Food and Agricultural Immunology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713422299

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Online Publication Date: 01 September 2009

To cite this Article Racedo, Silvia, Villena, Julio, Salva, Susana and Alvarez, Susana(2009)'Influence of yogurt consumption on the respiratory immune response',Food and Agricultural Immunology,20:3,231 — 244
To link to this Article: DOI: 10.1080/09540100903061659
URL: http://dx.doi.org/10.1080/09540100903061659

Food and Agricultural Immunology
Vol. 20, No. 3, September 2009, 231–244

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Influence of yogurt consumption on the respiratory immune response

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(Received 14 November 2008; final version received 21 May 2009)

The influence of two yogurts, made with different starter strains, on the immune response against a respiratory pathogen was studied. Yogurt P (YP) was prepared with the immune-enhancing strains *Lactobacillus bulgaricus* CRL 423 and *Streptococcus thermophilus* CRL 412. Control yogurt (YC) was prepared with *L. bulgaricus* AV31 and *S. thermophilus* AV4 strains with no intrinsic immuno-enhancing activity. Swiss albino mice were fed YP or YC for five days and infected intranasally with *Streptococcus pneumoniae* on day 6. The resistance to the infection and innate and specific immune responses were studied. Results showed that both yogurts were able to improve the resistance against pneumococcal infection when compared with control mice. However, YP was more effective than YC in protecting the mice against the infection. Both yogurts improved the activation and recruitment of phagocytes in the respiratory tract, but only YP was capable of increasing the number of IgA+ cells in the bronchus and the levels of pathogen specific IgA and IgG in the airways and serum. Our results show that the consumption of yogurt is able to enhance immunity in the respiratory tract. In addition, we demonstrate that the protective effect of yogurt could be improved if it is prepared with starter strains of *L. bulgaricus* and *S. thermophils* that have intrinsic immunomodulatory activity.

Keywords: probiotic; yogurt; respiratory infection; *Streptococcus pneumoniae*

Introduction

Probiotics are defined as viable non-pathogenic microorganisms which, when ingested, exert a positive influence on host health or physiology (Sanders, 2003). Since yogurt consumption has been associated with health benefits, yogurt can be considered a probiotic (Guarner et al., 2005). Several studies have demonstrated that yogurt is able to stimulate the immune system and increase the resistance against intestinal pathogens (Meydani & Ha, 2000). Although yogurt has long been known to improve host defence mechanisms against invading pathogens, the components responsible for these effects have been not fully defined (Meydani & Ha, 2000). The immunostimulatory effects of yogurt are believed to be due to it's bacterial components. Moreover, it has been shown that the viability of the lactic acid bacteria (LAB) present in yogurt is an important factor to achieve an optimal protective effect (Guarner et al., 2005; Van de water, Keen, & Gershwin, 1999).

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ISSN 0954-0105 print/ISSN 1465-3443 online

DOI: 10.1080/09540100903061659
http://www.informaworld.com

However, there are no studies that compare the immunological effects of yogurts made with different strains of LAB as starters. These studies would be of great importance as it is known that the immunostimulating activity of LAB differs depending on the properties of a particular strain rather than on the common characteristics of the species (Alvarez, Herrero, Bru, & Perdigón, 2001; Racedo et al., 2006; Villena et al., 2005). Thus, the use of *Lactobacillus bulgaricus* or *Streptococcus thermophilus* strains with intrinsic immunomodulatory activity, in the preparation of yogurt, would be able to improve its immunostimulatory activity.

Although most research concerning probiotic-mediated enhanced immune protection is focused on gastrointestinal tract pathogens, a few recent studies centred on whether probiotics might sufficiently stimulate the common mucosal immune system to provide protection to other mucosal sites as well (Alvarez, Villena, Racedo, Salva, & Agüero, 2007; Cross, 2002). In this sense, our lab studied the potential effect of probiotics on the improvement of the immune response against respiratory pathogens (Alvarez et al., 2007). We demonstrated that the administration of *Lactobacillus casei* CRL 431 or *Lactococcus lactis* NZ9000 were able to increase *S. pneumoniae* clearance rates in lung and blood, improved survival of infected mice and reduced lung injuries (Racedo et al., 2006; Villena, Medina, Vintiñi, & Alvarez, 2008). It has been demonstrated that gut-associated lymphoid tissue is stimulated by *L. bulgaricus* CRL 423 and *S. thermophilus* CRL 412, resulting in enhanced production of cytokines and secretory IgA (Dogi, Galdeano, & Perdigón, 2008; Perdigón, Vintiñi, Alvarez, Medina, & Medici, 1998). However, it has not been studied whether these strains can increase immunity against respiratory pathogens.

