The Journal of Microbiology (2012) Vol. 50, No. 6, pp. 1003–1008
DOI 10.1007/s12275-012-2365-1

Immunoprophylactic Effects of Shiitake Mushroom (*Lentinula edodes*) against *Bordetella bronchiseptica* in Mice

Bock-Gie Jung, Jin-A Lee, and Bong-Joo Lee*

College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Republic of Korea

(Received July 17, 2012 / Accepted September 5, 2012)

Antimicrobials are used as feed additives to improve growth performance and to prevent subclinical disease challenge in industrial animals. However, these drugs can lead to the development of resistant strains of bacteria. Shiitake mushrooms (SM) (*Lentinula edodes*) have long been popular as a health food in East Asia. Moreover, SM-derived polysaccharides are well-known as immunostimulants that possess antimicrobial properties. The aim of the present study was to evaluate the immunoprophylactic effects of SM against *Bordetella bronchiseptica* infection in mice as an initial step towards the development of eco-friendly feed additives to reduce the use of antimicrobials. Although SM had no effect on body weight gain under the un-infected conditions, SM alleviated progressive weight loss and helped in the recovery of body weight in *B. bronchiseptica* infected mice. Dietary supplementation with SM reinforced bacterial clearance in the infected mice. Of note, SM markedly increased the percentage of various T lymphocytes and the relative mRNA expression levels of tumor necrosis factor-α and interferon-γ in the bronchial lymph node early in the infection. Taken together, these findings suggest that SM could help in the improvement of body weight gain during *B. bronchiseptica* infection and may enhance the protective immune activity against a subclinical disease challenge, such as *B. bronchiseptica* infection in mice, probably by a strong stimulation of non-specific immune responses. Hence, SM may provide an alternative to reduce use of antimicrobials. Confirmation of the beneficial effects of SM as a feed additive is now required in industrial animals.

Keywords: *Bordetella bronchiseptica*, *Lentinula edodes*, immune enhancement

Introduction

Veterinary drugs, especially antimicrobials, were discovered over 50 years ago and have since been widely used as feed additives to improve growth performance and to prevent subclinical diseases in industrial animals (Cromwell, 2002). However, there is worldwide concern about the overuse of antimicrobials, including the development and spread of antimicrobial-resistant strains of bacteria and resistance genes from animals to humans through the food chain (Gustafson and Bowen, 1997; Hardy, 2002). For these reasons, the restriction of antimicrobials has become a worldwide trend. Under Regulation 1831/2003/EEC, the European Union initiated a progressive ban on the use of antimicrobials in animal feed beginning January 1, 2006 (European Union, 2003). This political decision has focused increasing attention on the development of eco-friendly alternatives to reduce the use of antimicrobials. One of the most promising methods of reducing antimicrobials in industrial animals is improving the immune status of animals through the prophylactic administration of natural immunostimulants such as mushrooms, herbs, and probiotics (Guo *et al*., 2004; Jung *et al*., 2010a, 2010b, 2010c).

Shiitake mushrooms (SM) (*Lentinula edodes*) have long been popular as a traditional drug and health food in East Asian countries (Chang, 1996). Presently, it is the second most cultivated edible mushroom in the world, comprising about 25% of the worldwide production (Boa, 2004). SM is a good source of carbohydrate, protein and essential amino acids. It is low in fat, has a high percentage of poly-unsaturated fatty acids and also contains many vitamins and minerals (Bisen *et al*., 2010). It is the source of well-studied and widely-approved polysaccharide medicines such as lentinan, LEM, KS-2, and eritadenine (Chang, 1996; Mattila *et al*., 2000). These compounds are well-known as a type of immunostimulant that possesses antitumor and antiviral properties (Maeda *et al*., 1998; Ng and Yap, 2002; Ngai and Ng, 2003), as well as antimicrobial potential (Komemushi *et al*., 1995, 1996; Hatvani, 2001; Ishikawa *et al*., 2001). Hypocholesterolimic (Sugiyama *et al*., 1995) and hypoglycaemic (Yang *et al*., 2002) activities have also been reported. Lentinan has been found to activate macrophages, T lymphocytes and other immune effector cells that modulate the release of cytokines, which may in turn account for its indirect antitumor and antimicrobial properties (Hamuro and Chihara, 1985). These collective observations suggest that SM possess a number of beneficial biological activities. Of particular relevance to the present study, SM provide an alternative way to reduce use of antimicrobials and simultaneously may help avoid the development of antimicrobial resistant bacteria through the promotion of immune activity and prevention of diseases.

