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ORIGINAL ARTICLE

The effects of *Spirulina platensis (Arthrospira platensis)* and *Saccharomyces cerevisiae* on the distribution and cytokine production of CD4+ and CD8+ T-lymphocytes in rabbits

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ABSTRACT. Natural additives have become one of the most alternative immune enhancer nowadays. In particular, *Spirulina platensis* (*Arthrospira platensis)* (SP) and *Saccharomyces cerevisiae* (SC) have been used for improving the immune system and quality of life. The aim of this study was to regulate the immune effect of *S. cerevis*iae and *S. platensis (A. platensis)* combination. Forty male New Zealand white rabbits, aged 5-6 weeks, were analysed in 4 groups: Control (basal diet); SC (added 3 g/kg diet), SP (added 5% of the diet); SC and SP (added 3 g/kg SC and 5% SP of the diet). The entire experiment lasted 90 days. Blood samples were obtained by ear venipuncture on the 90th day. The CD4+ and CD8+ T lymphocyte values were determined by flow cytometry and cytokines (IFN-γ and IL-4) were determined by ELISA. According to the results, there were no significant differences in the expression of cytokines, but serum CD4+/CD8+ increased in the animals fed SP and SC+SP supplemented diets (3 g/kg and 5% of the diet, respectively). It was concluded that *S. platensis (A. platensis)* may be used as an immune enhancer, although further studies are needed to clarify the effects of spirulina supplement on immunity.

Key words: rabbits*, Saccharomyces cerevisiae, Spirulina platensis, Arthrospira platensis,* immunity.

RESUMEN. Los aditivos naturales se han convertido en uno de los mayores potenciadores alternativos de inmunidad actualmente. *Spirulina platensis* (SP) y *Saccharomyces cerevisiae* (SC) se han usado para mejorar el sistema inmunológico y la calidad de vida. El objetivo de este estudio fue regular el efecto inmune de la combinación de *S. cerevisiae* y *S. platensis* (*A. platensis*). Se analizaron 40 conejos blancos machos de Nueva Zelanda, de 5 a 6 semanas de edad, en 4 grupos: Control (dieta basal); SC (añadido 3 g / kg de dieta); SP (añadido 5% de la dieta); SC y SP (se añadieron 3 g/kg SC y 5% SP de la dieta), respectivamente. El experimento completo duró 90 días. Muestras de sangre fueron obtenidas mediante punción venosa de oído en el día 90. Los valores de linfocitos T CD4+ y CD8+ se determinaron mediante citometría de flujo y las citoquinas (IFN-γ e IL-4) se determinaron por ELISA. De acuerdo con los resultados, no hubo diferencias significativas en la expresión de citoquinas, pero el suero CD4+/CD8+ aumentó en los animales alimentados con SP y SC + SP dietas suplementadas (3 g/kg y 5% de la dieta, respectivamente). Se concluye que *S. platensis* (*A. platensis*) se puede utilizar como un potenciador immune, aunque se necesitan más estudios para aclarar los efectos de la suplementación de espirulina en la inmunidad.

Palabras clave: conejos, *Saccharomyces cerevisiae, Spirulina platensis*, *Arthrospira platensis,* inmunidad.

INTRODUCTION

Natural foods are a complementary part of a healthy immune system. Recently, immunomodulation properties of these supplements have been widely studied in animals and humans. Researchers attempt to establish the immune-promoting effects of these supporting ingredients with immune parameters. Broadly speaking, cellular and humoral immunity parameters are contemplated, similar as a T lymphocyte subpopulation and cytokines. The T cells coreceptors CD4+ and CD8+ play an important role in modulating immune responses to pathogens and tumor cells, and are important in orchestrating overall immune responses (Arstila *et al* 1994, Broere *et al* 2011). Activation of these T lymphocyte subpopulations stimulate plasma cells for producing antibodies. The CD4+ T lymphocytes help in homeostasis of T helper cells while CD8+ T lymphocytes play a central part in processing the cellular immunity.

Previously, using the additives such as antibiotics, hormones and drugs was beneficial but had an adverse effect leading to cause resistance of the organism, therefore the natural additives are the protein source to replace them. There are many different types of natural supplements and it is recommended to use these additives separately. At present, research is focused on determining how these supplements improve immune system function and their benefits to animals.