The aim of this work was to study the influence of yogurt consumption on the innate and adaptive immune response of mice against a respiratory pathogen infection. We performed a comparative study of the effect of two yogurts, made with starter strains of *L. bulgaricus* and *S. thermophilus* with or without intrinsic immunomodulatory activity.

Materials and methods

Animals

Six-week-old Swiss albino mice (22–28 g) were obtained from the random-bred colony kept in our research centre (CERELA). Each experimental group consisted of 25–30 mice (5–6 for each day before and after infection). The Ethical Committee for Animal Care at CERELA approved experimental protocols.

Yogurt preparation and feeding procedures

Two simulated commercial yogurts were prepared by the Laboratory of Experimental Foods at CERELA. Yogurts were prepared from a stock culture of LAB containing *L. bulgaricus* and *S. thermophilus*. Yogurt P (YP) was prepared with the immune-enhancing strains *L. bulgaricus* CRL 423 and *S. thermophilus* CRL 412 (Dogi et al., 2008; Perdigón et al., 1998). Control yogurt (yogurt C) was prepared with *L. bulgaricus* AV31 and *S. thermophilus* AV4, strains with no intrinsic immunoenhancing activity (unpublished data). LAB were mixed at a ratio of 1:1, incubated in 10% non-fat milk (NFM) at 42°C for 4 h and then at 4°C for 24 h. At the end of this process the total

bacterial count was 2×10^8 cells/ml. The yogurts were prepared every 48 h in order to ensure a constant number of bacteria. A balanced conventional diet was supplemented with yogurts and administered for five consecutive days to different groups of mice (YP and YC groups, respectively). The control group was fed the balanced conventional diet without supplemental yogurt.

Pneumococcal infection

Capsulated *Streptococcus pneumoniae* serotype 14 was isolated from the respiratory tract of a patient from the Department of Clinical Bacteriology of the Niño Jesús Children's Hospital in San Miguel de Tucumán, Argentina. This is one of the most frequent serotypes of *S. penumoniae* isolated in pneumococcal infections in Argentina.

S. pneumoniae was first grown on blood agar for 18 h. Freshly grown colonies were suspended in Todd Hewitt broth (Oxoid) and incubated overnight at 37°C, harvested and washed three times with sterile PBS. Cell density was adjusted to 4×10^7 CFU/l. Challenge with *S. pneumoniae* was performed on the day after the end of each yogurt treatment (on the sixth day). Mice were challenged intranasally with the pathogen by dripping 25 µl of an inoculum containing 10^6 CFU of *S. pneumoniae* (log-phase) in PBS into each nostril. Animals were held in a head-up vertical position for 2 min to facilitate migration of the inoculum to the alveoli.

Bacterial cell counts in lung homogenates and blood

For bacterial cell counts in lung, mice were sacrificed on day 1, 5, 10 and 15 post-infection and their lungs were excised, weighed and homogenised in 5 ml of sterile peptone water. Homogenates were diluted appropriately, plated in duplicate on blood agar and incubated for 18 h at 37°C. *Streptococcus pneumoniae* colonies were counted and the results were expressed as $\log_{10}$ CFU/g of organ. Progression of bacterial growth to the bloodstream was monitored by blood samples obtained by cardiac puncture with a heparinised syringe. Samples were plated on blood agar and bacteremia was reported as negative or positive hemocultures after incubation for 18 h at 37°C.

Total and differential number of blood and bronchoalveolar lavages (BAL) leukocytes

Bronchoalveolar lavages (BAL) samples were obtained according to the technique previously described (Villena et al., 2005, 2008). Briefly, the trachea was exposed and intubated with a catheter and two sequential BAL were performed in each mouse by injecting 0.5 ml of sterile PBS; the recovered fluid was centrifuged for 10 min at 900 *g*; the pellet was used to determine total and differential counts of BAL leukocytes and the fluid was frozen at -70°C for subsequent antibody analyses. The total number of leukocytes in blood and BAL were determined with a hemocytometer. Differential cell counts were performed by counting 200 cells in blood or BAL smears stained with May Grünwald-Giemsa.