Bordetella bronchiseptica is a small (0.2–0.5 μm by 1.5 μm), Gram-negative, piliated coccobacillus and is important as a cause of diseases in the respiratory tract of dogs, pigs, laboratory rodents and a variety of wild mammalian species (Hagan and Bruner, 1988). It is a primary etiological agent, or a predisposing factor, in atrophic rhinitis (Harris and

*For correspondence. E-mail: bjlee@chonnam.ac.kr; Tel.: +82-62-530-2850; Fax: +82-62-530-2857

Switzer, 1968), pneumonia (Palzer *et al.*, 2008) and porcine reproductive and respiratory disease complex (Brockmeier *et al.*, 2001). *B. bronchiseptica*-related infections produce economic losses of millions of dollars from mortality, sacrificed pigs, medicated feed, veterinary fees, sales losses from deformed pigs, reduced weight gain and poor feed conversion (Goodnow, 1980; Boessen *et al.*, 1988).

The aim of the present study was to evaluate the immunoprophylactic effects of SM against *B. bronchiseptica* in mice as an initial step towards the development of eco-friendly feed additives to reduce the use of antimicrobials. To this aim, clearance of the bacteria in bronchio-alveolar lavage (BAL) was assessed and body weight changes were monitored in mice experimentally infected with *B. bronchiseptica*. In addition, the present study evaluated several immunological criteria, including the lymphocyte subpopulation and relative mRNA expression levels for cytokines in the bronchial lymph node (BLN).

Materials and Methods

Experimental diet preparation

SM used in the present study were harvested from a local farm in Chonnam province, Republic of Korea. The fresh mushrooms were cleaned thoroughly, chopped into small pieces in a biosafety hood and lyophilized. Following lyophilization, the mushrooms were further processed into fine powder and incorporated into the diets.

Animals and diets

Specific pathogen-free, female, 4-week-old, ICR mice (DBL, Korea) were randomly divided into three groups consisting of 15 mice each. The control group received a commercial, nutritionally complete, extruded, dry, rodent feed (Feedlab, Gyeonggi province, Korea). The experimental groups received the same extruded dry rodent feed supplemented with either 1% (wt/wt) SM (1% SM-fed group) or 3% (wt/wt) SM (3% SM-fed group). All mice were housed in an air-conditioned room, kept in polypropylene cages and allowed free access to their particular diet and tap water from drinking bottles with stainless steel sipper tubes. All animal procedures were approved by the Institutional Animal Care and Use Committee of Chonnam National University (Approval number: CNU IACUC-YB-2010-1).

Experimental *B. bronchiseptica* infection

All mice were acclimatized to their particular diet for 9 weeks before experimental bacterial infection. *B. bronchiseptica* was prepared as previously described (Smith and Baskerville, 1979; Goodnow, 1980; Hagan and Bruner, 1988) with some modifications. Briefly, the original stock of *B. bronchiseptica* was cultured overnight on MacConkey agar (BD Biosciences, USA). A single colony was selected and inoculated into Brain Heart Infusion broth (BD Biosciences) in a universal bottle and incubated at 250 rpm for 16 h at 37°C. Viable counts of the broth culture were made using the pour plate method. Inoculated broth was diluted with phosphate-buffered saline (PBS) to obtain the infective dose of 1×10^9 colony-forming units (CFU)/ml, which was the optimal dose as determined in our previous study (Jung *et al.*, 2010a). Ten microliters of the *B. bronchiseptica* culture were inoculated intranasally into each nostril of each mouse. Body weight was monitored throughout the experimental period. Five mice from each group were randomly sacrificed for collection of BAL and BLN at 3, 6, and 9 days post-infection (DPI).

Viable bacterial cell count in BAL

A viable-bacteria cell count was carried out in BAL samples collected from each sacrificed mouse at 3, 6, and 9 DPI. BAL was collected as previously described (Jung *et al.*, 2010a). Briefly, the samples were collected by inserting a suitable cannula towards the lungs into the exposed trachea. A 2 ml syringe was attached to the cannula and the lungs were washed with 1 ml of PBS by flushing the solution in and out of the lungs several times. The wash solutions were collected into a suitable tube. One hundred microliters of the wash solutions were serially diluted 10-fold in PBS and 100 µl of each dilution was spread onto a MacConkey agar plate (BD Biosciences) and incubated at 37°C for 48 h. Colonies that were characteristically small, pale, with a pinkish hue were counted and expressed as log CFU/ml, but only for those plates having between 30 and 300 colonies per plate. In addition, representative colonies were subjected to Gram staining and biochemical tests for identification purposes (Smith and Baskerville, 1979; Goodnow, 1980; Hagan and Bruner, 1988). Each sample was tested in duplicate.