Natural additives such as *Saccharomyces cerevisiae* (SC) and *Spirulina platensis* (SP) are seen as natural protein sources in animal feeds. Out of these two products, *S. platensis (A. platensis)* is a cyanobacterial species that has been speculated to be associated with modulation of the immune system (Qureshi and Ali 1996). *S. platensis (A. platensis)* has been approved as a health food by the World Health Organization (WHO) and it is set to become

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one of the most alternative treatments in the 21st century (Annapuna *et al* 1991, Hasler 2002). The immunmodulatory function of spirulina was first described in mice (Cheng-Wu 1994). The researchers demonstrated that spirulina enables antibody production due to the effects of its contents, polysaccharide and phycocyanin. A study at the University of Missisippi, School of Pharmacy, reported the extraction of a polysaccharide from spirulina called *Immulina*. A powerful immunostimulating activity of this polysaccharide due to activation of monocytes and macrophages was demonstrated (Pugh *et al* 2001). Mao *et al* (2000) showed that spirulina induced the secretion of IFN-γ in human blood cells, suggesting that spirulina plays an important role in the balance of the Th1 and Th2 cytokine producing. The positive effects of *S. cerevisiae* can originate from either their direct nutritional or their health promoting effects such as behaving as a bioregulator of the intestinal microflora and reinforcing the natural defenses of the host (Hassanein and Soliman 2010). Even so, this yeast cell wall component effects on immunity has been considered for a long time. The yeast cell wall component beta glucan works as an immunstimulant by activating functional activities of macrophage (Pillemer *et al* 1954). There are several studies focused on immune system modulation by yeast and beta glucan (Ortuno *et al* 2002, Robertsen *et al* 1990, Shen *et al* 2009).

These natural products*, S. cerevisiae* and *S. platensis (A. platensis)* support the preventive medicine in animals due to their macrophage activity and positive effects on the immune system. Although some studies have been performed on the immunity of *S. cerevisiae* and *S. platensis (A. platensis)* in animals (Cheng-Wu 1994, Hassanein and Soliman 2010, Salazar-Monroy *et al* 2012, Watanuki *et al* 2008), the combined impression of *S. cerevisiae* and *S. platensis (A. platensis)* have not been investigated so far. This study aimed to evaluate the combined effect of *S. cerevisiae* and *S. platensis (A. platensis)* on immune system in animals.

MATERIAL AND METHODS

ANIMALS, GROUPS AND FEEDING

Forty male New Zealand white rabbits, aged 5-6 weeks with a mean body weight of 1,000.9 g were randomly allocated on a weight basis to four groups: Control, I. SC (added 3 g/kg diet), II. SP (added 5% of the diet), III. Combination of SC and SP (added 3 g/kg SC and 5% SP of the diet, respectively). The rabbits were housed individually in purpose-built metal cages. Feed and water were offered *ad libitum* throughout the 90 day test. Basal diet (pelleted) was formulated to contain 2,500 kj ME/kg metabolizable energy, 16% crude protein and was projected to take on maintenance requirements according to the NRC (1997). Chemical composition and ingredients of the diet are provided in tables 1 and 2. Chemical analyses of diets were carried out according to AOAC (2000). Basal diet was supplemented with *S. cerevisiae* live yeast culture (Yea Sacc^{1026} Altech, Nicholasville: 1x10^{9} CFU g^{-1}) and/or *S. platensis (A. platensis)* (Egert, Izmir-Turkey). Doses were provided and modified according to literature (Cheng-Wu 1994, Hassanein and Soliman 2010, Salazar-Monroy *et al* 2012, Watanuki *et al* 2008), and the supplements were provided together for the study.

Table 1. Chemical composition of basal diet (%DM).

	Diet
Dry matter%	88.89
Crude protein%*	16.00
Ether extracts%*	3.52
Crude fiber%*	10.95
Ash	7.68

* Based on Dry Matter percentage.

Table 2. Ratio of feed ingredients.

Ingredients	Usage rate (%)
Barley	30.00
Alfalfa meal	25.00
Corn14	17.61
Soybean meal 46	10.83
Rice bran	10.08
Corn bran	3.60
Limestone	1.40
Salt	0.80
Dicalcium phosphate 18	0.28
Vitamin premix*	0.25
Methionine	0.09
Anticoccidial	0.03
Antioccidial	0.03
Total	100.00

*Premix: Vit A 4,800,000 IU, Vit D 800,000 IU, Vit E 14,000 mg, Biotin 18 mg, CH-CL 50,000 mg, Folic acid 400 mg, Niacin 8,000 mg, Pant. Acid 4,000 mg, Riboflavin 2,800 mg, Thiamin 1,200 mg, Pyridoxine 2,000 mg, Vit K 1,600 mg, Zinc 24,000 mg, Iron 2,000 mg, Iodine 400 mg, Manganese 32,000 mg, Selenium 60 mg, Copper 24,000 mg.

