb readily on the negative microbial cell membrane. These results in the formation of an impermeable layer on the cell surface. So as to prevent nutrients from entering the cell and interfere with the normal physiological metabolism of bacteria (Tsai et al., 2002; Kim & Rajapakse, 2005). COS could chelate heavy metals and nutrients (Naveed et al., 2019). COS can polymerize with the cell wall components of Gram-positive pathogenic bacteria to form positive ion precipitation, exposing the cell membrane to osmotic pressure, dissolving the cytoplasm, and lysing the cell (Lee et al., 2002). COS penetrates into the cells of pathogenic bacteria and flocculates with negatively charged solutes, and disrupts cell metabolism (Zhang, 2019) and DNA synthesis of pathogenic bacteria, and blocks RNA transcription (Figure 1).

The main mechanisms of COS promote probiotics proliferation are as follows:

The macromolecule COS cannot be absorbed into the blood (Zeng et al., 2008), and enters the back part of the small intestine to play a role (Chen et al., 2020), and finally is degraded in the intestine (Fernandes et al., 2008). However, the fully deacetylated COS cannot be digested by biological enzymes, thus increasing the applicability of COS to probiotics (Nurhayati et al., 2016), thereby promoting the growth of probiotics.

2)COS indirectly acts on microorganisms

COS could reduce the susceptibility to pathogenic bacteria by improving the immune activity of the body (Li et al., 2019). COS absorbed into the blood can cause a series of biological reactions (Chae et al., 2005; Fernandes et al., 2008), and promote the growth of probiotics, increase the secretion of short-chain fatty acids (SCFA) and lactic acid, and reduce the number of pathogenic bacteria (Selenius et al., 2018). COS improves the integrity of intestinal structure: increase the height of intestinal villi, improve intestinal structure, and effectively improve the absorption of nutrients in the small intestine. Increase CLDN3 expression, reduce DAO and endotoxin levels, and maintain the integrity of the intestinal barrier (Vishu Kumar et al., 2005), promote healthy intestinal environment.

The current research on the effect of COS on the regulation of intestinal flora, the molecular weight is mainly focused on the COS with a molecular weight of <3000 Da, and 0.01-0.5% COS *in vitro* experiments can exert the effect of inhibiting pathogenic bacteria (Table 2). *In vivo* experiments among them, the working concentration of COS is 30-500 mg/kg (0.003%~0.05%), which can regulate animal intestinal flora.

Table 1. Advantages and disadvantages for preparation methods.

Methods	Advantage	Disadvantage
Chemical method	Easy operation	1. difficulty products separation 2. serious environmental pollution
Physical method	Easy purification	Low yield
Non-specific enzymatic hydrolysis method	1. Friendly environment 2. Higher efficiency 3. Controllable operation and monitoring	Lower degradability and productivity
Chitosanase hydrolysis method	Directly produce the desired degradation products without degrading other groups of the substrate	1. Limited sources of enzymes 2. Limited volume-produce

Source: Aam et al. (2010); Liang et al. (2018); Zhou et al. (2020).

4 Direct effects of COS on immunity

In addition to acting on the intestinal flora and the way of immune response, COS can also regulate the body's immunity by entering the blood. Immune cells include T\B lymphocytes, natural killer (NK) cells, and mononuclear phagocytes. The immune response mainly includes humoral immunity, phagocytosis and cellular immunity. Non-specific immunity mainly includes the phagocytosis and elimination of pathogens by macrophages and white blood cells. Macrophages and dendritic cells play a key role in the immune system, activating the immune