Determination of lymphocyte subpopulation in BLN

BLN was obtained from each sacrificed mouse at 3, 6, and 9 DPI, and single cell suspensions were prepared by pushing the tissue through a 40 µm nylon mesh (BD Biosciences). Isolated cells were analyzed to determine of the percentage of various lymphocytes including $CD3^-CD19^+$ B lymphocyte, $CD3^+CD19^-$ T lymphocyte, $CD4^+CD8^-$ T lymphocyte, and $CD4^-CD8^+$ T lymphocyte as previously described (Jung *et al.*, 2010a). To determine the percentage of $CD3^-CD19^+$ B lymphocyte and $CD3^+CD19^-$ T lymphocyte, the cells were stained with both phycoerythrin (PE)-conjugated anti-mouse CD3 (BD Biosciences) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD19 (BD Biosciences). To determine the percentage of $CD4^+CD8^-$ T lymphocyte and $CD4^-CD8^+$ T lymphocyte, the cells were stained with both FITC-conjugated anti-mouse CD4 (BD Biosciences) and PE-conjugated anti-mouse CD8 (BD Biosciences). After incubation at 4°C for 30 min in the dark, the cells were washed twice with PBS and the lymphocyte subpopulation was analyzed using a FACSort flow cytometer (BD Biosciences). Viable lymphocytes were gated by forward and side-scatter characteristics (FSC/SSC), and 10,000 events were analyzed for positive staining with FITC or PE. Results for each lymphocyte subpopulation were expressed as percentages of events in the FSC/SSC lymphocyte gate.

Evaluation of relative mRNA expression levels for TNF-α and IFN-γ in BLN

Total RNA extraction from BLN was performed using an

Table 1. Primer and probe sequences for murine cytokines

		Sequence (5′-3′)	Length (bp)[a]	Accession[b]
TNF-α	FW	CATCTTCTCAAAATTCGAGTGACAA	175	M13049 Y00467
	RV	TGGGAGTAGACAAGGTACAACCC		
	TP	CACGTCGTAGCAAACCACCAAGTGGA		
IFN-γ	FW	TCAAGTGGCATAGATGTGGAAGAA	92	K00083 M74466 M28381
	RV	TGGCTCTGCAGGATTTTCATG		
	TP	TCACCATCCTTTTGCCAGTTCCTCCAG		
β-Actin	FW	AGAGGGAAATCGTGCGTGAC	148	V01217 J00691
	RV	CAATAGTGATGACCTGGCCGT		
	TP	CACTGCCGCATCCTCTTCCTCCC		

FW, forward primer; RV, reverse primer; TP, TaqMan probe dual-labeled with 5′FAM (report dye) and 3′TAMRA (quencher dye); IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor alpha; β-actin, beta-actin. [a]Amplicon length in base pairs. [b]GenBank accession no. of cDNA and corresponding gene available online at http://www.ncbi.nlm.nih.gov/.

RNeasy Mini kit (QIAGEN, USA) and target RNA was reverse transcribed using a QuantiTect® reverse transcription kit (QIAGEN) according to the manufacturer's instructions. To minimize variations in reverse transcriptase efficiency, all samples were transcribed simultaneously. Primers and probes for murine tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and β-actin were designed as previously described (Overbergh *et al.*, 1999). Their sequences are shown in Table 1. The probes were dual labelled with the reporter dye 6-carboxyfluorescein (FAM) at the 5′ end and the quencher dye 6-carboxytetramethyrhodamine (TAMRA) at the 3′ end. TNF-α and IFN-γ mRNA levels were determined by a real-time polymerase chain reaction (PCR) assay using MyiQ™2 (Bio-Rad Laboratories, USA) with 0.5 μg of cDNA. The threshold cycle (Ct; the cycle number at which the amount of amplified gene of interest reaches a fixed threshold) was determined subsequently. Relative quantitation of TNF-α and IFN-γ mRNA expression was calculated by a comparative Ct method as previously described (Livak and Schmittgen, 2001). The relative quantitation value of the target (TNF-α or IFN-γ) was normalized to an endogenous control β-actin gene and relative to a calibrator. It was expressed as $2^{-\Delta\Delta Ct}$ (fold), where ΔCt = Ct of target gene – Ct of endogenous control gene and ΔΔCt = ΔCt of samples for target gene – ΔCt of the calibrator for the target gene.

Statistical analyses

The data are expressed as mean±standard deviation (SD) and the means of the different parameters were compared between groups by analysis of variance (ANOVA). Significant differences between the groups were evaluated by Tukey's multiple comparison tests using Minitab Statistical Software 13.20 (Minitab, State College, USA), and $P<0.05$ was considered as the level of significance.

