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Dietary intervention with sialylated lactulose affects the immunomodulatory activities of mice

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ABSTRACT

Four sialylated lactuloses [*N*-acetylneuraminic acid-α2,3-lactulose (Neu5Acα2,3lactulose), *N*-acetylneuraminic acid-α2,6-lactulose (Neu5Acα2,6lactulose), deaminoneuraminc acid-α2,3-lactulose (Kdnα2,3lactulose), and deaminoneuraminc acid-α-2,6-lactulose (Kdnα2,6lactulose)] were reported to modulate the immunity of mice. The influences of cytokine expression, cell immunity, humoral immunity, and nonspecific immunity were investigated in our study using several techniques. Analysis via ELISA showed that cytokine expression was induced by sialylated lactulose treatment consistently in the serum and spleen. Among the 4 tested sialylated lactuloses, Neu5Acα2,6lactulose performed the best, simultaneously and appropriately promoting the expression of proinflammatory and anti-inflammatory factors in the serum and spleen. Kdnα2,3lactulose showed the best antioxidant activity according to detection of the activity of superoxide dismutase, myeloperoxidase, peroxidase, and alkaline phosphatase. Flow cytometry revealed that only Kdnα2,3lactulose significantly boosted the $CD3^+$ T lymphocyte ratio similarly to that of lactulose. Analysis of the hemolysin content to characterize humoral immunity revealed that Kdnα2,3lactulose notably increased hemolysin content compared with that in the control group. To evaluate the nonspecific immune effects of the 4 sialylated lactuloses, a fluorescence microsphere phagocytosis assay was used to analyze the phagocytosis of macrophages. Kdnα2,3lactulose still performed the best in enhancing the phagocytosis of macrophages, showing markedly increased phagocytic percentage and phagocytic index values compared with those in the control and lactulose groups. Comparing the differences of these 4 sialylated lactuloses in affecting immunity in mice revealed that Kdnα2,3lactulose had the best overall performance in influencing cytokine expression, cell immunity, humoral immunity, and nonspecific immunity. This study provides critical support for use of sialylated lactuloses as potential immunomodulators in foods.

Key words: sialylated lactuloses, antioxidant enzyme, cytokine level, immunomodulation, dietary intervention

INTRODUCTION

Functional oligosaccharides are prebiotics. Some reports have shown that functional oligosaccharides promote the proliferation and growth of bifidobacteria, regulate intestinal microbiota, reduce blood lipid levels, benefit nutrient digestion and absorption, resist oxidation, and enhance immunity (Lu et al., 2002; Mussatto and Mancilha, 2007; Wang et al., 2013). Sialylated oligosaccharides, a group of oligosaccharides containing sialic acid structures, are abundant in breast milk and present important health benefits to neonates because they exert substantial effects on immune function enhancement, gut maturation, pathogens, and cognitive development (Angata and Varki, 2002; McNicholl and McNicholl, 2001; Simon et al., 1997; Zeng et al., 2019). Based on their potential benefits, sialylated oligosaccharides may be an interesting ingredient in the design of infant nutritional formulas. *N*-Acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and deaminoneuraminc acid (Kdn) are 3 basic forms of sialic acids. Exploring the functionality of these sialylated glycans in detail may make them potential supplements in infant formula (Martín-Sosa et al., 2003; Thongaram et al., 2017). Lactulose [4-O-(β-D-galactopyranosyl)-D-fructose] is a functional disaccharide that does not exist in nature and was first discovered as an isomerized lactose in heated milk in 1929 (Montgomery and Hudson, 1930). It can promote the proliferation of bifidobacteria and lactobacilli, reduce the pH value in the colon environment, and inhibit the growth of harmful bacteria (Nooshkam et al., 2018). The pharmacological properties of lactulose make it widely used to

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treat chronic constipation, inhibit *Salmonella* growth, treat enteritis, reduce blood ammonia values, regulate osmotic pressure, prevent tumors, and enhance immunity in the field of medicine (Llamas et al., 2010; Jiang et al., 2015). Sialylated lactuloses are a category of sialylated oligosaccharides derived from lactulose as receptors, which can regulate the structure of intestinal flora, proliferate beneficial flora, and inhibit pathogenic bacteria (Song et al., 2019). Two types of sialylated lactuloses, Neu5Ac-derived and Kdn-derived lactuloses, have been successfully synthesized (Zeng et al., 2018; Pham et al., 2019). They showed antibacterial activity against *Staphylococcus aureus* and affected immunomodulation in mice (Zeng et al., 2018, 2019). Although we have previously investigated these biological effects on 4 sialylated lactuloses, their effects on the immunological functions of mice will be further studied and are expected to be applied in the food industry.

The immune organs of the body comprise central and peripheral immune organs. The spleen is the largest peripheral immune organ of the body and contains numerous lymphocytes and macrophages. It is the center of cellular immunity and humoral immunity of the body and the main site of antibody generation (Bronte and Pittet, 2013). The spleen also contains many natural killer T cells, which sense lipid antigens and participate in diverse immune responses by secreting cytokines such as interleukins (e.g., IL-1, IL-2, IL-6, and IL-7), stem cell factor, and tumor necrosis factor (**TNF-α**) and activate downstream adaptive immune cell types (Bronte and Pittet, 2013; Li et al., 2019). Hemolysin is a sensitive, complementarily immobilized antibody that specifically binds to the erythrocyte antiprototype and is produced by stimulating the surface antigen. To some extent, the analysis of the serum hemolysin lever can reflect the proliferation, differentiation, and antibody secretion of B cells (Ma et al., 2020). Antioxidation is an important parameter to evaluate the body's health and immune status, as well as the body's ability to scavenge excess oxygen free radicals under normal conditions (Dong et al., 2015). Macrophages, an important component of the innate immune system, act as "scavengers" in the immune response (Bolego et al., 2013). They can phagocytose, digest, and process cell debris and pathogens and activate other immune cells to induce an immune response (Fukahori et al., 2014; Liang et al., 2018). After activation, they exert immunomodulatory effects by releasing diverse immune molecules (Rae et al., 2007; Pan et al., 2017).