Rabbits were preferred as research material because they are commonly used for research on the immune system and production of polyclonal antibodies. At the same time, they are less belligerent in nature and have few health problems (Zutphen *et al* 1993). The experimental protocols were approved by the Animal Care and Use Committee of Uludag University and are in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The study was carried

out with the permission of Uludag University Animal Experimentation Local Ethics Committee (Approval No: 2010-09/01).

MEASUREMENTS

CD4+ and CD8+ T Lymphocytes. Blood samples were collected from all rabbits at the end of the study. Subpopulations of T lymphocytes (CD4+, CD8+ and CD4+/CD8+) were analysed by flow cytometry according to method of Davis *et al* (2008). All reagents used in flow cytometry protocols were purchased from Becton Dickinson while the antibodies were from Santa Cruz (USA) and Novus Biological (LLC, USA). Blood samples were obtained by ear venipuncture using acid citrate dextrose (ACD) blood tubes. Two flow cytometry tubes were used for each sample and 50 µl blood added in all tubes. In first tube, whole blood (50 µl) samples were stained with 10 µl Isotype control antibody mouse IgG2a:sc3878 (Santa Cruz). 50 µl of diluted primary antibody (Anti CD4, Ken-4, Novus Biologicals, LLC, USA) added in second tube and incubated 15 minutes on ice. After incubation, the tubes washed three times with cell wash (Becton Dickinson, BD) and centrifuged for 5 min at 1500 rpm. 50 µl of the diluted conjugate (Goat anti Mouse IgG2a-FITC, Santa Cruz Biotechnology, USA) were added in all tubes, then they were incubated on ice in the darkness. Afterwards that, lyse solution (FACS Louis, 2 ml) was added for lysing for 10 min incubation time. After incubation, samples were sentrifugated for 5 min at 1500 rpm. Finally, 200 µl cell wash were added before analysis with FACSCanto cytometry (BD FACSCantoTM, BD Biosciences, ABD).

Cytokines. Plasma IL-4 and IFN-γ were measured using a commercially available rabbit ELISA kit (USCN, USA). The minimum detectable dose was 6.1 pg/ml for IFN-γ and 14.2 pg/ml for IL-4. Assays were performed colorimetrically using a plate reader (Sun *et al* 2010). As a result of readings, the standard optical denstiy values were obtained from control and other groups.

STATISTICAL ANALYSIS

Statistical analyses were performed with SPSS (Version 17.0; Chicago, IL). Data were examined for normality distribution and variance homogeneity assumptions. All the values were grouped and the means and standard errors were calculated. One-way ANOVA was applied to the all parameters to analyse the difference between groups. Differences were considered significant at $P<0.05$. If the difference between groups was significant ($P<0.05$), differences were evaluated by Tukey's test (Dowdy and Wearden 1981). On the other hand, in non-homogenous groups, differences between means were analysed by Kruskal Wallis and following Mann Whitney U test between groups one by one (Dawson and Trapp 2001).

RESULTS

Immunity parameters of the control and experimental groups (SC, SP and SC+SP) are shown in figure 1. The percentage of CD4+ were 34.39%, 35.73%, 36.04%, 35.98% and the percentage of CD8+ were 19.37%, 20.50%, 16.36%, 16.54%, respectively in control, SC, SP and SC+SP. The CD4+/CD8+ was significantly higher in SP and SC+SP group compared to control ($P<0.05$). The percentage of CD4+ in the SP and SC+SP groups was higher than the control group, but not significantly. There were no significant alterations in the T lymphocyte subpopulation of the groups. Also, FSC/SSC histograms and gates in working sample, and other linear histograms are presented in figure 2.

DISCUSSION

The CD4+ T lymphocytes help in homeostasis of T helper cells and CD8+ T lymphocytes play a central part in processing the cellular immunity. However, the CD4+/CD8+ T lymphocytes reveals the action of T lymphocytes. On the other hand, CD4+T lymphocytes activate the type 1 T helper (Th1) and the type 2 T helper (Th2) cells, thereby, Th1 secretes IFN-gamma (IFN-γ) and Th2 secretes IL-4 cytokines. This basic immune mechanism refers to the specific protective response and calls for balance and harmony.