Results

Body weight changes

The changes in body weights of the mice over the whole experimental period are shown in Fig. 1. The body weights of all mice gradually increased for 9 weeks before experimental *B. bronchiseptica* infection. No significant differences in body weight changes were observed among the groups in this period. After the bacterial infection, the body weight of all infected mice markedly decreased. However, the 1% and 3% SM-fed groups showed re-increasing body weight changes

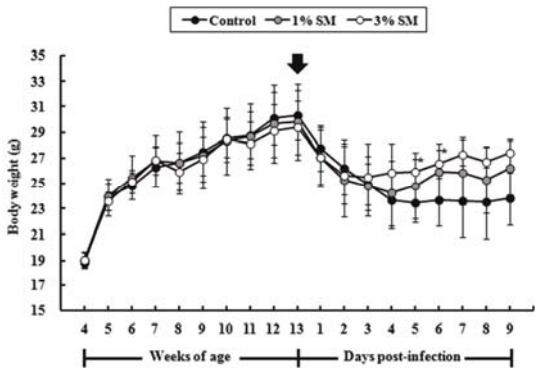

Fig. 1. Effect of shiitake mushrooms on body weight changes in mice. All mice were acclimatized to their particular diet for 9 weeks before *B. bronchiseptica* infection. Body weight was monitored throughout the experimental period. Arrow indicates the day of the experimental *B. bronchiseptica* infection. Control: control group; 1% SM: 1% SM-fed group; 3% SM: 3% SM-fed group. For each group, data values represent the mean±SD. *$P<0.05$ vs. control.

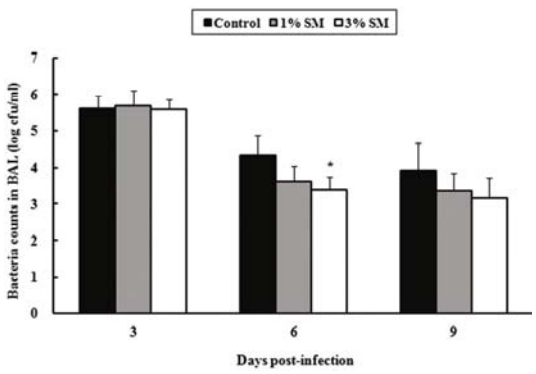

Fig. 2. Viable bacterial cells counted in bronchio-alveolar lavage (BAL) of experimentally *B. bronchiseptica* infected mice. BAL samples were collected from each sacrificed mouse at 3, 6, and 9 days post-infection. Control: control group; 1% SM: 1% SM-fed group; 3% SM: 3% SM-fed group. For each group, data values represent the mean±SD. *$P<0.05$ vs. control.