In this study, we investigated the effects of 4 sialylated lactuloses—*N*-acetylneuraminic acid-α2,3-lactulose (**Neu5Acα2,3lactulose**), *N*-acetylneuraminic acid-α2,6-lactulose (**Neu5Acα2,6lactulose**), deaminoneuraminc acid-α2,3-lactulose (**Kdnα2,3lactulose**), and deaminoneuraminc acid-α-2,6-lactulose (**Kdnα2,6lactulose**)—on immune function in mice. The levels of cytokines in the serum and spleen, antioxidant enzyme [superoxide dismutase (**SOD**), myeloperoxidase (**MPO**), peroxidase (**POD**), and alkaline phosphatase (**AKP**)] activity of the serum, ratio of $CD3^+$ T lymphocytes in the spleen, hemolysin content, and peritoneal macrophage function of sialylated lactulose-treated mice were determined to evaluate the immunomodulatory effect.

MATERIALS AND METHODS

Materials

Lactulose was purchased from Carbosynth (Carbosynth Ltd.). Neu5Acα2,3lactulose, Neu5Acα2,6lactulose, Kdnα2,3lactulose, and Kdnα2,6lactulose were synthesized as described previously by our group (Zeng et al., 2018, 2019). The ELISA kits for TNF-α, IL-1β, serum IL-6, serum IL-10, and IL-12 were purchased from Wuhan Youersheng Trading Co. Ltd. The total SOD, POD, AKP, and MPO assay kits were purchased from Nanjing Jiancheng Bioengineering Institute. Analytical-grade NaCl, HCl, Na_2HPO_4, KCl, and KH_2PO_4 were obtained from Guangfu Science and Technology Development Co. Ltd.

A mouse peripheral blood lymphocyte isolation kit (TBD) was purchased from Beijing Ya Anda Biotechnology Co. Ltd. Anti-mouse CD3 fluorescein isothiocyanate (FITC) was purchased from Ebioscience Company. Alsever's Solution was purchased from Beijing Ji Mei Biotechnology Co. Ltd. Chickens and guinea pigs were provided by China Three Gorges University (Yichang, China). We purchased RPMI 1640 from Gibco. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich Corp. Microfiltration membranes (75 μm) and fluorescent microspheres (505/515) were purchased from Invitrogen. Six-well culture plates and cell scrapers were purchased from Corning Inc.

Animal Treatment

Specific-pathogen-free female Kunming mice (42 d old) were purchased from the China Three Gorges University (Yichang, China). The animals were tested by the disease control and prevention center of Hubei Province (permit number: SCXK 2017-0012). All animal experiments were approved by the Animal Care and Use Committee of Henan Institute of Science and Technology. The mice were housed at 22 ± 2°C under a 12-h light-dark cycle with free access to routine mouse

diet and water. After 1 wk of acclimation, the mice were randomly divided into 6 treatment groups and fed experimental diets for 14 consecutive days. The daily feeding designs were as follows: (1) 0.2 mL of normal saline (control group, **ck**, n = 10); (2) 0.2 mL of lactulose (5 mg/mL; lactulose group, n = 10); (3) 0.2 mL of Neu5Acα2,3lactulose (5 mg/mL; Neu5Acα2,3lactulose group, n = 10); (4) 0.2 mL of Neu5Acα2,6lactulose (5 mg/mL; Neu5Acα2,6lactulose group, n = 10); (5) 0.2 mL of Kdnα2,3lactulose (5 mg/mL; Kdnα2,3lactulose group, n = 10); (6) 0.2 mL of Kdnα2,6lactulose (5 mg/mL) treatment (Kdnα2,6lactulose group, n = 10); this dose was equivalent to a human dose of 3.2 mg/kg). All assays were performed on d 15.

Collection and Preservation of Eyeball Blood and Serum Samples

Eyeball blood was collected as described previously (Zeng et al., 2019). Blood was obtained from the eyeballs of experimental mice and collected in a serum separation tube, which was stored at 4°C overnight. The tube was centrifuged at 1,000 × *g* for 20 min at 4°C, and then the supernatant was collected and stored at −20°C.

Preparation of Spleen Cells and Spleen Homogenate

Spleen tissue was obtained and washed with precooled PBS (0.01 mol/L; pH = 7.0–7.2). The spleen tissue was cut into small pieces and placed into a glass homogenizer. The spleen homogenate was centrifuged at 10,000 × *g* for 5 min at 4°C; the precipitate was discarded, and the supernatant was retained.