Peroxidase activity in blood and lung

Myeloperoxidase (MPO) activity of blood neutrophils was determined through a cytochemical method (Washburn test) using benzidine as a MPO chromogen (Racedo

et al., 2006). Cells were graded as negative or weakly, moderately or strongly positive and were used to calculate the score. In addition, neutrophil infiltration in lung tissue was quantified by measurement of MPO in lung homogenates. Lungs were cleared of blood, removed and homogenised in 50 mM acetate buffer, pH 5.4 (MPO-assay buffer). Homogenates were frozen at -70°C for 15 min, thawed, sonicated for 60 s and centrifuged at 3600 $\times g$ for 15 min at 4°C. MPO was evaluated by adding 200 µl of an appropriate dilution of the lysate to 20 mM 3,3′, 5,5′-tetramethylbenzidine in dimethylphormamide and 30 µl of 2.7 mM of hydrogen peroxide in MPO-assay buffer. The reaction mix was incubated for 3 min at 37°C and stopped with ice-cold 200 mM sodium acetate buffer (pH 3). Absorbance was read at 655 nm against a standard curve made with commercial MPO (Sigma). The results were expressed as specific activity of MPO (MPO units/mg of total proteins in lung homogenate). Total protein concentration was determined in the cellular lysates by Bradford's method.

Alveolar phagocytes activation

The bactericidal activity (oxidative burst) of macrophages and neutrophils was measured in the pellet of BAL fluid using the nitro blue tetrazolium (NBT) reduction test (Sigma-Aldrich) (Villena et al., 2005, 2008). NBT was added to each sample with (positive control) or without addition of the bacterial extract; then samples were incubated at 37°C for 20 min. In the presence of oxidative metabolites, NBT (yellow) is reduced to formazan, which forms a blue precipitate. Smears were prepared and, after staining, samples were examined under a light microscope for blue precipitates. A hundred cells were counted and the percentage of NBT positive (+) cells was determined.

Anti-pneumococcal antibodies determination by ELISA

To evaluate the adaptative immune response, a previously developed ELISA technique was used for the determination of anti-pneumococcal IgA and IgG in serum and BAL (Villena, Racedo, Agüero, & Alvarez, 2006; Villena et al., 2005). Briefly, plates were coated with a heat killed *S. pneumoniae*–sodium carbonate–bicarbonate buffer (1:100) suspension, pH 9.6. Non-specific protein binding sites were blocked with PBS containing 5% NFM. Samples were diluted (serum 1:20; BAL 1:2) with PBS containing 0.05% (v/v) Tween 20 (PBS-T). Peroxidase-conjugated goat anti-mouse IgA or IgG (Fc specific, Sigma) were diluted (1:500) in PBS-T. Antibodies were revealed with a substrate solution [3–3′, 5–5′-tetramethylbenzidine (Sigma)] in citrate–phosphate buffer (pH 5, containing 0.05% H_2O_2) and the reaction was stopped by addition of H_2SO_4 1M. Readings were carried out at 493 nm. Antibody concentration was expressed as mg/l determined from a standard curve made with commercial mouse IgA or IgG (Sigma).

Immunofluorescence test for IgA+cells in bronchus-associated lymphoid tissue (BALT)

The number of IgA producing cells was determined on lung histological slides by a direct immunofluorescence technique according to Agüero, Villena, Racedo, Haro, and Alvarez (2006). The immunofluorescence test was performed using the

respective monospecific antibodies (α-chain specific) conjugated with fluorescein isothiocyanate (FITC; Sigma, St. Louis, MO 61378, USA). Histological samples were incubated with the appropriate antibody dilution (1/100 for IgA in 0.01 m Na PBS solution (pH 7.2) for 30 min at 37°C. Then, samples were washed three times with PBS solution and examined using a fluorescent light microscope. The results were expressed as the number of positive fluorescent cells per 10 fields (magnification 100×). They represent the mean of three histological slices per animal ($n = 5$) for each treatment and time point.

Histopathological studies of the lung

To evaluate tissue damage induced by infection, histopathological studies were performed. Mice were sacrificed and lungs were aseptically removed, fixed in 4% formalin and embedded in histowax (Leica Microsystems Nussloch GmbH). Histopathological assessment was performed on five-micron tissue sections stained with hematoxylin–eosin for light microscopy. All slides were coded and evaluated blindly.

