

Effect of oral supplementation of bamboo grass leaves extract on cellular immune function in dairy cows

Hiromichi Ohtsuka¹, Hidemi Fujiwara¹, Ai Nishio¹, Yasushi Sakai², Kei-ichi Matsuda², Yukiko Muramatsu³, Hideo Hara³, Yuzo Nakagawa-Yagi⁴, Taka-aki Hara³

¹Kitasato University School of Veterinary Medicine, Towada, Aomori, Japan

²Chuo Domestic Animal Clinic, Miyagi Agricultural Mutual Aid Association, Furukawa, Ohsaki, Miyagi, Japan

³Hakuju Institute for Health Science Co. Ltd., Shibuya-ku, Tokyo, Japan

⁴Unial Co. Ltd., Itabashi-ku, Tokyo, Japan

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Abstract

Beta glucans extracted from bamboo (*Sasa senanensis*) grass leaves are known to have an immune-modulatory effect in animals. These glucans have been used for the treatment of diseases such as viral infections, inflammation, and cancer. The aim of this study was to evaluate the immuno-modulatory effect of SanSTAGE™ (pure compounds obtained from the bamboo grass leaves; 25% of bamboo grass extract and 75% of dextrin) on peripheral blood leukocyte population and mRNA expression of immune related molecules of 20 dairy cows. Ten cows were orally administered 30 mg/kg/day of SanSTAGE™ for first two weeks; the other 10 cows were control without supplementation. The blood samples were collected in tubes containing dipotassium-EDTA for analysis of leukocyte population, and in tubes containing heparin for analysis of cytokine production. Cows supplemented with SanSTAGE™ showed an increased number of CD8⁺ T cells and expression of perforin (cytotoxicity factor to virally infected cells) and MX-2 (anti-virus factor). The study describes for the first time that oral administration of supplement extracted from Kumaizasa bamboo grass leaves affects cellular immune function of dairy cows, and can be recommended as part of diet for prevention of infectious diseases.

Immune modulatory, leukocyte population, oral administration, Sasa senanensis

Infectious diseases, such as mastitis and metritis, cause loss of productivity in dairy cows (Gunay and Gunay 2008; Könyves et al. 2009). Since impaired immunity can increase the susceptibility of cows to these infections, maintaining good immune function is important for reducing infectious diseases. Immunomodulatory supplements including vitamins, minerals, probiotic or beta glucans enhanced the immune function of dairy cows (Politis et al. 2004; Kohiruimaki et al. 2008; Salman et al. 2013).

Kumaizasa bamboo (*Sasa senanensis*) is a giant, woody grass mainly distributed in China, Japan, and Southeast Asia. The leaves of this bamboo have been used for treating fever, stomachache, diarrhoea or chest diaphragm inflammation as a traditional Chinese medicine for a long time (Lu et al. 2005). Previous study demonstrated that serum concentrations of interleukin-12 (IL-12) and interferon- γ (IFN- γ) were significantly increased in mice fed with beta glucans isolated from bamboo leaf extracts (Seki et al. 2010). In addition, glucans from the Kumaizasa bamboo leaf activated anti-tumour efficacy in mice (Ren et al. 2004), which is why they are widely used to improve immunity in animals. However, the effect of oral administration of glucans from Kumaizasa bamboo leaf extracts on the immune function of food animals has not been clarified.

SanSTAGE™ is a commercial supplement that contains only the extract from Kumaizasa bamboo grass leaves produced by Hakuju Institute for Health Science Co. Ltd. (Japan). Significant decrease in the somatic cell count was observed after oral administration

Address for correspondence:

Hiromichi Ohtsuka
School of Veterinary Medicine
Kitasato University
Higashi 23-35-1
Towada, Aomori 034-8628, Japan

Phone: +008-0176-64-2710
Fax: +008-0176-64-9748
E-mail: ohtsuka@vmas.kitasato-u.ac.jp
<http://actavet.vfu.cz/>

of this supplement to Holstein cows with chronic mastitis in a field study (Sakai et al. 2012). However, it is unknown whether the oral administration of SanSTAGE™ feed supplementation affects the cellular immune function in dairy cows.

The aim of this study was to evaluate the immune modulatory effect of the SanSTAGE™ bamboo extract in dairy cows.

Materials and Methods

Animals

Twenty healthy lactating Holstein cows in their mild to late lactation kept in tie-stalls barns at two farms (10 cows in each farm) were used in this study. The cows were divided into two groups (experimental and control) of 10 animals. Cows from experimental group (4 and 6 cows from first and second farm) were orally administered 30 mg/kg/day of SanSTAGE™ (pure compounds obtained from the bamboo grass leaves; 25% of bamboo grass leaves extract and 75% of dextrin) for first two weeks, and cow from control group (6 and 4 cows from first and second farm) did not receive SanSTAGE™.