Measurement of TNF-α, IL-1β, IL-6, IL-10, and IL-12 Cytokine Levels in Mice

The TNF-α, IL-1β, IL-6, serum IL-10, and IL-12 levels were measured using ELISA kits according to the manufacturer's protocols. Briefly, standard or samples were added to micropores. Next, the plates were washed 3 times with PBS containing 0.05% Tween 20. Each well was blocked with PBS containing 5% skim milk for 1 h at 37°C. After washing the plate 3 times, biotinylated TNF-α, IL-1β, IL-6, IL-10, or IL-12 antibody was added and incubated at 37°C for 1 h. The unbound biotinylated antibodies were then washed off, and horseradish peroxidase (HRP)-labeled avidin was added. Finally, 3,3′,5,5′-tetramethylbenzidine was added to the plate, which was then incubated for approximately 15 min. The absorbance (optical density) was measured at 450 nm using a microplate analyzer (BioTeK Instruments Inc.), and the concentrations of the samples were calculated.

Measurement of Total SOD, POD, AKP, and MPO Activities in Serum

The SOD, POD, AKP, and MPO activities in serum were determined using assay kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocols.

Activity of SOD was determined by measuring the inhibition rate of the enzyme to O_2^-. The sample and reagents provided in the kit were mixed thoroughly according to the manufacturer's protocol. After incubation at 37°C for 40 min, all the mixtures were analyzed by detecting the absorbance at 550 nm. One unit of SOD activity (U) was defined as the quantity of SOD required to produce 50% inhibition of nitrite reduction in 1 mL of reaction solution by measuring the change in absorbance at 550 nm.

Activity of POD was measured based on the change in absorbance at 420 nm by catalyzing H_2O_2. The sample and reagents provided in the kit were mixed according to the manufacturer's protocols. The mixtures were incubated at 37°C for 30 min. Finally, reagent IV was added to each tube, and the absorbance of each sample was measured at 420 nm.

Activity of AKP was determined using an alkaline phosphatase assay kit according to the manufacturer's instructions. The samples were added to 96-well plates, and substrates and p-nitrophenol from the Alkaline Phosphatase Assay Kit were subsequently added and incubated for 30 min at 37°C. Finally, the AKP activity was determined at a wavelength of 405 nm.

Activity of MPO was measured according to the assay kit manufacturer's instructions. Briefly, 10 μL of the supernatant was transferred to PBS (pH 6.0) containing 3,3′-dimethoxybenzidine and H_2O_2. The MPO activity of the supernatant was determined by measuring the H_2O_2-dependent oxidation of 3,3′-dimethoxybenzidine and was expressed as units per gram of total protein (u/g).

Determination of $CD3^+$ T Cells

Lymphocytes were isolated from the eyeball peripheral blood of mice using Ficoll (Sigma-Aldrich Corp.). Briefly, 5 mL of Ficoll was added to a 15-mL centrifuge tube. Next, peripheral blood diluted with PBS was placed on top of the Ficoll layer. After that, the tube was centrifuged at 400 to 500 × *g* for 30 min at 4°C, and the liquid in the tube was divided into 4 layers. The second layer (lymphocyte layer) was removed into

another 15-mL tube. The lymphocytes were washed with PBS 3 times. Finally, the cells were resuspended in 1 mL of RPMI 1640 medium for further use.

The $CD3^+$ T cells were detected as follows. The cell concentration was adjusted to 1×10^6 cells in 100 μL of PBS containing 0.5% BSA. Next, 0.25 μg of anti-mouse CD3-FITC was added to the tube and incubated at 4°C for 30 min in the dark. The samples were washed twice with PBS containing 0.5% BSA. After that, the cells were resuspended in 0.5 mL of PBS buffer containing 0.5% BSA and analyzed using a CytoFLEX flow cytometer (Beckman Coulter).

Hemolysin Determination

Hemolytic activity was determined as described previously (De Carli and Tasca, 2002; Arslan and Çelik, 2013; Imran et al., 2020). Fresh chicken blood was washed with normal saline 3 times by centrifugation at 352 × *g* at 4°C for 5 min to collect erythrocytes. After the last treatment with sialylated lactulose, each mouse was immunized by intraperitoneal injection of 0.2 mL of chicken erythrocytes suspended in 5% (vol/vol) normal saline. On the seventh day, the eyeball peripheral blood of the mice was obtained, and the serum was diluted 100-fold with normal saline. Next, the diluted serum (1 mL) was mixed with 0.5 mL of 5% chicken erythrocyte suspension and 0.5 mL of 10% complement (guinea pig serum) and incubated at 37°C for 30 min. The absorbance of the supernatant of each sample was measured at 540 nm. A blank control without serum was used as a negative control.

Measurement of Peritoneal Macrophage Function

The phagocytic capacity of macrophages was detected as described by Zhang et al. (2020) with some modifications. After treatment with sialylated lactulose for the last time, 0.2 mL of 2% chicken erythrocyte suspension was injected into the enterocoelia of each mouse to activate the macrophages. Four days later, peritoneal macrophages were collected as follows. The mice were euthanized by cervical dislocation, and resident peritoneal macrophages were harvested by peritoneal cavity lavage with 4 mL of PBS per mouse. Next, the intraperitoneal fluid was collected and centrifuged at 500 × *g* for 10 min. The cell pellet was washed with PBS once, followed by centrifugation at 500 × *g* for 10 min. The cell number of macrophages was adjusted to 4 to 6 × 10^5/mL with Hanks' solution. One milliliter of the cell suspension was added to each well of a 6-well plate. Fluorescent microspheres (1×10^7/well) pretreated with 1% BSA at a volume ratio of 1:100 were added to each well. Next, the mixture was incubated at 37°C for 120 min in the dark. After incubation, the supernatant was discarded, and the plate was washed gently twice with PBS buffer. The adherent cells were scraped, resuspended, and then analyzed by flow cytometry (Beckman Coulter). The phagocytic percentage (**PP%**) and phagocytic index (**PI**) were calculated according to the following formulas:

$$\text{PP\%} = \text{Number of macrophages engulfing fluorescent microspheres/Total macrophages} \times 100\%;$$

$$\text{PI} = \text{Total number of fluorescent microspheres that were engulfed/Total macrophages.}$$

Statistical Analysis

Each experiment was repeated 3 times, and the average was calculated. The data were expressed as means ± standard deviation. The data were analyzed using the SPSS 16.0 software package (SPSS Inc.) for one-way ANOVA by pairwise comparisons, and the significant differences of the means ($P < 0.05$) were analyzed using Duncan's multiple-range test.