Statistical analysis

Experiments were performed in triplicate and results were expressed as mean ± standard deviation. After verification of a normal distribution of data, two-way ANOVA was used. Tukey's test (for pairwise comparisons of the means) was used to test for differences between the groups. Differences were considered significant at $p < 0.05$.

Results

Resistance against pneumococcal infection

The capacity of YC and YP to improve respiratory immunity was evaluated in a *S. pneumoniae* infection model. After the challenge, the pathogen was detected in lung and blood samples throughout the assayed period (15 days post-infection) in the control group (Figure 1). Mice treated preventively with YC and YP showed lower lung bacterial counts than the control group. However, bacterial cell counts in the YP group were lower than in the YC mice. Moreover, YP treatment allowed the complete elimination of the pathogen on day 15 post-infection. Hemocultures from the YP and YC groups were negative after days 7 and 10 post-infection, respectively (data not shown).

Lung tissue injury

In order to establish whether the reduction in pneumococcal cell counts was correlated with lower tissue damage in lungs, histological studies were performed in the respiratory tract of infected mice. The histopathological examination of control mice lungs revealed an intense inflammatory response with progressive parenchymal involvement, including widespread cellular infiltration, haemorrhage (Figure 2A), increased fibrosis in bronchial walls (Figures 2A and 2E) and vessels and reduction of the alveolar airspaces (Figures 2A and 2C). In contrast, the YP group revealed focal cellular infiltration with a significant delay in tissue injury in comparison with

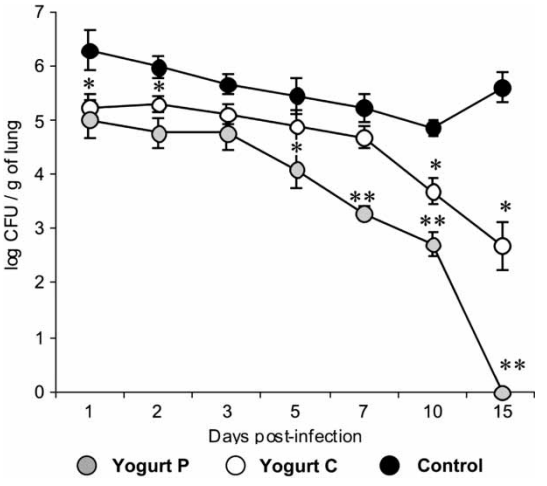

Figure 1. Bacterial cell counts in lung homogenates after the challenge with *Streptococcus pneumoniae*. Mice were treated preventively with yogurt P (containing the immunoenhancing strains *L. bulgaricus* CRL 423 and *S. thermophilus* CRL 412) or yogurt C (containing *L. bulgaricus* AV31 and *S. thermophilus* AV4, strains with no intrinsic immunomodulatory activity) for five consecutive days and then challenged nasally with 10^6 *S. pneumoniae* cells. Control mice were infected without previous treatment. Shown are mean $\pm$ SD ($n = 6$ mice/group). *Significant differences compared to the control group ($P < 0.05$). **Significant differences compared to the yogurt C and control groups ($P < 0.05$).

the infected control group (Figure 2B). The lungs of the YP mice showed moderate fibrosis in bronchial walls and conserved alveolar airspaces (CAA) (Figures 2D and 2F). Mice administered YC showed histological signs intermediate to those of YP and control mice.

Total and differential leukocyte counts in blood and bronchoalveolar lavages (BAL)

After challenge with *S. pneumoniae,* an increase in the total number of blood and BAL leukocytes was observed in treated and control mice (Table 1). There were no significant differences between the groups concerning the number of blood leukocytes throughout the assayed period. In contrast, BAL leukocytes values were significantly higher in treated mice than in the control group on day 1 post-infection, without differences between YC and YP groups (Table 1). In all groups, BAL leukocytes reached a peak on day 5 and from then on they gradually decreased. Mice treated with YC or YP showed significantly lower numbers of BAL leukocytes than control mice on days 10 and 15 post-infection.