Blood samples were collected from the tail vein before supplementation and in weeks 1, 2 (with supplementation) and week 3 and 4 (without supplementation) after supplementation. The samples were collected first into tubes containing dipotassium-EDTA for the analysis of leukocyte population, and into tubes containing heparin for the analysis of cytokine production. White blood cell (WBC) counts were determined by a blood cell counter (Celltac α MEK-6358, NIHON KOHDEN, Tokyo, Japan).

Flow cytometry

Two ml of each blood sample with dipotassium EDTA were mixed with 4 ml of 0.83% ammonium chloride solution, and the leukocytes were obtained. The leukocyte samples were washed twice in phosphate-buffered saline (PBS) and incubated with each monoclonal antibody (mAb). The following antibodies (VMRD, Pullman, WA, U.S.A.) were used: CACT183A (mAb for CD4 antigen expressed on helper lymphocyte), BAT82A (mAb for CD8 antigen expressed on cytotoxic lymphocyte), CAT82A (mAb for MHC class II antigen expressed on monocyte/B lymphocyte), B7A1 (mAb for WC1-N1 antigen expressed on dy T lymphocyte), MCA2365EL (mAb for CD335 antigen expressed on NK cell; AbD Serotec, Int., Ltd, U.K.) and MY4 (mAb for CD14 antigen expressed on monocyte/macrophage; Coulter Immunology, Florida, U.S.A.). The cells were incubated at 4 °C for 60 min, and washed with phosphate buffered saline (PBS). In order to visualize cell surface markers, goat anti-mouse IgM-fluorescein isothiocyanate conjugated antibody and goat anti-mouse IgG-phycoerythrin conjugated antibody (Cappel, Durham, NC, U.S.A.) were added. After treating for 30 min at 4 °C, the samples were washed with PBS and then analyzed. The data were acquired by 10,000 counts per sample using Cytomics FC500 software (Beckman Coulter, Fullerton, CA, U.S.A.). The percentage of stained cells was determined using Flow Jo software (Tree Star, Inc. Oregon, U.S.A.).

Real-time PCR

The peripheral blood mononuclear cell (PBMC) (2×10^6) in 1 ml of Roswell Park Memorial institute (RPMI) 1640 medium (Invitrogen, Tokyo, Japan) supplemented with 10% foetal calf serum (Cansera International, Rexdale, Canada) were placed in a 24 well plate, and stimulated with 5 mg/ml of phytohaemagglutinin (PHA; AppliChem GmbH, Germany) for 12 h at 37 °C. After incubation, the supernatants were removed, and the cells were re-suspended using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). The PBMCs were then subjected

Table 1. Primers used for real-time PCR expression analysis.

Gene	Accession number	Product length	Primer designation	Sequence (5'-3')
IFN- γ	NM_174086	108	Forward	TCAAATTCGGTGGATGATCT
			Reverse	CTTCTCTCCGCTTTCTGAGG
IL-4	NM_173921	117	Forward	GCCCCAAGAACAACAACACTGA
			Reverse	GAGATTCCTGTCAAGTCCGC
Granulysin	NM_001075143.1	136	Forward	GAGATCAGCCCGATGAGAATAC
			Reverse	TTTCCAGCTACGATGTCCTCA
Perforin	NM_001143735.1	105	Forward	ACTCCGAGGATGCCAACTTC
			Reverse	TGTGACCAGGTGAAAACCTGTA
MX-2	NM_173941.2	80	Forward	CCTACAAGTGCACAGGTGACAAC
			Reverse	CTCTACGCTTCCACGGGAGA

to RNA extraction and real-time PCR using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, U.S.A), as described previously in detail by Maeda et al. (2013).

Total RNA was transcribed into cDNA by using the GoScript reverse Transcription System (Promega, Mannheim, Germany) combined with oligoDT₁₈ primers, recombinant RNasin ribonuclease inhibitor and GoScript™ reverse transcriptase according to the manufacturer's specification and protocol. The primers used for the evaluation of gene expression for β-actin, IFN-γ, IL-4, granulysin, perforin, and myxovirus resistance protein (MX)-2 were designed as described in the literature (Table 1). Real-time PCR was set up using 2 μl first-strand cDNA template, 7.4 μl deionized H₂O, 0.3 μM of upstream and downstream primers and 10 μl Power SYBR Green I master mix with ROX as reference dye (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A). The thermal cycling conditions were 3 min at 95 °C followed by 15 s at 95 °C (40 cycles) and 1 min at 60 °C. Melting curve analysis was constructed to verify the presence of gene-specific peak and the absence of the primer dimer.