RESULTS

Effect of Sialylated Lactulose on Serum Cytokine Levels in Mice

Cytokines, including proinflammatory factors (TNF-α, IL-1β, and IL-6) and anti-inflammatory cytokines (IL-10 and IL-12), in serum were measured by ELISA. The levels of TNF-α, IL-1β, and IL-6 in the lactulose group were significantly ($P < 0.05$) higher than those in the ck group, whereas the levels of IL-10 and IL-12 were notably lower than those in the ck group (Table 1). In the sialylated lactulose-treated groups, the TNF-α, IL-1β, IL-6, IL-10, and IL-12 levels in the Neu5Acα2,3lactulose group were not significantly different from those in the ck group. In the Neu5Acα2,6lactulose oral gavage-treated group, the levels of proinflammatory factors (TNF-α, IL-1β, and IL-6) were higher than those in the ck group and lower than those in the lactulose group ($P < 0.05$).

The levels of TNF-α, IL-1β, and IL-6 in the serum Kdnα2,3lactulose group were markedly higher than those in the ck group and considerably lower than those in the lactulose group ($P < 0.05$). The IL-6 levels between the Kdnα2,3lactulose group and the lactulose group were not different. However, the levels of IL-10 and IL-12 in the Kdnα2,3lactulose group were markedly lower than those in the ck group. However, the IL-10 and IL-12 concentrations in the Kdnα2,3lactulose

Table 1. Effects of sialylated lactuloses on mouse serum cytokine levels (data expressed as mean ± SD)[1]

Group	Cytokine level (pg/mL)				
	TNF-α	IL-1β	IL-6	IL-10	IL-12
ck	11.19 ± 0.89^{c}	17.72 ± 1.45^{d}	39.53 ± 4.39^{b}	26.04 ± 2.44^{b}	31.19 ± 3.78^{b}
Lactulose	22.70 ± 1.60^{a}	28.70 ± 1.39^{a}	60.54 ± 6.28^{a}	15.59 ± 1.72^{c}	25.15 ± 1.44^{c}
Neu5Acα2,3lactulose	11.11 ± 1.05^{c}	18.01 ± 1.09^{d}	39.90 ± 3.17^{b}	26.25 ± 2.27^{b}	31.63 ± 3.55^{b}
Neu5Acα2,6lactulose	17.98 ± 1.63^{b}	24.85 ± 1.61^{bc}	53.33 ± 5.31^{a}	31.37 ± 2.31^{a}	40.35 ± 4.38^{a}
Kdnα2,3lactulose	19.09 ± 1.72^{b}	26.14 ± 1.66^{b}	56.50 ± 5.90^{a}	13.23 ± 1.24^{c}	21.63 ± 3.34^{c}
Kdnα2,6lactulose	12.25 ± 0.70^{c}	22.97 ± 1.17^{c}	44.00 ± 4.01^{b}	28.92 ± 1.92^{ab}	37.62 ± 4.04^{a}

$^{a–d}$In the same column, different lowercase superscript letters indicate significant differences ($P < 0.05$).

[1]ck = control group; TNF = tumor necrosis factor; Neu5Acα2,3lactulose = *N*-acetylneuraminic acid-α2,3-lactulose; Neu5Acα2,6lactulose = *N*-acetylneuraminic acid-α2,6-lactulose; Kdnα2,3lactulose = deaminoneuraminc acid-α2,3-lactulose; Kdnα2,6lactulose = deaminoneuraminc acid-α-2,6-lactulose.

group were 15.14% and 14.0% lower than those in the lactulose group, respectively.

The levels of TNF-α and IL-6 in the serum of the Kdnα2,6lactulose group were not significantly different from those in the ck group, but the level of IL-1β was significantly higher than that in the ck group ($P < 0.05$). The levels of TNF-α, IL-1β, and IL-6 in the Kdnα2,6 lactulose group were significantly lower than those in the lactulose group ($P < 0.05$), and the level of IL-10 was not significantly different from that in the ck group; however, the concentration of IL-12 was significantly higher than that in the ck group ($P < 0.05$). Additionally, the concentrations of IL-10 and IL-12 in the Kdnα2,6lactulose group were higher than those in the lactulose group ($P < 0.05$).

Effect of Sialylated Lactulose on Spleen Cytokine Levels in Mice

Lactulose oral gavage treatment significantly increased the levels of proinflammatory cytokines (TNF-α, IL-1β, and IL-6; $P < 0.05$) and decreased the levels of IL-10 and IL-12 ($P < 0.05$) compared with those in the ck group (Table 2). Similar to serum results, cytokine expression in the Neu5Acα2,3lactulose group was not significantly different from that in the ck group. However, the levels of proinflammatory factors in the Neu5Acα2,6lactulose, Kdnα2,3lactulose, and Kdnα2,6lactulose groups were partially or markedly increased ($P < 0.05$) compared with those in the ck group. Specifically, compared with the lactulose group, the levels of IL-10 and IL-12 in the Neu5Acα2,3lactulose group significantly increased ($P < 0.05$), whereas those of IL-10 and IL-12 in the Kdnα2,3lactulose group significantly decreased ($P < 0.05$) compared with those in the ck group and were not different from those in the lactulose group.