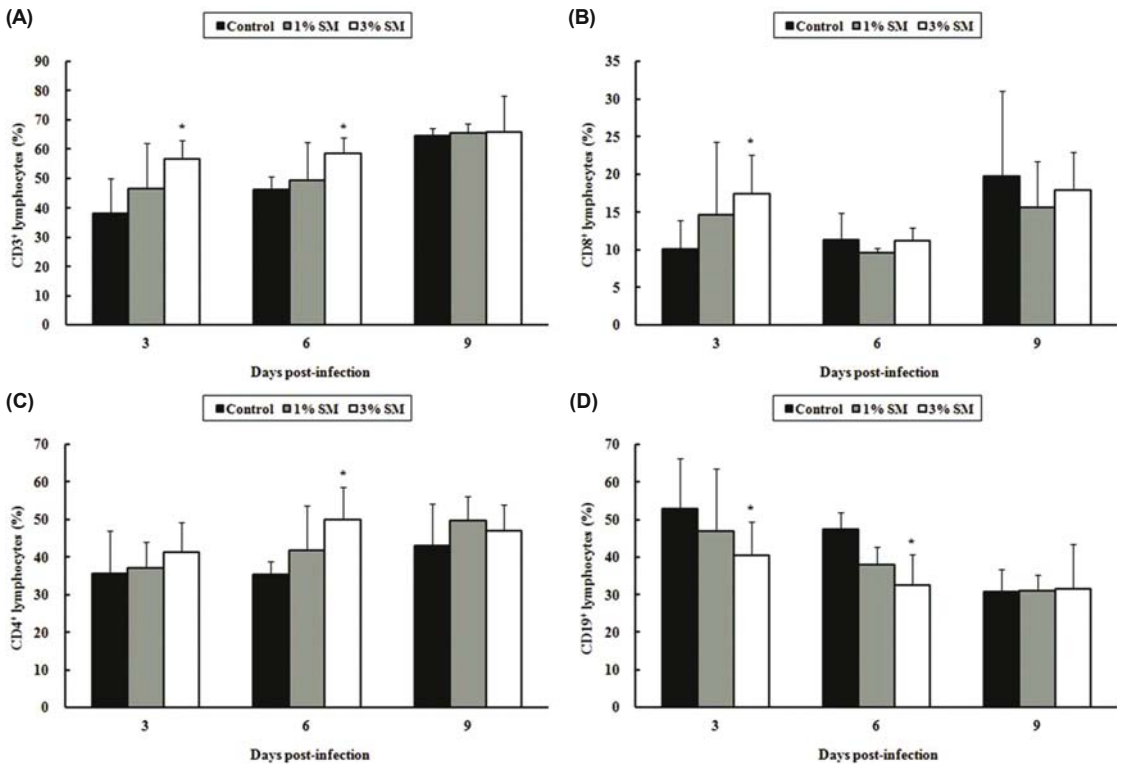

Fig. 3. Effect of shiitake mushrooms on the lymphocyte subpopulation (%) in the bronchial lymph node (BLN) of mice experimentally infected with *B. bronchiseptica*. BLN was obtained from each sacrificed mouse on 3, 6, and 9 days post-infection. Those were used to determine (A) $CD3^+$ T lymphocyte, (B) $CD8^+$ T lymphocyte, (C) $CD4^+$ T lymphocyte, and (D) $CD19^+$ B lymphocyte components and their percentage. Control: control group; 1% SM: 1% SM-fed group; 3% SM: 3% SM-fed group. For each group, data values represent the mean±SD. $^*P<0.05$ vs. control.

from 5 and 4 DPI, respectively. In contrast, the body weight of mice in the control group decreased or remained constant. In particular, between 5 and 6 DPI the body weight in the 3% SM-fed group significantly increased compared with the control group ($P<0.05$).

Bacterial clearance in BAL

The number of the viable bacterial cells counted in BAL is shown in Fig. 2. The viable bacterial cells in the BAL of mice in all three groups were maximum at 3 DPI, after which there was a precipitous drop until 6 DPI followed by a moderate decline until 9 DPI. There were no significant differences in bacterial cell counts among the groups at 3 DPI. The number of viable bacterial cells in BAL was the highest in the control group, while in the 3% SM-fed group it remained the lowest among the groups from 6–9 DPI. In particular, the number of viable bacterial cells in BAL significantly decreased in the 3% SM-fed group, compared with those the control

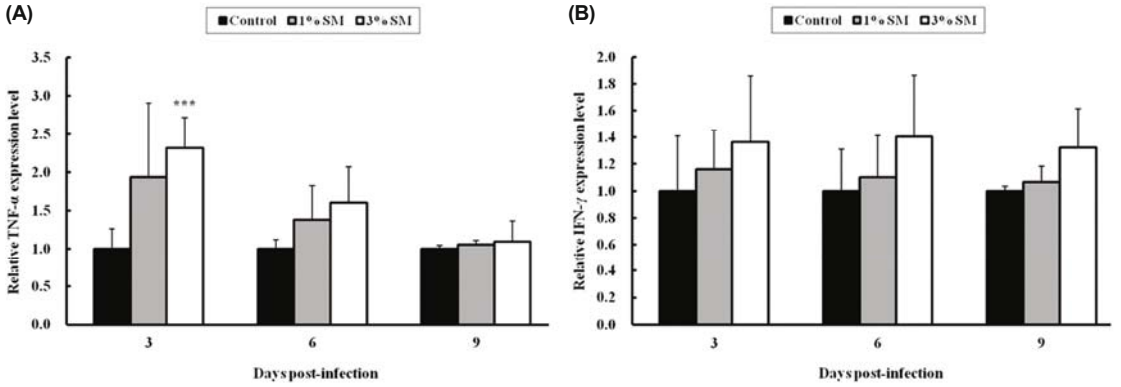

Fig. 4. Effect of shiitake mushrooms on relative mRNA expression levels of (A) TNF-α and (B) IFN-γ in the bronchial lymph node (BLN) of mice experimentally infected with *B. bronchiseptica*. Control, control group; 1% SM, 1% SM-fed group; 3% SM, 3% SM-fed group. For each group, data values represent the mean±SD. $^{***}P<0.001$ vs. control.

group at 6 DPI ($P<0.05$). There was a small, but not statistically significant, reduction in the number of viable bacterial cells at 9 DPI.

Lymphocyte subpopulation in BLN

The percentage of $CD3^+CD19^-$ T lymphocyte in BLN of the 3% SM-fed group was significantly increased compared with the control group at 3 and 6 DPI ($P<0.05$). The percentage of $CD4^-CD8^+$ T lymphocytes and $CD4^+CD8^-$ T lymphocytes in BLN of the 3% SM-fed group were also significantly increased compared with the control group at 3 and 6 DPI, respectively ($P<0.05$). Conversely, the percentage of $CD3^-CD19^+$ B lymphocyte in BLN of the 3% SM-fed group was significantly decreased compared with the control group ($P<0.05$) at 3 and 6 DPI ($P<0.05$) (Fig. 4).