The final quantification of immune related molecules mRNA was carried out using the comparative CT (threshold cycle) method previously described by Livak and Schmittgen (2001). This method was used after a validation experiment, which demonstrated that the efficiencies of the target and reference (β-actin) genes were approximately equal.

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Difference between two groups of cows was tested using the Mann-Whitney's U-test. For all analyses, values of $P < 0.05$ were considered significant.

Results

The time-course changes in peripheral CD4⁺, CD8⁺ and WC1-N1⁺ T cell showed a trend of increasing in numbers up to week 2 in the experimental group followed by a decrease after the end of supplementation. At week 2, the numbers of CD4⁺ and CD8⁺ T cells reached the peak, and there was a significant ($P < 0.05$) difference in CD8⁺ T cells between the two groups (Fig. 1 A, B). In both groups, numbers of CD335⁺, MHC class-II⁺CD14⁺ and CD14⁺ cells showed similar changes without significant differences (Fig. 1 D, E, F).

The concentrations of IFN-γ and IL-4 varied without significant differences between groups. The IFN-γ mRNA concentration was lowest at week 2 (Fig. 2 A, B). The time-course changes in granulysin concentration were similar in both groups without a significant difference (Fig. 2 C). Perforin concentration reached the peak at week 2 in the experimental group, when it was significantly ($P < 0.05$) higher than in the control group. After week 2, perforin concentration in the experimental group decreased (Fig. 2 D). Although MX-2 concentration in the control group was stable, in the experimental group it increased slightly and peaked at week 2 followed by gradual decrease. No significant ($P < 0.05$) difference was found between the two groups in the MX-2 concentrations (Fig. 2 E).

Discussion

SanSTAGE™ supplementation in our study showed a significant increase in the peripheral CD8⁺ T cell number and a slight increase (non-significant) in CD4⁺ T cell number at week 2. The CD8⁺ T cells have cytotoxic ability for foreign as well as modified autoantigens, such as transplants and tumour cells and Th1 CD4⁺ T cells activate CD8⁺ T cells to obtain cytotoxic ability in protective immunity (Hsu et al. 1998). Increased numbers of peripheral CD4⁺ and CD8⁺ T cells might be due to the connection of enhanced proliferation of CD8⁺ and CD4⁺ T cells by SanSTAGE™ supplementation. In a previous report, the extracts from Kumaizasa bamboo significantly suppressed tumour growth via activation of NK cells and macrophages as well as following the increased serum IL-12 concentrations (Seki et al. 2010). In our study, we did not observe changes in the peripheral CD14⁺ and CD335⁺ cells. The SanSTAGE™ supplementation may affect CD8⁺ rather than NK cells in dairy cows.

The CD8⁺ T cells and NK cells contain granules rich in perforin and granulysin molecules that contribute to lysis of virally infected cells or tumours (Peña and Krensky 1997). Perforin-mediated cytotoxicity is well documented in CD8⁺ T cells and NK