The TNF-α and IL-6 levels in the Kdnα2,6lactulose group were not significantly different from those in the ck group, but the IL-1β concentration was significantly lower ($P < 0.05$) than that in the lactulose group and significantly higher ($P < 0.05$) than that in the ck group. The level of IL-10 was not significantly different from that in the ck group but was significantly ($P < 0.05$) higher than that in the lactulose group; however, the IL-12 level was significantly ($P < 0.05$) higher than that in the lactulose and ck groups. These results

Table 2. Effects of sialylated lactuloses on mouse spleen cytokine levels (data expressed as mean ± SD)[1]

Group	Cytokine level (pg/mL)				
	TNF-α	IL-1β	IL-6	IL-10	IL-12
ck	48.90 ± 4.05^{d}	140.07 ± 17.17^{d}	85.62 ± 13.01^{d}	46.38 ± 6.95^{b}	58.15 ± 4.90^{c}
Lactulose	85.31 ± 7.51^{a}	240.26 ± 27.70^{a}	139.48 ± 16.02^{a}	34.53 ± 4.60^{c}	41.58 ± 4.42^{d}
Neu5Acα2,3lactulose	47.92 ± 5.26^{d}	149.89 ± 22.80^{cd}	85.31 ± 9.68^{d}	47.24 ± 5.22^{b}	58.06 ± 5.67^{c}
Neu5Acα2,6lactulose	73.84 ± 5.89^{b}	185.07 ± 16.76^{b}	119.47 ± 10.58^{b}	60.09 ± 6.81^{a}	81.85 ± 5.52^{a}
Kdnα2,3lactulose	76.62 ± 6.55^{b}	220.84 ± 26.77^{a}	129.33 ± 10.81^{ab}	28.09 ± 4.39^{c}	34.41 ± 3.81^{e}
Kdnα2,6lactulose	59.06 ± 5.73^{c}	169.98 ± 26.37^{bc}	102.09 ± 7.30^{c}	51.88 ± 8.50^{b}	69.84 ± 6.56^{b}

$^{a–e}$In the same column, different lowercase superscript letters indicate significant differences ($P < 0.05$).

[1]ck = control group; TNF = tumor necrosis factor; Neu5Acα2,3lactulose = *N*-acetylneuraminic acid-α2,3-lactulose; Neu5Acα2,6lactulose = *N*-acetylneuraminic acid-α2,6-lactulose; Kdnα2,3lactulose = deaminoneuraminc acid-α2,3-lactulose; Kdnα2,6lactulose = deaminoneuraminc acid-α-2,6-lactulose.

Table 3. Effects of sialylated lactulose on serum antioxidant enzyme activity in mice (data expressed as mean ± SD)[1]

Group	Antioxidant enzyme			
	SOD (U/mL)	MPO (U/mL)	POD (U/mL)	AKP (King unit/dL)
ck	161.21 ± 17.23^{c}	38.94 ± 8.80^{c}	11.92 ± 1.46^{c}	7.82 ± 0.86^{c}
Lactulose	232.67 ± 22.10^{b}	57.52 ± 6.26^{b}	16.16 ± 2.25^{b}	11.47 ± 1.30^{b}
Neu5Acα2,3lactulose	168.27 ± 14.47^{c}	38.50 ± 5.92^{c}	11.30 ± 1.61^{c}	7.87 ± 0.86^{c}
Neu5Acα2,6lactulose	121.74 ± 15.22^{d}	27.43 ± 6.19^{d}	7.07 ± 1.12^{d}	5.34 ± 1.17^{d}
Kdnα2,3lactulose	281.28 ± 27.86^{a}	71.68 ± 10.80^{a}	22.96 ± 2.77^{a}	13.45 ± 1.93^{a}
Kdnα2,6lactulose	129.35 ± 17.28^{d}	31.86 ± 8.80^{cd}	8.30 ± 1.64^{d}	6.84 ± 0.63^{c}

$^{a–d}$In the same column, different lowercase superscript letters indicate significant differences ($P < 0.05$).

[1]ck = control group; SOD = superoxide dismutase; MPO = myeloperoxidase; POD = peroxidase; AKP = alkaline phosphatase; Neu5Acα2,3lactulose = *N*-acetylneuraminic acid-α2,3-lactulose; Neu5Acα2,6lactulose = *N*-acetylneuraminic acid-α2,6-lactulose; Kdnα2,3lactulose = deaminoneuraminc acid-α2,3-lactulose; Kdnα2,6lactulose = deaminoneuraminc acid-α-2,6-lactulose.

indicated that Kdnα2,6lactulose was appropriate to activate immunity while maintaining reasonable levels of secretion of proinflammatory and anti-inflammatory cytokines.