Relative mRNA expression levels of TNF-α and IFN-γ in BLN

The relative mRNA expression levels of TNF-α and IFN-γ in BLN of the 1% and 3% SM-fed groups showed a increasing, dose-dependent tendency compared with the control group during the whole experimental infection period, although the difference was not significant between the groups (Fig. 5). Of note, the relative mRNA expression levels of TNF-α in the BLN of the 3% SM-fed group was significantly increased compared with the control group at 3 DPI ($P<0.05$).

Discussion

Antimicrobials are widely used as feed additives to improve growth performance and to prevent subclinical disease in industrial animals (Cromwell, 2002). The present study monitored body weight changes and evaluated whether SM is an effective immunostimulant that can protect against *B. bronchiseptica*, which causes massive economic loss from mortality, reduced weight gain and poor feed conversion (Goodnow, 1980; Boessen *et al*., 1988).

The present results demonstrate that dietary supplementation with SM has no effect on body weight gain in mice for the 9 weeks before the bacterial infection. This finding is similar to previous reports that SM or SM-derived compounds had no significant effects on body weight gain in rats and mice (Nieminen *et al*., 2009; Yoshioka *et al*., 2010). Although SM had no effect on body weight gain in the absence of infection, SM did alleviate progressive weight loss and aided in the recovery of body weight in *B. bronchiseptica* infected mice. These results suggest that continuous ingestion of SM could help improve growth performance in environments with a risk of exposure to contaminating pathogens, such as conventional farms.

The percentage of T lymphocytes in BLN of the 3% SM-fed group was significantly increased as compared to the control group early in infection. The $CD4^-CD8^+$ T lymphocyte percentage and $CD4^+CD8^-$ T lymphocyte percentage in BLN of the 3% SM-fed group were significantly increased compared with the control group at 3 and 6 DPI, respectively. These results imply that continuous ingestion of SM markedly reinforces mitogenicity and immune responses of T lymphocytes early in infection. In particular, SM stimulates $CD8^+$ T lymphocytes and then $CD4^+$ T lymphocytes after bacterial infection. This agrees with the findings of Kupfahl *et al*. (2006), who noted that the SM-derived compound(s) enhances the protective $CD8^+$ T lymphocyte responses against bacterial infection. Moreover, this may corroborate previous studies that reported that fruiting body and mycelial extracts of SM are able to enhance the proliferation of rat thymocytes directly and act as co-stimulators in the presence of the T-mitogen PHA (Israilides *et al*., 2008).

Relative mRNA expression levels of TNF-α and IFN-γ in BLN of the 1% and 3% SM-fed groups showed an increasing tendency compared with the control group in a dose-dependent manner during the entire infection period. Of note, relative mRNA expression levels of the TNF-α in BLN of the 3% SM-fed groups were significantly increased compared with the control group at 3 DPI. TNF-α and IFN-γ are mainly produced by activated macrophages and stimulate T lymphocytes (Jeong *et al*., 2004). These cytokines play an important role in the host defence against infection by microbial pathogens, and induce a variety of physiologically significant responses that contribute to immunity (Branellec and Chouaib, 1991; Samuel, 2001). These results suggest that continuous ingestion of SM could improve host immunity and increase resistance against bacterial infection. The present study also demonstrates that dietary supplementation of SM reinforces bacterial clearance in *B. bronchiseptica* infected mice. These findings echo those of Zhou *et al*. (2009), in that pre-treatment of mice with SM-derived compounds significantly decreases parasitemia after infection by *Plasmodium yoelii* 17XL. Moreover, the present data may relate to previous studies that reported that SM-derived compounds enhance the expression of MHC II, CD80/CD86 and Toll-like receptors (TLR2/TRL4), and increase the production of IL-12 in spleen dendritic cells co-cultured with parasitized red blood cells (Maeda *et al*., 1994; Zhou *et al*., 2009).

Taken together, these findings suggest that some component(s) of SM could help improve body weight gain in the presence of infection and enhance the protective immune activity against subclinical disease challenge, such as *B. bronchiseptica* infection in mice, probably via a strong stimulation of non-specific immune responses. Hence, SM may provide an alternative way to reduce use of antimicrobials. However, SM contains a complex array of compounds. Therefore, precise knowledge of the major component(s) of SM responsible for the beneficial effects is needed. Studies to that end are currently in progress. Confirmation of the beneficial effects of SM as a feed additive is now required in industrial animals.

Acknowledgements

The authors acknowledge a graduate fellowship provided by the Korean Ministry of Education and Human Resources Development through the Brain Korea 21 project.