Phagocytes activity in blood and lungs

Blood peroxidase score and lung MPO activity were not modified by the treatments before challenge with the pathogen (Table 2). After infection, these parameters showed a gradual increase in all experimental groups; however, mice treated with YC and YP showed significantly higher values than the control group. After a peak on

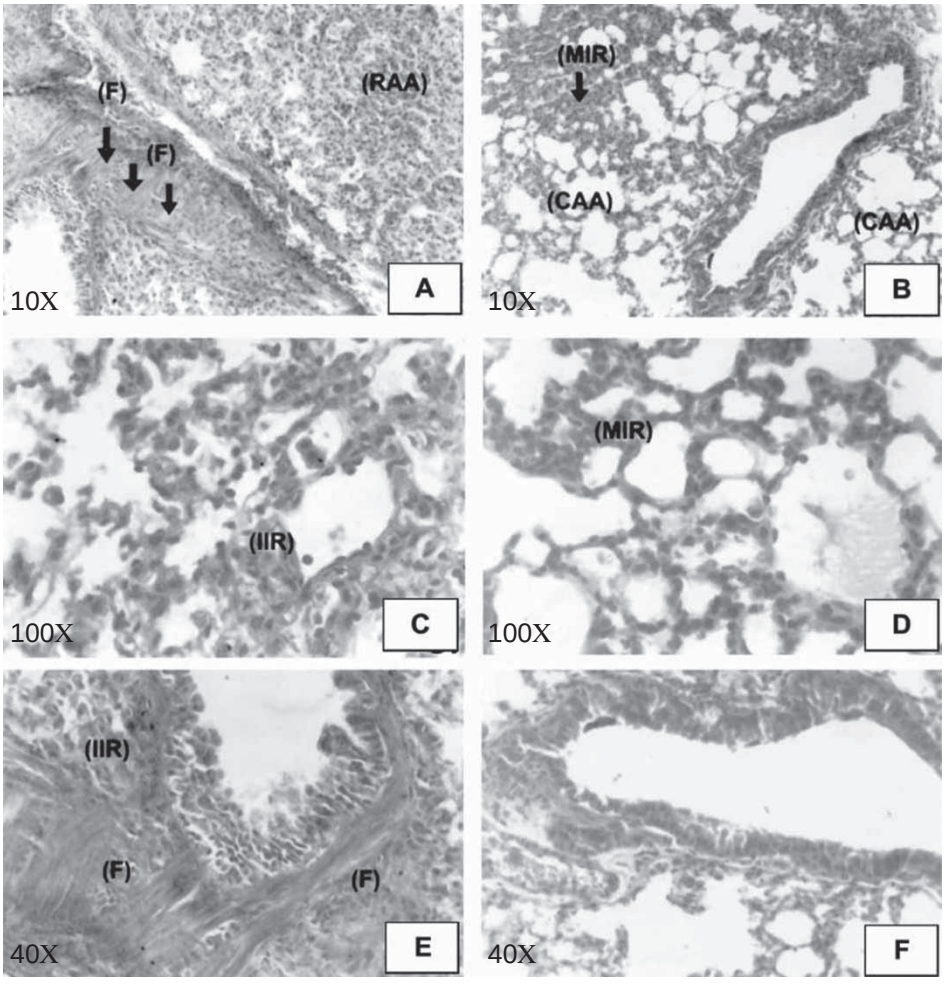

Figure 2. Histological changes in lungs. Mice were treated preventively with yogurt P (containing the immunoenhancing strains *L. bulgaricus* CRL 423 and *S. thermophilus* CRL 412) or yogurt C (containing *L. bulgaricus* AV31 and *S. thermophilus* AV4, strains with no intrinsic immunomodulatory activity) for five consecutive days and then challenged nasally with 10^6 *S. pneumoniae* cells. Control mice were infected without previous treatment. Intensive inflammatory response (IIR), fibrosis (F) and reduction of alveolar airspaces (RAA) are evident in the control group (A, C, E). Mice treated with yogurt P showed signs of a moderate inflammatory response (MIR) without haemorrhage and conserved alveolar airspaces (CAA) in comparison with control mice (B, D, F). Magnification: $\times 200$ (A, B), $\times 1000$ (C, D), $\times 400$ (E, F). Photographs of day 10 post-infection are shown.

day 3 post-infection, blood and lung MPO activity gradually decreased but, in contrast with the early stages of infection, the groups treated with yogurts showed significantly lower peroxidase activity than the control groups at the late stage of infection. No differences were observed between YC and YP groups (Table 2). The stronger activation of blood and lung neutrophils observed in the yogurts treated mice would be involved in the more effective blood and lung pneumococcal