Effect of Sialylated Lactulose on Serum Antioxidant Enzyme Activity in Mice

The activity levels of 4 antioxidant enzymes (SOD, MPO, POD, and AKP) in the serum of each group of mice were measured using commercial kits according to the manufacturer's instructions. The activity of the 4 antioxidant enzymes in the lactulose group was significantly ($P < 0.05$) higher than that in the ck group (Table 3). No difference was found in the activity of the 4 antioxidant enzymes in the serum between the Neu5Acα2,3lactulose and ck groups. In the Neu5Acα2,6lactulose group, the activities of the 4 antioxidant enzymes were significantly ($P < 0.05$) lower than those in the ck group. In the Kdnα2,3lactulose group, the activities of the 4 antioxidant enzymes were much stronger than those in the lactulose and ck groups. Specifically, the activities of SOD, MPO, POD, and AKP in the Kdnα2,3lactulose group were increased by 74.48, 84.08, 92.62, and 71.99%, respectively, compared with those in the ck group and were increased by 20.89, 24.62, 42.08, and 17.26%, respectively, compared with those in the lactulose group. The activity of SOD in the Kdnα2,6lactulose group was significantly ($P < 0.05$) decreased compared with that in the ck group; however, no obvious difference was found in the MPO, POD, and AKP enzyme activities among the Kdnα2,6lactulose and ck groups.

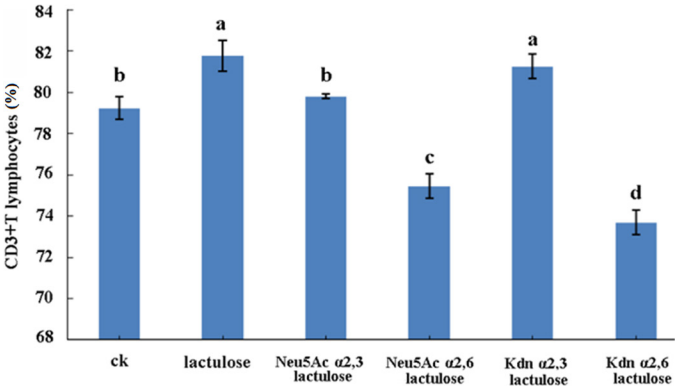

Figure 1. Effects of sialylated lactuloses on the ratio of CD3^{+} T lymphocytes isolated from the eyeball peripheral blood in mice by flow cytometry. ck = control group. Bars with different letters were statistically different ($P < 0.05$). The daily feeding designs were as follows: 0.2 mL of normal saline fed for control group; 0.2 mL of lactulose (5 mg/mL) fed for lactulose group; 0.2 mL of *N*-acetylneuraminic acid-α2,3-lactulose (Neu5Acα2,3lactulose), *N*-acetylneuraminic acid-α2,6-lactulose (Neu5Acα2,6lactulose), deaminoneuraminc acid-α2,3-lactulose (Kdnα2,3lactulose), and deaminoneuraminc acid-α-2,6-lactulose (Kdnα2,6lactulose; 5 mg/mL) fed for each test group (n = 10), respectively. The assays were performed after 14 consecutive days of feeding (on d 15). Error bars indicate SD.

Effect of Sialylated Lactulose on the Proportion of CD3^{+} T Cells in Mice

CD3^{+} T cells are mature lymphocytes and the main active cells involved in cellular immunity. The increase in the CD3^{+} T cell ratio indicates the enhancement of cellular immune function (Fukahori et al., 2014; Jin et al., 2017). Here, flow cytometry was used to analyze the effect of sialylated lactulose on the proportion of CD3^{+} T cells in mice. The ratio of CD3^{+} T lymphocytes in the lactulose group was significantly ($P < 0.05$) higher than that in the ck group (Figure 1). Among the 4 sialylated lactulose-treated groups, only in the Kdnα2,3lactulose group was the ratio of CD3^{+} T lymphocytes remarkably higher than that in the ck group and approximately the same as that in the lactulose-treated group (Figure 1). The ratios of CD3^{+} T lymphocytes in mice fed Neu5Acα2,6lactulose and Kdnα2,6lactulose were markedly lower than those in ck mice.

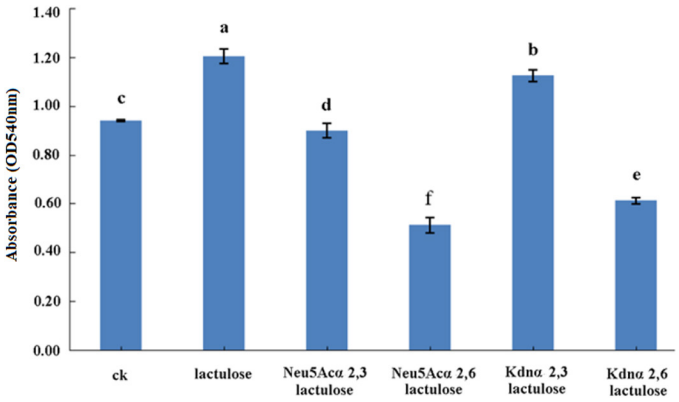

Figure 2. Effects of sialylated lactuloses on the hemolysin content in serum from eyeball peripheral blood in mice. ck = control group; OD = optical density at 540 nm. Bars with different letters were statistically different ($P < 0.05$). Feeding design as follows: 0.2 mL of normal saline for control group; 0.2 mL of lactulose (5 mg/mL) for lactulose group; 0.2 mL of *N*-acetylneuraminic acid-α2,3-lactulose (Neu5Acα2,3lactulose), *N*-acetylneuraminic acid-α2,6-lactulose (Neu5Acα2,6lactulose), deaminoneuraminc acid-α2,3-lactulose (Kdnα2,3lactulose), and deaminoneuraminc acid-α-2,6-lactulose (Kdnα2,6lactulose; 5 mg/mL) for each test group (n = 10), respectively. The assays were performed after 14 consecutive days of feeding (on d 15). Error bars indicate SD.