Animal studies have shown *S. platensis* (*A. platensis*) to effect on the immune system in chickens, carp and mouse (Baojiang 1994, Qureshi *et al* 1996, Watanuki *et al* 2008). In a mice study (Cheng-Wu 1994), it was found an association between the polysaccharide and phycocsiyanin metabolites of spirulina and effects on the improving the development of the white blood cells, bone cell products and the percentage of T lymphocytes. On the other hand, Hirarashi *et al* (2002) found that addition of the extract of *S. platensis* (Immulina) did not affect the CD4+ and CD8+ T lymphoctyes in humans, and suggested that spirulina had no effect on cellular immunity. Perhaps the discrepancy is to be explained by the mutations in strains, protocols, and spirulina proportions used in each experiment. Løbner *et al* (2008) studied Immulina in humans and observed this extract of spirulina increases the CD4+ T cell proliferation. Also, there are some studies on the spirulina effect in both humoral and cellular immunity (Baojiang 1994, Qureshi *et al* 1996). These manuscripts showed that dietary spirulina has immune-stimulatory effects on the immune system.

The positive effect of the probiotic *S.cerevisiae* can originate from either their direct nutritional effect or their health promoting effects such as behaving as bioregulator of the intestinal microflora and reinforcing the natural defenses of the host (Hassanein and Soliman 2010). Lately, studies proved that live yeast *S. cerevisiae* enhances immunity. Yeasts support the immunity by increasing the migration of T-lymphocytes to Peyer's patches in blood when the yeast