Table 1. Blood and bronchoalveolar lavages (BAL) leukocytes. Mice were treated preventively with yogurt P (containing the immunoenhancing strains *L. bulgaricus* CRL 423 and *S. thermophilus* CRL 412) or yogurt C (containing *L. bulgaricus* AV31 and *S. thermophilus* AV4, strains with no intrinsic immunomodulatory activity) for five consecutive days and then challenged nasally with 10^6 *S. pneumoniae* cells. Control mice were infected without previous treatment. Results of blood and BAL leukocytes are expressed as 10^9 and 10^7 cell/l, respectively. Shown are mean $\pm$SD ($n = 6$ mice/group).

	Blood leukocytes			BAL leukocytes		
Days	Control	Yoghurt C	Yoghurt P	Control	Yoghurt C	Yoghurt P
0	6.9 ± 0.2	6.5 ± 0.6	6.7 ± 0.7	2.0 ± 0.1	1.9 ± 0.6	2.1 ± 0.5
1	8.9 ± 0.1	8.8 ± 0.7	9.0 ± 0.8	20.1 ± 0.1	24.8 ± 1.0[a]	25.6 ± 0.6[a]
5	9.7 ± 0.9	9.5 ± 0.9	9.5 ± 0.9	29.6 ± 1.2	27.1 ± 0.5	29.5 ± 0.5
10	8.5 ± 0.4	8.1 ± 0.1	8.2 ± 0.2	25.3 ± 0.4	21.7 ± 0.4[a]	20.3 ± 1.0[a]
15	7.2 ± 0.7	7.2 ± 0.8	7.4 ± 0.8	23.3 ± 0.5	18.0 ± 0.4[a]	17.4 ± 0.5[a]

[a]Significantly different from the control group at the same time point ($p < 0.05$).

clearance, which would allow these groups to return earlier to basal levels of peroxidase activity.

Prior to infection, yogurt treatments induced a significant increase in BAL NBT+ cells compared to the control group (Table 2). Challenge with *S. pneumoniae* increased the bactericidal function of BAL phagocytes in all experimental groups. Both yogurt treatments induced significantly higher phagocyte activity on days 1 and 2 post-infection than the control group, and no differences were observed between YP and YC groups.

Anti-pneumococcal antibodies in serum and bronchoalveolar lavages (BAL)

Challenge with the pathogen increased the levels of specific IgG and IgA antibodies in serum and BAL in all experimental groups, a peak being reached at day 15 post-infection. YP treatment induced a significant increase in specific pneumococcal BAL IgA and IgG antibodies compared to controls (Figures 3A,B). In contrast, levels of BAL anti-pneumococcal IgG and IgA in mice that received YC were similar to those in the control group. Serum anti-pneumococcal IgA levels were similar in all groups (Figure 3D). However, levels of serum anti-pneumococcal IgG were significantly higher in YP group compared to the control (Figure 3C). No differences were found in serum specific IgG between YC and control groups.

Number of IgA+ cells in bronchus-associated lymphoid tissue (BALT)

The higher amount of BAL IgA would indicate that YP administration is able to stimulate the IgA cell cycle. Thus, we next determined the number of IgA+ cells in the bronchus. Figure 4A shows that treatment with YP enhanced the number of bronchus IgA+ cells prior to challenge with *S. pneumoniae*. The infection induced an increase of the number of bronchus-associated lymphoid tissue (BALT) IgA+ cells in all groups (Figure 4A). However, the YP mice showed significantly higher numbers of IgA producing cells in the BALT at days 10 and 15 post-infection compared to the

Table 2. Blood and bronchoalveolar lavages (BAL) phagocytes activity. Mice were treated preventively with yogurt P (containing the immunoenhancing strains *L. bulgaricus* CRL 423 and *S. thermophilus* CRL 412) or yogurt C (containing *L. bulgaricus* AV31 and *S. thermophilus* AV4, strains with no intrinsic immunomodulatory activity) for five consecutive days and then challenged nasally with 10^6 *S. pneumoniae* cells. Control mice were infected without previous treatment. Results of blood peroxidase, lung myeloperoxidase (MPO) and BAL NBT+ cells are expressed as score number, MPO units/mg and percentage, respectively. Shown are mean ± SD ($n = 6$ mice/group).