References

Bisen, P.S., Baghel, R.K., Sanodiya, B.S., Thakur, G.S., and Prasad, G.B. 2010. *Lentinus edodes*: a macrofungus with pharmacological

activities. *Curr. Med. Chem.* **17**, 2419–2430.
Boa, E. 2004. Wild edible fungi: A global overview of their use and importance to people, p. 148. FAO, Rome, Italy.
Boessen, C.R., Kliebenstein, J.B., Cowart, R.P., Moore, K.C., and Burbee, C.R. 1988. Effective use of slaughter checks to determine economic losses from morbidity in swine. *Acta. Vet. Scand. Suppl.* **84**, 366–368.
Branellec, D. and Chouaib, S. 1991. TNF: antitumoral agent at the border lines of immunity and inflammation. *Pathol. Biol. (Paris).* **39**, 230–239.
Brockmeier, S.L., Palmer, M.V., Bolin, S.R., and Rimler, R.B. 2001. Effects of intranasal inoculation with *Bordetella bronchiseptica*, porcine reproductive and respiratory syndrome virus, or a combination of both organisms on subsequent infection with *Pasteurella multocida* in pigs. *Am. J. Vet. Res.* **62**, 521–525.
Chang, R. 1996. Functional properties of edible mushrooms. *Nutr. Rev.* **54**, S91–S93.
Cromwell, G.L. 2002. Why and how antibiotics are used in swine production. *Anim. Biotechnol.* **13**, 7–27.
European Union. 2003. Regulation (EC) No. 1831/2003 of the European Paliament and of the council of 22 September 2003 on additives for use in animal nutrition (text with EEA relevance). L 268, pp. 29–43. Off. J. Eur. Union.
Goodnow, R.A. 1980. Biology of *Bordetella bronchiseptica*. *Microbiol. Rev.* **44**, 722–738.
Guo, F.C., Kwakkel, R.P., Williams, B.A., Li, W.K., Li, H.S., Luo, J.Y., Li, X.P., Wei, Y.X., Yan, Z.T., and Verstegen, M.W. 2004. Effects of mushroom and herb polysaccharides, as alternatives for an antibiotic, on growth performance of broilers. *Br. Poult. Sci.* **45**, 684–694.
Gustafson, R.H. and Bowen, R.E. 1997. Antibiotic use in animal agriculture. *J. Appl. Microbiol.* **83**, 531–541.
Hagan, W.A. and Bruner, D.W. 1988. Hagan and Bruner's microbiology and infectious diseases of domestic animals, pp. 117–120. Cornell University Press, New York, N.Y., USA.
Hamuro, J. and Chihara, G. 1985. Lentinan, a T-cell oriented immunopotentiator: its experimental and clinical applications and possible mechanism of immune modulation, pp. 409–436. *In* Fenichel, R.L. and Chirigos, M.A. (eds.), Immune Modulation Agents and Their Mechanisms. Marcel Dekker, New York, N.Y., USA.
Hardy, B. 2002. The issue of antibiotic use in the livestock industry: what have we learned? *Anim. Biotechnol.* **13**, 129–147.
Harris, D.L. and Switzer, W.P. 1968. Turbinate atrophy in young pigs exposed to *Bordetella bronchiseptica*, *Pasteurella multocida*, and combined inoculum. *Am. J. Vet. Res.* **29**, 777–785.
Hatvani, N. 2001. Antibacterial effect of the culture fluid of *Lentinus edodes* mycelium grown in submerged liquid culture. *Int. J. Antimicrob. Agents* **17**, 71–74.
Ishikawa, N.K., Kasuya, M.C.M., and Vanetti, M.C.D. 2001. Antibacterial activity of *Lentinula edodes*. *Braz. J. Microbiol.* **32**, 206–210.
Israilides, C., Kletsas, D., Arapoglou, D., Philippoussis, A., Pratsinis, H., Ebringerová, A., Hríbalová, V., and Harding, S.E. 2008. *In vitro* cytostatic and immunomodulatory properties of the medicinal mushroom *Lentinula edodes*. *Phytomedicine* **15**, 512–519.
Jeong, H.J., Chung, H.S., An, H.J., Seo, S.W., Kim, T.G., Won, J.H., Shin, J.Y., Ahn, K.S., and Kim, H.M. 2004. The immune-enhancing effect of the herbal combination Bouum-Myunyuk-Dan. *Biol. Pharm. Bull.* **27**, 29–33.
Jung, B.G., Ko, J.H., Cho, S.J., Koh, H.B., Yoon, S.R., Han, D.U., and Lee, B.J. 2010a. Immune-enhancing effect of fermented Maesil (*Prunus mume* Siebold & Zucc.) with probiotics against *Bordetella bronchiseptica* in mice. *J. Vet. Med. Sci.* **72**, 1195–1202.