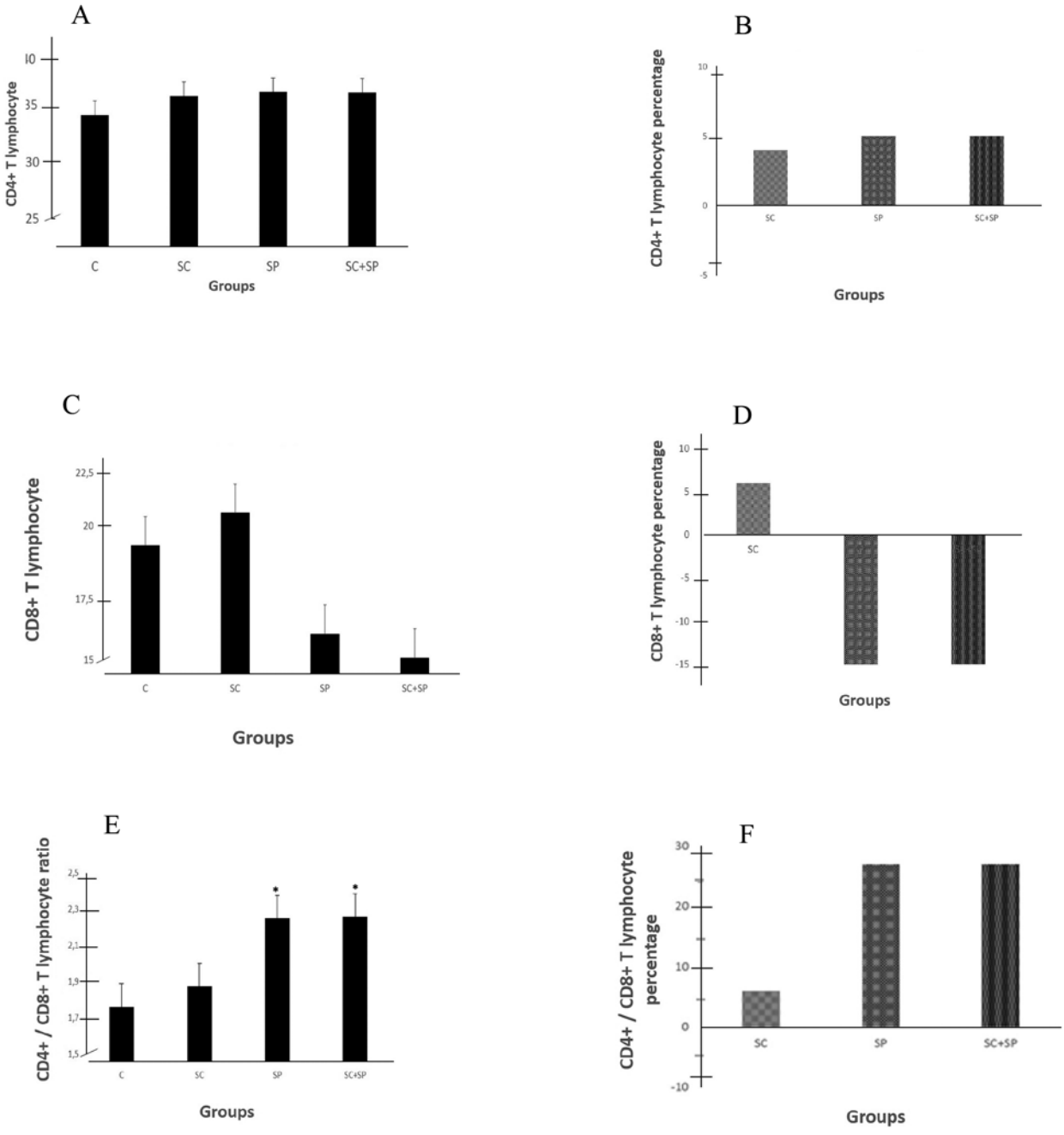

Figure 1. CD4+ and CD8+ T lymphocytes subpopulation count and percentage difference. A) CD4+ T lymphocyte subpopulation count ; B) CD4+ T lymphocyte percentage difference ; C) CD8+ T lymphocyte subpopulation count; D) CD8+ T lymphocyte percentage difference; E) CD4+/CD8+ count; F) CD4+/CD8+ percentage difference. Data plotted as mean±standard error, n = 40. *C*, control group (basal diet); *SC*, *S.cerevisiae* group (added 3 g/kg); *SP*, *S.platensis* group (added 5%); *SC+SP*, *S.cerevisiae* and *S.platensis* group (added 3 g/kg SC and 5% SP of the diet, respectively).

**P*<0.05 different from the control group.

fused with ileum M cells (Salazar-Monroy *et al* 2012). On the contrary, in that location there is not a precise information about how the yeast affects blood T lymphocyte subpopulations. The yeast cell wall component effects on immunity has been considered for a long time; its component beta glucan works as an immunstimulant by activating functional activities of macrophage (Pillemer *et al* 1954). On the other hand, beta glucan activates B-lymphocytes, and interacts with macrophages though beta-glucan receptors on macrophages, thereby induces release of cytokines. The yeast *S. cerevisiae* contains immunstimulants such as beta glucan, nucleic acids, mannanoligosacharides and

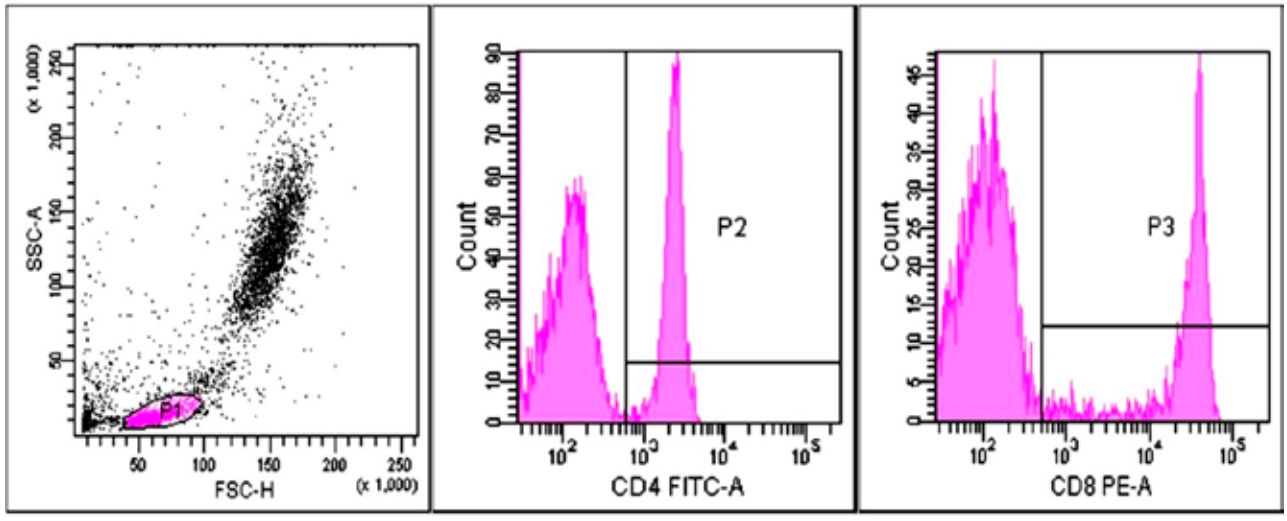

Figure 2. Receiving in view of lymphocytes in FSC/SSC histograms and gated in a working sample, and other linear histograms are showing the expression of CD4+ and CD8+.