Effect of Sialylated Lactulose on the Hemolysin Content in Mice

An increased hemolysin content indicates that humoral immunity in the body is improved. Lactulose treatment significantly increased the hemolysin content ($P < 0.05$) of mice compared with that of ck mice (Figure 2). Following sialylated lactulose treatment, only Kdn-α2,3-lactulose significantly ($P < 0.05$) enhanced the hemolysin level, although the degree of improvement was slightly lower than that with lactulose. In the mouse serum of the other 3 sialylated lactulose-treated groups, the hemolysin content was significantly ($P < 0.05$) lower than that in the ck group. These results indicate that sialylated lactulose (Kdnα2,3lactulose) can improve the body's humoral immunity.

Effect of Sialylated Lactulose on Peritoneal Macrophage Function in Mice

The phagocytic capacity of macrophages is an important index of nonspecific immune evaluation. In this study, the phagocytosis of fluorescent microspheres was used to detect the phagocytosis ability of macrophages. Lactulose treatment did not affect the phagocytic capacity of macrophages because the PP% and PI values showed no difference between the lactulose and ck groups (Table 4). However, the PP% and PI values of the Neu5Acα2,3lactulose group and the Kdnα2,3lactulose group were significantly increased ($P < 0.05$) compared with those of the ck and lactulose groups, and Kdnα2,3lactulose performed better. The PP% and PI values of the mice fed Neu5Acα2,3lactulose were 35.82% higher than those of the ck and lactulose groups, and the PP% and PI values of the mice in the Kdnα2,3lactulose group were 191.04% higher than those of the ck and lactulose groups.

DISCUSSION

Human milk oligosaccharides are important components of human milk, including neutral and acidic oligosaccharides (ten Bruggencate et al., 2014). Human milk oligosaccharides have a variety of functions, such as stimulating immunity, anti-inflammation, anti-cancer, anticoagulation, and antiviral activities (Mussatto and Mancilha, 2007; Triantis et al., 2018). Neutral oligosaccharides, such as galacto-oligosaccharides and fructo-oligosaccharides, have been widely used to fortify infant formula (Giovannini et al., 2014; ten Bruggencate et al., 2014; Vandenplas et al., 2018). Synthetic acidic oligosaccharides, such as sialylated oligosaccharides, have not been designed for infant formula, to date (ten Bruggencate et al., 2014). However, acidic oligosaccharides have recently received increased attention because they also modulate immune functions (Kurakevich et al., 2013; Yu et al., 2016). In our previous study, we successfully synthesized 4 sialylated lactuloses (Neu5Acα2,3lactulose, Neu5Acα2,6lactulose, Kdnα2,3lactulose, and Kdnα2,6lactulose; Yu et al., 2016; Zeng et al., 2018). Neu5Acα2,3lactulose and Neu5Acα2,6lactulose showed inhibitory effects on *Staph. aureus*. Analysis of the thymus and spleen index and immunoglobulin levels revealed that Kdnα2,3lactulose

Table 4. Effects of sialylated lactulose on phagocytosis of fluorescent microspheres by peritoneal macrophages in mice (data expressed as mean ± SD)[1]

Group	PP%	PI
ck	0.67 ± 0.18^{c}	0.0067 ± 0.0018^{c}
Lactulose	0.67 ± 0.06^{c}	0.0067 ± 0.0006^{c}
Neu5Acα2,3lactulose	0.91 ± 0.03^{b}	0.0091 ± 0.0003^{b}
Neu5Acα2,6lactulose	0.62 ± 0.05^{c}	0.0062 ± 0.0005^{c}
Kdnα2,3lactulose	1.95 ± 0.10^{a}	0.0195 ± 0.0010^{a}
Kdnα2,6lactulose	0.49 ± 0.17^{c}	0.0049 ± 0.0017^{c}

$^{a–c}$In the same column, different lowercase superscript letters indicate significant differences ($P < 0.05$).

[1]ck = control group; PP% = phagocytic percentage; PI = phagocytic index; Neu5Acα2,3lactulose = *N*-acetylneuraminic acid-α2,3-lactulose; Neu5Acα2,6lactulose = *N*-acetylneuraminic acid-α2,6-lactulose; Kdnα2,3lactulose = deaminoneuraminc acid-α2,3-lactulose; Kdnα2,6lactulose = deaminoneuraminc acid-α-2,6-lactulose.

performed the best in regulating the immune function of mice and was relatively safe (Zeng et al., 2019). However, the differences in the roles of these 4 sialylated lactuloses in humoral, cellular, and innate immunity have not yet been elucidated. The present study was designed to elucidate the differences.