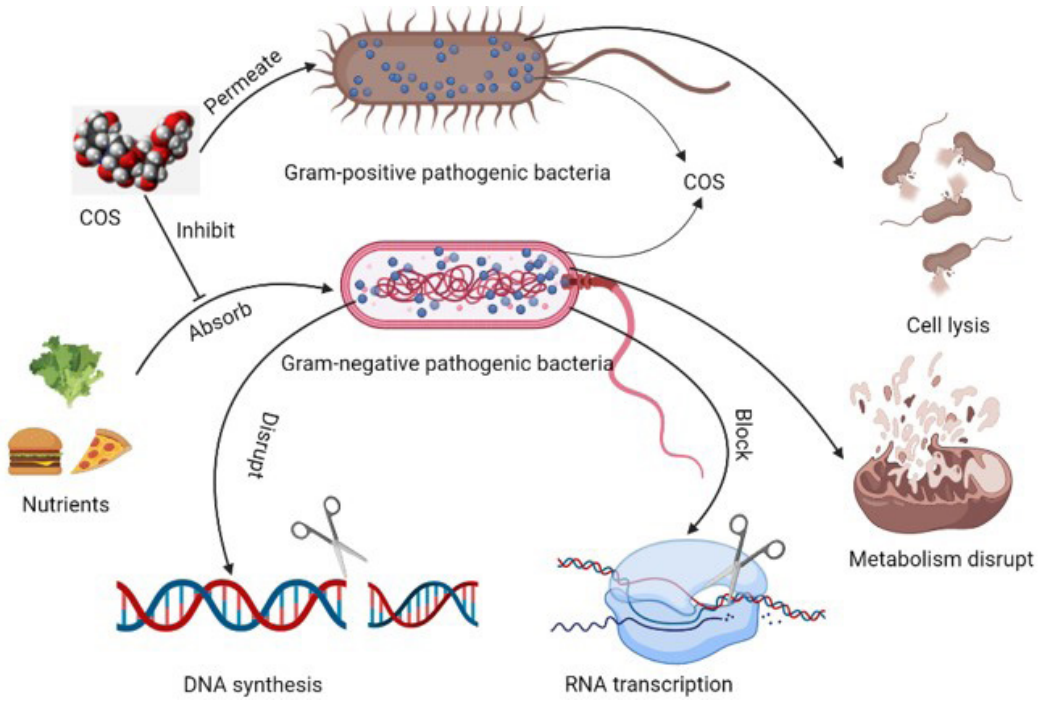

Figure 1. The mechanisms of COS againsts pathogenic bacteria. COS enables bind and adsorb readily on the negative microbial cell membrane to interfere normal physiological metabolism of bacteria. COS penetrates into the cells of pathogenic bacteria and disrupts cell metabolism and DNA synthesis, blocking RNA transcription.

Table 2. Inhibition and Promotional effect of COS on Pathogenic Bacteria and Probiotics.

MW/DP	Content	Effect strain
2000-3000Da	0.1%	*Actinomycetes*
—	0.1%	*Streptococcus mutans*
<3000Da	0.25%	*Staphylococcus aureus*
<1500Da	0.5%	*Escherichia coli*
		Lactobacillus rhamnosus
DP=2~6	0.3%	*Bacillus cereus*
<1000Da	5 mg/mL (0.5%)	*Listeria monocytogenes*
DP=4~5	100 mg/mL (0.01%)	*Bacillus subtilis*
		Staphylococcus aureus
		Escherichia coli
DP=2~8	0.1%	*Lactobacillus brevis*
		Lactobacillus casei
<1000 Da	100mg/kg (0.01%)	*Bifidobacterium*

Source: Ying et al. (2002); Koppová et al. (2012); Zhang (2019); Li et al. (2019); Chae et al. (2005); Sánchez et al. (2017); Wu et al. (2013); Wan et al. (2017).

response through cytokine release, phagocytosis, and antigen presentation. The activation of macrophages is the key to promote immune activity. Stimulated macrophages release a variety of pro-inflammatory mediators and cytokines, including nitric oxide (NO), tumor necrosis factor-a (TNF-a), prostaglandin E2 (PGE2), interleukin-1β (IL- 1β), Interleukin-6 (IL-6) (Shi et al., 2022) . COS can be recognized by immune cells and exert immunostimulatory properties, thereby improving the body's immunity (Table 3), and resisting diseases.

The current researches have revealed that COS mainly improves the body's immunity through the following ways:

COS activates AMP-activated protein kinase (AMPK) through calcium-sensing receptor (CaSR) to enhance tight junction in epithelial cells (Muanprasat & Chatsudthipong, 2017), increasing the production of intestinal SCFA and enhancing the ability of SCFA to bind to G-coupled protein receptors on leukocytes.