kitins. The enhancing effects of the yeast and yeast beta glucan on immune system cellular phone functions were demonstrated in both *in vitro* and in animal experiments. However, these works were based on the yeast beta glucan (Devonta *et al* 2009, Guo *et al* 2003, Robertsen *et al* 1990). Guo *et al* (2003) added beta glucan obtained from *S. cerevisiae* to broiler starting and developing fields, and they covered the increase of CD4+, CD8+, and CD4+/CD8+ in the intestinal intraepithelial leukocytes.

In our study, CD4+/CD8+ T lymphocyte ratio of rabbits in the groups of SP and SC+SP was significantly increased ($P<0.05$). Our results demonstrate that, similarly to the findings of Salazar *et al* (Salazar-Monroy *et al* 2012), an increase in CD4+, CD8+ and CD4+/CD8+ in peripheral blood in pigs and the increase of these T lymphocyte subpopulation stimulates the antibody yield in the pigs fed 0.3% *S. cerevisiae*. In other studies on pigs (Shen *et al* 2009) the researchers added 2.5, 5, 10 and 20 g *S. cerevisiae* to feed, and the animals expressed a substantial development in the CD4+/CD8+ ratio in experimental groups compared to control. According to our findings and previous studies it was identified that *S. cerevisiae* may enhance the T lymphocyte subpopulations (CD4+, CD8+, CD4+/CD8+) and thereby improves the immunity. On the other hand, although statistically not significant, the percentage of difference of the CD4+ T lymphocytes in SP and SC+SP group tended to increase 5%, while in that of CD8+ T lymphocytes it decreased 16% and 15%, respectively. However the percentage difference between the control and experiment groups for the percentage of the CD4+/CD8+ increased 27% when fed with both SC and SC+SP. CD8+ T lymphocytes authorise the regulation and differentiation of CD4+ T lymphocytes. Meanwhile CD8+ T lymphocytes can also work in an indirect manner which by changing the behaviour of antigen-presenting cells and kill infected and damaged cells. Therefore, the CD4+/CD8+ ratio is just one of the indicators to check if the immunity is strong or weak (Arstila *et al* 1994, Broere *et al* 2011).

Previous studies have demonstrated an effect of products derived from *S. cerevisiae* and *S. platensis (A. platensis)*, activating immune functions and on adaptive immune responses in chickens (Hasanein and Soliman 2010), mice (Cheng-Wu 1994), carp (Watanuki 2008) and mouse (Baojiang 1994). To our knowledge, this is the first study to assess the natural immune effects of SP, SC and SP+SC combinations in rabbits. Following the outcomes of this study, the cellular immunity of the CD4+/CD8+ T lymphocyte of rabbits fed by *S. platensis (A. platensis)* and combination *S. platensis (A. platensis)* and *S. cerevisiae* were significantly increased. Although not statistically significant, supplementing rabbit with *S. platensis (A. platensis)* tended to increase the CD4+ T lymphocyte. This may be the immune effects of polysaccharide and phycocsiyanin of spirulina on growing leukocyte and bone cell.

Some studies have suggested that *S. platensis (A. platensis)* and *S. cerevisiae* could improve immunity by increasing the concentration of cytokines IFN-γ and IL-4 in newborn pigs (Shen *et al* 2009) and humans (Hirahashi *et al* 2002). However, there were no significant differences in expressions of cytokines between experimental groups were found in our study. This may be ascribable to the contribution rate of these natural supplements. There are several studies focused on the immune system modulation by spirulina and yeast (Ortuno *et al* 2002, Robertsen *et al* 1990, Watanuki *et al* 2008, Shen *et al* 2009).

In conclusion, in this study, *S. platensis (A. platensis)* can positively affect the immune system. On the other hand, *S. platensis (A. platensis)* and *S. cerevisiae* combination improved the immune system components

and more studies would be necessary to clear up the effects of supplementing spirulina and its combinations on immunity.

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