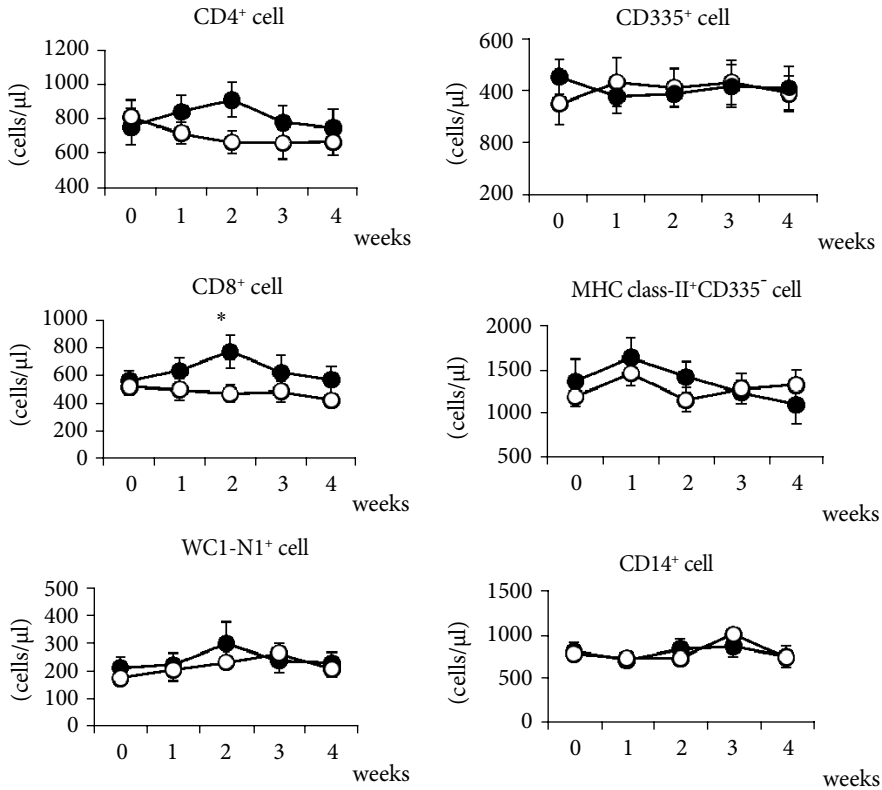


Fig. 1. Changes of peripheral leukocytes in dairy cows treated with SanSTAGE™ (black circles) and untreated control (white circles). Asterisks indicate the time point where significant differences ($P < 0.05$) between SanSTAGE™-treated and control animals were observed.

cell-mediated defense that produces pores on target membranes (Liu et al. 1995), and granulysin has a spectrum of antimicrobial activity including the creation of holes and induction of apoptosis in the target cells (Okada et al. 2003). In our study, perforin mRNA concentration in PBMC increased in the group supplemented with SanSTAGE™. It suggests that the supplementation of SanSTAGE™ might enhance the protective immunity by forming a membrane-attack-complex against infected cells in dairy cows. In addition, a slight increase in MX-2 mRNA concentration was observed in the group supplemented with SanSTGAE™. The MX proteins identify the antiviral activities against RNA viruses in cells (Asano et al. 2003). Although it was not significant, antiviral activities may be one of the effects of the SanSTAGE™ supplementation.

In conclusion, we observed that commercial supplement extracted from Kumaizasa bamboo grass leaves induced perforin mRNA expression followed by an increase in peripheral CD8⁺ T cells in dairy cows. Activated CD8⁺ T cells migrate to the site of infection and kill virus-infected cells (Peña and Krensky 1997). Since, CD8⁺ T cells are key mediators in the immune response to many viral infections, bamboo leaf extract may have the potential to enhance antiviral effects in dairy cows.

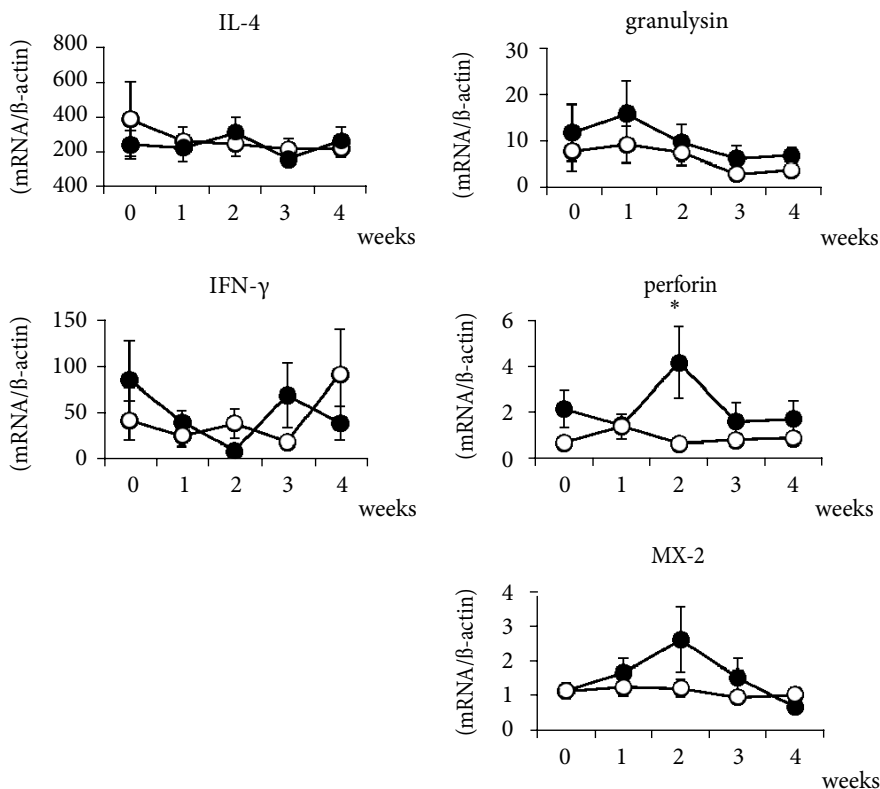


Fig. 2. Changes of immune factors in dairy cows treated with SanSTAGE™ (black circles) and untreated control (white circles). Asterisks indicate the time point where significant differences ($P < 0.05$) between SanSTAGE™-treated and control animals were observed.

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