COS interacts with carbohydrate receptors on intestinal epithelial cells and immune cells (Muanprasat & Chatsudthipong, 2017). and produces IFN-γ through intestinal epithelial cells, activate macrophages (Deng et al., 2008) and improve the activity of NK cells (Zhai et al., 2018). Promoting humoral immunity and releasing antibody production such as IgA, IgM and IgG (Kong et al., 2018) to enhance the body's resistance.

In immune cells, COS inhibits the suppression of P38 MAPK, extracellular signal-regulated kinase (ERK1/2) and C-Jun N-terminal kinase (JNK1/2) in mitogen-activated protein kinase (MAPK)-dependent pathways to block the nuclear translocation of activator protein 1 (AP-1). And COS promotes inhibitory kappa B (IKB) degradation in the nuclear factor-kappa B (NF-KB) pathways. As a result, the pro-inflammatory mediators, such as tumor necrosis factor-α(TNF-α), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), prostaglandin E 2 (PGE 2) and nitric oxide (NO), has been inhibited (Muanprasat & Chatsudthipong, 2017) (Figure 2).

5 Direct effects of COS on tumor growth inhibition

Immune cells play important roles in either anti-tumor or pro-tumor responses. The balance of immune cell is related to tumor progression and recurrence. A large number of immune cells produce various interactions between the interaction and the activation and inhibition of anti-tumor response.

Table 3. COS improves the immune function of the body.

MW/DP	Content	Model	Item
1000 Da	0.04 g/kg	Immunodeficient mice	NK cell activity
			Immune organ index
			Phagocytic index
DP=3~7	0.6 g/kg	Mouse	Serum IgG content
DP=4~11	0.25, 0.5, 1 g/kg	Cy-treated mice	IL-2
			IL-12
			IFN-γ
			IL-10
			Immune organ indices
			DTH reaction, Footpad thickness
			Phagocytic activity
			ACP activities
			LDH activities
1000 Da, 3000 Da and 8000 Da	0.8 g/kg	Tilapia	Phagocytic activity of leucocytes, Phagocytic index of leucocytes
_	0.1, 0.2, 0.3 g/kg	mice genital tract infected by Chlamydia trachomatis	IgG, IL-11,spleen and thymus indexes
DP=3~5	0.1, 1, 10, and 100 μg/mL,	RAW 264.7	Macrophage phagocytosis capacity assay
	0.1, 1, 10, or 100 μg/mL	Mouse splenic lymphocyte	Splenic lymphocyte proliferation
		Splenic natural killer cell	NK cells activity
	33, 100 and 333 mg/kg	Rats	Thickness of the paw, Spleen and thymus indices, Mononuclear phagocytic system (MPS) function

Source: Zhai et al. (2018); Kong et al. (2018); Mei et al. (2013); Guan et al. (2019); Xing et al. (2017).

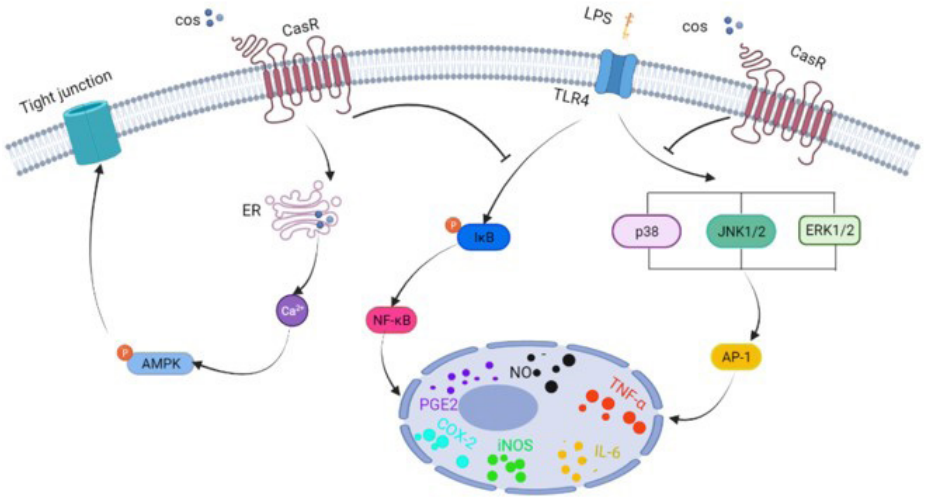

Figure 2. The mechanisms of COS improve immunity. COS activates AMPK through CaSR to enhance tight junction in epithelial cells, inhibits the suppression of MAPK and NF-KB pathways to inhibit secrete pro-inflammatory mediator.