Jung, B.G., Ko, J.H., and Lee, B.J. 2010b. Dietary supplementation with a probiotic fermented four-herb combination enhances immune activity in broiler chicks and increases survivability against *Salmonella* Gallinarum in experimentally infected broiler chicks. *J. Vet. Med. Sci.* **72**, 1565–1573.
Jung, B.G., Toan, N.T., Cho, S.J., Ko, J.H., Jung, Y.K., and Lee, B.J. 2010c. Dietary aluminosilicate supplement enhances immune activity in mice and reinforces clearance of porcine circovirus type 2 in experimentally infected pigs. *Vet. Microbiol.* **143**, 117–125.
Komemushi, S., Yamamoto, Y., and Fujita, T. 1995. Antimicrobial substance produced by *Lentinus edodes*. *J. Antibact. Antifung. Agents* **23**, 81–86.
Komemushi, S., Yamamoto, Y., and Fujita, T. 1996. Purification and identification of antimicrobial substances produced by *Lentinus edodes*. *J. Antibact. Antifung. Agents* **24**, 21–25.
Kupfahl, C., Geginat, G., and Hof, H. 2006. Lentinan has a stimulatory effect on innate and adaptive immunity against murine *Listeria* monocytogenes infection. *Int. Immunopharmacol.* **6**, 686–696.
Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**, 402–408.
Maeda, Y.Y., Takahama, S., and Yonekawa, H. 1998. Four dominant loci for the vascular responses by the antitumor polysaccharide, lentinan. *Immunogenetics* **47**, 159–165.
Maeda, Y.Y., Yonekawa, H., and Chihara, G. 1994. Application of lentinan as cytokine inducer and host defence potential in immunotherapy of infectious diseases, pp. 261–279. *In* Mashi, K.N. (ed.), Immunotherapy of Infections, Marcel Dekker, New York, N.Y., USA.
Mattila, P., Suonpää, K., and Piironen, V. 2000. Functional properties of edible mushrooms. *Nutrition* **16**, 694–696.
Ng, M.L. and Yap, A.T. 2002. Inhibition of human colon carcinoma development by lentinan from shiitake mushrooms (*Lentinus edodes*). *J. Altern. Complement. Med.* **58**, 1–9.
Ngai, P.H. and Ng, T.B. 2003. Lentin, a novel and potent antifungal protein from shitake mushroom with inhibitory effects on activity of human immunodeficiency virus-1 reverse transcriptase and proliferation of leukemia cells. *Life Sci.* **73**, 3363–3374.
Nieminen, P., Kärjä, V., and Mustonen, A.M. 2009. Myo- and hepatotoxic effects of cultivated mushrooms in mice. *Food. Chem. Toxicol.* **47**, 70–74.
Overbergh, L., Valckx, D., Waer, M., and Mathieu, C. 1999. Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. *Cytokine* **11**, 305–312.
Palzer, A., Ritzmann, M., Wolf, G., and Heinritzi, K. 2008. Associations between pathogens in healthy pigs and pigs with pneumonia. *Vet. Rec.* **162**, 267–271.
Samuel, C.E. 2001. Antiviral actions of interferons. *Clin. Microbiol. Rev.* **14**, 778–809.
Smith, I.M. and Baskerville, A.J. 1979. A selective medium facilitating the isolation and recognition of *Bordetella bronchiseptica* in pigs. *Res. Vet. Sci.* **27**, 187–192.
Sugiyama, K., Arashi, T., and Yamakawa, A. 1995. Eritadenine-induced alteration of hepatic phospholipid metabolism in relation to its hypocholesterolemic action in rats. *Nutrit. Biochem.* **6**, 80–87.
Yang, B.K., Kim, D.H., Jeong, S.C., Das, S., Choi, Y.S., Shin, J.S., Lee, S.C., and Song, C.H. 2002. Hypoglycemic effect of a *Lentinus edodes* exo-polymer produced from a submerged mycelia culture. *Biosci. Biotechnol. Biochem.* **66**, 937–942.
Yoshioka, Y., Tamesada, M., and Tomi, H. 2010. A repeated dose 28-day oral toxicity study of extract from cultured *Lentinula edodes* mycelia in Wistar rats. *J. Toxicol. Sci.* **35**, 785–791.
Zhou, L.D., Zhang, Q.H., Zhang, Y., Liu, J., and Cao, Y.M. 2009. The shiitake mushroom-derived immuno-stimulant lentinan protects against murine malaria blood-stage infection by evoking adaptive immune-responses. *Int. Immunopharmacol.* **9**, 455–462.