	Blood peroxidase			Lung MPO			BAL NBT+ cells		
Days	Control	Yoghurt C	Yoghurt P	Control	Yoghurt C	Yoghurt P	Control	Yoghurt C	Yoghurt P
0	112.1 ± 7.1	113.6 ± 3.2	119.3 ± 4.3	1.21 ± 0.27	1.25 ± 0.17	1.23 ± 0.15	25.8 ± 0.8	42.1 ± 2.7[a]	44.9 ± 3.1[a]
1	120.2 ± 7.3	178.5 ± 5.4[a]	177.5 ± 8.1[a]	5.70 ± 0.87	7.64 ± 0.19[a]	7.87 ± 0.22[a]	63.9 ± 1.1	75.8 ± 2.2[a]	77.2 ± 2.5[a]
2	152.4 ± 2.5	190.4 ± 4.5[a]	192.6 ± 5.3[a]	7.49 ± 0.47	9.67 ± 0.54[a]	9.80 ± 0.33[a]	70.8 ± 1.5	77.9 ± 1.1[a]	78.6 ± 0.9[a]
3	197.3 ± 5.3	218.4 ± 6.0[a]	220.2 ± 8.1[a]	5.41 ± 0.68	8.25 ± 0.28[a]	8.10 ± 0.42[a]	71.8 ± 1.8	75.3 ± 3.7	78.2 ± 1.1
5	194.2 ± 5.5	162.8 ± 4.1[a]	160.0 ± 6.8[a]	4.46 ± 0.56	4.45 ± 0.49	4.30 ± 0.10	63.8 ± 2.1	62.4 ± 3.1	63.2 ± 1.1
10	167.4 ± 8.4	94.7 ± 5.1[a]	92.3 ± 8.2[a]	2.94 ± 0.66	2.32 ± 0.51	2.10 ± 0.21	63.3 ± 0.5	60.9 ± 0.8	62.6 ± 0.8
15	157.6 ± 7.0	92.8 ± 6.3[a]	94.7 ± 7.4[a]	2.34 ± 0.16	2.21 ± 0.98	2.00 ± 0.12	65.4 ± 3.3	66.7 ± 3.4	67.1 ± 1.7

[a]Significantly different from the control group at the same time point ($p < 0.05$).