Tumor metastasis is one of the main complications of cancer (Nam et al., 2007). NO promotes metastasis by inducing angiogenesis and inhibiting platelet aggregation. Inhibition of iNOS can help anti-inflammatory and inhibit tumor metastasis. In addition, matrix metalloproteinase MMPS is also involved in tumor growth and invasion. And apoptosis is caused by the complex synergy of multiple genes and multiple proteins (Han et al., 2016). A wide range of studies revealed that COS can effectively inhibit tumor cell metastasis and growth, and promote tumor cell apoptosis (Shen et al., 2009).

The current researches on the anti-tumor effect of COS mainly focuses on the following aspects (Figure 3):

1) Tumor cell's glycolysis and energy will be inhibited by COS through the inhibition of tumor-specific variant of pyruvate kinases;

2)Inhibition of DNA synthesis in cancer cells.

COS can inhibit DNA synthesis and metastasis in cancer cells by increasing the expression of p21, reducing the expression of cyclin A, cdk2 and PCNA, and inhibiting the action of ornithine decarboxylases (Nam et al., 2007).

3) Start apoptotic factors to promote cancer cell apoptosis.

Activate the apoptotic promoter Caspase-3, successfully block cancer cell proliferation and Gz/M phase arrest (Xing et al., 2017), up-regulate pro-apoptotic gene Bax, down-regulate pro-apoptosis the expression of gene Bcl-2 (Beerheide et al., 2000; Tsujimoto & Shimizu, 2000), increase the radiosensitivity of cancer cells and promotes cancer cell apoptosis.

4) Inhibit the synthesis of genes and proteins related to tumor metastasis and block tumor metastasis.

COS can inhibit CD147, MMP-9 (Van Ta et al., 2006), MMP-2 by inhibiting the CD147/MMP-2 pathway activation, thereby inhibiting the expression of matrix protease MMPS, inhibiting the metastasis and invasion of cancer cells (Nam & Shon, 2009).

There are many studies on the use of low-molecular-weight COS for anti-tumor, which may be related to the ability of low-molecular-weight COS to be absorbed into the blood. The research results show that COS has a good inhibitory effect on many tumors (Table 4).

6 Safety profile of COS

In order to fully understand the potential therapeutic applications of COS, many studies have used *in vitro* and *in vivo* models to investigate the safety of COS, especially its mutagenicity, cytotoxicity and systemic toxicity have been evaluated (Table 5).

Mutagenicity is the ability of a substance to induce mutations, which can cause alterations in cell function and promote the development of diseases, especially cancer. It has been proved that COS has no mutagenic potential in both *in vitro* and *in vivo* models.