Cytokines, synthesized and secreted by immune cells or nonimmune cells, are important immunoactive molecules that regulate the immune response by regulating immune cell growth, differentiation, and function. Many studies have reported that oligosaccharides show proinflammatory or anti-inflammatory properties by regulating cytokine levels in treated mice (Laparra et al., 2013; Algieri et al., 2014; Plaza-Díaz et al., 2018). Chitosan oligosaccharide exerts anti-allergic effects against shrimp tropomyosin-induced food allergies by affecting Th1 and Th2 cytokines (Kong et al., 2018). Azuma et al. (2015) reported that oral chito-oligosaccharides reduced serum levels of proinflammatory cytokines (TNF-α and IL-6). Corbin et al. (2008) suggested that the balance between proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and anti-inflammatory cytokines (IL-10 and IL-12) determines the process by which the body produces inflammation. The biological activity of some oligosaccharides derived from lactulose has been studied. Algieri et al. (2014) assessed the preventative effects of oligosaccharides derived from lactulose (OsLu) in the trinitrobenzenesulfonic acid model of rat colitis and found that OsLu showed excellent anti-inflammatory properties compared with lactulose and inhibited various proinflammatory markers, such as IL-1, IL-6, IL-12, and IL-23 and other chemokines. Laparra et al. (2013) deduced that galactooligosaccharides obtained from transgalactosylation of lactulose (GOS-Lu) significantly reduced pathogen adhesion and inhibited the production of IL-1β by intestinal cells stimulated by the pathogens tested. The effect of GOS-Lu on the *Listeria monocytogenes*-mediated TNF-α production was more discrete and did not affect TNF-α production in cultures exposed to *Salmonella enterica*. In the present study, compared with the other 3 tested sialylated lactuloses, Neu5Acα2,6lactulose performed the best in simultaneously promoting the expression of proinflammatory (TNF-α, IL-1β, and IL-6) and anti-inflammatory cytokines (IL-10 and IL-12) to an appropriate extent in the serum and spleen of mice. Kdnα2,3lactulose markedly enhanced the expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) but decreased the expression of anti-inflammatory cytokines (IL-10 and IL-12). Thus, in terms of regulating cytokine expression, Kdnα2,3lactulose may perform the best to help the body resist pathogens by promoting the release of proinflammatory cytokines.

As biocatalysts produced by living cells, antioxidant enzymes can inhibit free radical production, balance metabolism, and slow the rate of oxidation (Kong et al., 2018). Essential enzymes in the animal body, an increase in the contents of SOD, POD, AKP, and MPO can improve the body's disease resistance (Dong et al., 2015; Oshi et al., 2018). Antioxidant metabolism is a critical mechanism involved in immunoregulation (Pilipow et al., 2018). Many oligosaccharides have been reported to possess both antioxidant and immunomodulatory activities. For example, alginic acid oligosaccharide accelerates weaned pig growth by regulating antioxidant capacity and immunity (Wan et al., 2016). *Ulva lactuca* oligosaccharides are highly efficient at preventing apoptosis and exert substantial antiaging effects in mice (Liu et al., 2019). Fructo-oligosaccharide can modulate the immune system (Franco-Robles and López, 2015) and increase catalase (antioxidant) levels (Galdino et al., 2018). In the present study, dietary intervention with Kdnα2,3lactulose obviously improved the activities of antioxidant enzymes in the serum of mice. These results indicate that the improved immune function of Kdn-α2,3-lactulose may be attributable to its antioxidant activity.

The T cells (T lymphocytes) comprise a group of heterogeneous lymphocytes with different functions. The CD3 molecule is a critical marker of T cells. CD3 cells are mature lymphocytes and the main active cells involved in cellular immunity; $CD3^+$ T cells are the main active cells involved in cellular immunity. They play an essential role in regulating inflammation and participate in the immune response. An increased proportion of $CD3^+$ T cells indicates enhanced cellular immunity (Fukahori et al., 2014; Jin et al., 2017). Some oligosaccharides are adjuvants that bind to the surfaces of some exogenous antigens (viruses, toxins, and eukaryotic cells) to improve humoral and cellular immunity mediated by B lymphocytes and T lymphocytes (Ngo et al., 2008; Wang et al., 2010; Marcobal et al., 2013). In our study, as shown in Figure 1, among the 4 sialylated lactulose groups, only the Kdnα2,3lactulose group had a $CD3^+$ T lymphocyte ratio markedly higher than that in the ck group and the same as that in the lactulose-treated group. These results indicate that Kdnα2,3lactulose can effectively enhance cellular immunity.

The generation of hemolysin under blood stimulation is a crucial indicator to evaluate immune function, and an increase in hemolysin indicates improved humoral immunity (Ma et al., 2020). Ma et al. (2017) reported that soybean oligosaccharides significantly induced hemolysin production in mice and suggested that soybean oligosaccharides as prebiotics may improve the

human immune response. In our study, compared with that in the ck group, the hemolysin level of mice in the Kdnα2,3lactulose group was markedly increased, whereas those in the other 3 groups of mice treated with Neu5Acα2,3lactulose, Neu5Acα2,6lactulose, and Kdnα2,6lactulose were decreased. These results indicate that only Kdnα2,3lactulose can enhance the humoral immunity of mice.

Macrophages are a critical component of the innate immune system and play a vital role in the immune response (Gordon, 2003). Phagocytic capacity is an important indicator of macrophage function. Many oligosaccharides enhance the phagocytosis of macrophages (Yuan et al., 2006; Jiao et al., 2012). Unsaturated guluronate oligosaccharide augments the antibacterial activity of macrophages, and enhanced macrophage phagocytosis plays an important role in this process (Xu et al., 2014). In our study, the PP% and PI values (indicators that characterize the phagocytic capacity of macrophages) of the mice in the Kdnα2,3lactulose group were notably higher than those of the ck and lactulose groups. Thus, Kdnα2,3lactulose improves the nonspecific immunity of mice by enhancing the phagocytosis ability of macrophages.

CONCLUSIONS

Our results demonstrated that the 4 sialylated lactuloses had different effects on the immunological functions of mice. The Neu5Ac2,3lactulose group showed no significant effects on the immune activity of mice. Neu5Ac2,6lactulose and Kdnα2,6lactulose increased the concentrations of proinflammatory factors and anti-inflammatory factors in mice. Kdnα2,3lactulose contributed to improving cellular immunity, humoral immunity, and nonspecific immunity in mice. Thus, Kdnα2,3lactulose plays a prominent role in enhancing immune activity. This function warrants further investigation for its potential application in the food industry.

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