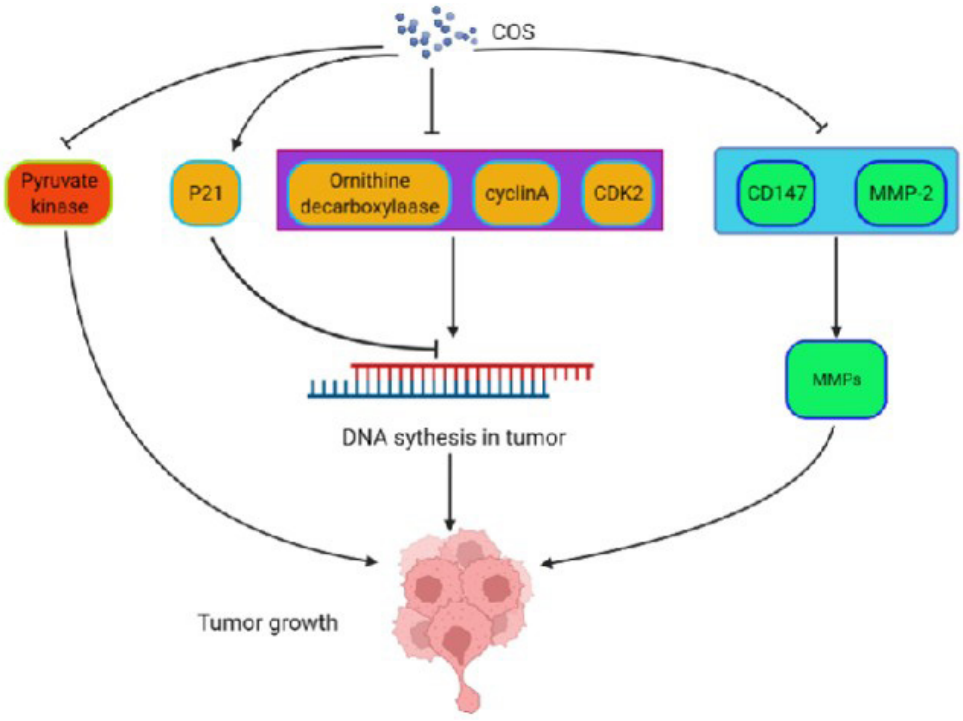

Figure 3. The mechanisms of COS anti-tumor effect. COS inhibits tumor cell's glycolysis and energy, DNA synthesis, and blocks tumor metastasis. In addition, COS promotes cancer cell apoptosis also.

Table 4. Anti-tumor effects of COS.

MW	Content	Model	Item
1000~3000Da	500 μg/mL	HT1080 cell	MMP-9
—	1, 2, 3, 4 and 5 mg/mL	SW480 cell	Inhibition rate
DP=2~6	100 pg/mL	ECs cell	IL-8
1000~3000Da	0.1, 0.5, 1, 3, 5 mg/mL	MDA-MB-231 cell	Invasive inhibition rate
1000~3000Da	0.1, 0.5, 1, 3, 5 mg/mL	HT-29 cell	NO inhibition rate
DP=3~8	500 mg/kg	Llc mice	Rate of lung metastases

Source: Van Ta et al. (2006); Han et al. (2016); Liu et al. (2011); Nam & Shon (2009); Nam et al. (2007); Shen et al. (2009).

Table 5. The safety of COS.

Item	Results
Ames test	Non-mutagenic
micronucleus assays	1 mg/mL did not induce any genetic alterations in human lymphocytes
comet assays	
micronucleus test and the chromosomal aberration test	5 g/kg/day for 180 days had no effects on the frequency of micronuclei and chromosomal aberrations in bone marrow cells in mice
Cytotoxicity	IC50 of 5 mg/mL
Acute toxicity	The median lethal dose (LD50) of COS is > 10 g/kg
Subacute toxicity studies	The no observed adverse effect level (NOAEL) of COS is > 3 g/kg/day for rats of either sex
	The NOAEL of COS is >500 mg/kg/day in mice
	The NOAEL of COS in the subchronic toxicity study is equal to 0.2% (w/w) or 124 mg/kg/day in male rats and 142 mg/kg/day in female rats

Source: Qin et al. (2006); Worku et al. (2011); Yoon et al. (2005); European Food Safety Authority (2007); Kim et al. (2001); Mattaveewong et al. (2016); Naito et al. (2007).

Further research is needed to determine the types of COS/constituents that induce dermal reactions, and subchronic and chronic toxicity of COS are necessary.

7 Conclusions and perspectives

Our studies have concluded advantages and disadvantages of different preparation methods for COS. We described how COS exerts its function on immune support through intestinal, humoral and cellular immunity, and how it plays an anti-cancer effect. The oral safety range of COS has been provided by numerous studies. In view of the 2-20 DP for COS, each DP of COS has its unique biological activity. Therefore, the biological activities of COS can be dozens of times that of chitosan. As an immunostimulatory agent, COS dues in part to their lower MW and easy absorption characteristics. We speculated that COS may have a certain application as a food ingredient in food, supplement products and pet nutrition to support daily health. Its biological activity and its molecular mechanism need to be confirmed by further studies. Clarifying the way of action of COS will promote the widely use in dietary supplements, food and medicine.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Author contributions

L. L., L. L. and S. W. designed the conception. Y. W. wrote the main manuscript text, K. Z. prepared figures 1-3. X. S., Y. H., and N. D. collected references. Z. L. designed the conception and approved the manuscript. All authors reviewed the manuscript.

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