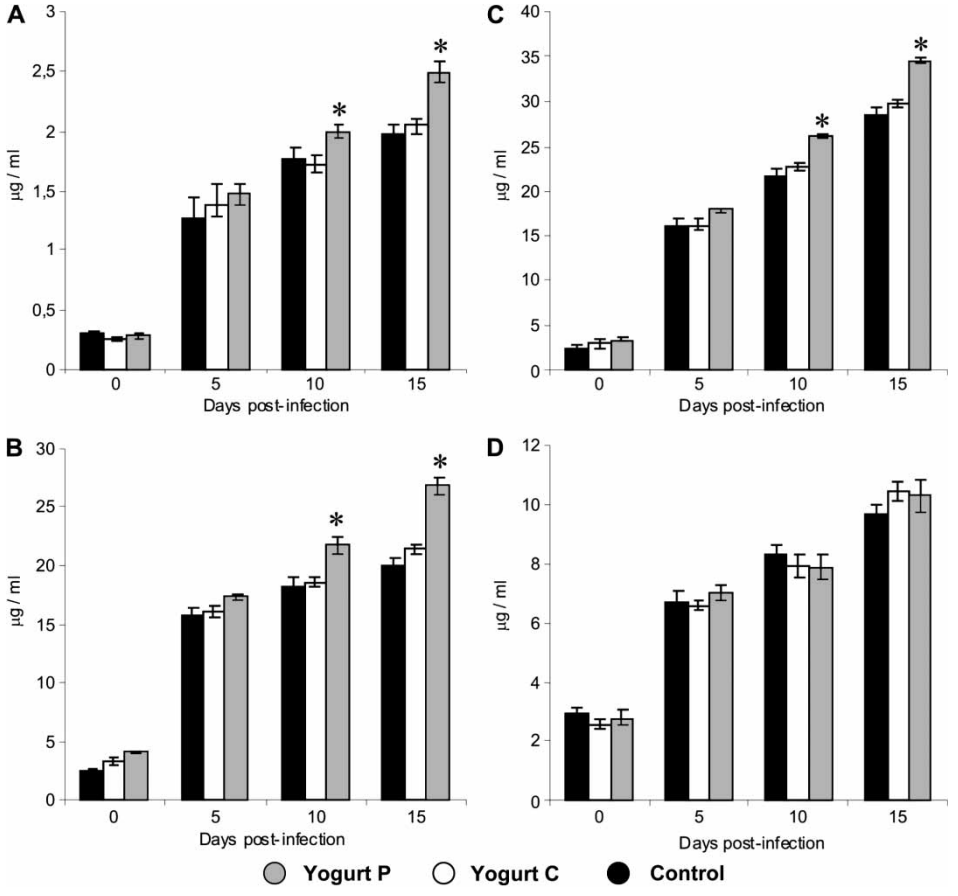

Figure 3. Anti-pneumococcal antibody levels in serum and broncho-alveolar lavage (BAL). Mice were treated preventively with yogurt P (containing the immunoenhancing strains *L. bulgaricus* CRL 423 and *S. thermophilus* CRL 412) or yogurt C (containing *L. bulgaricus* AV31 and *S. thermophilus* AV4, strains with no intrinsic immunomodulatory activity) for five consecutive days and then challenged nasally with 10^6 *S. pneumoniae* cells. Control mice were infected without previous treatment. BAL IgG (A), BAL IgA (B), serum IgG (C) and serum IgA (D). Shown are mean $\pm$SD ($n = 6$ mice/group). *Significant differences compared to the control group ($P < 0.05$).

control group (Figures 4A–C). The numbers of IgA+ cells in mice that received YC were similar to those in the control group all the assayed period (Figure 4A).

Discussion

In the present work, we studied the influence of two yogurts, made with different starter strains of *L. bulgaricus* and *S. thermophilus*, on the immune response against a respiratory pathogen. Results showed that both yogurts were able to improve the resistance against *S. pneumoniae* infection. The number of pneumococci found in lung of treated mice was lower and the period of septicemia shorter than those of the controls. However, the yogurt containing the immunoenhancing strains *L. bulgaricus*

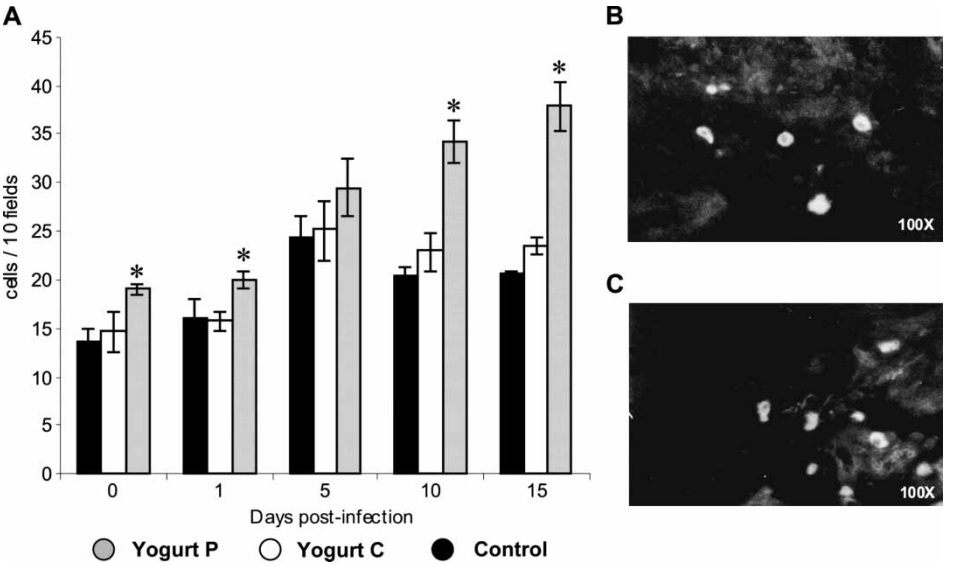

Figure 4. Number of IgA+ cells in bronchus-associated lymphoid tissue (A). Mice were treated preventively with yogurt P (containing the immunoenhancing strains *L. bulgaricus* CRL 423 and *S. thermophilus* CRL 412) or yogurt C (containing *L. bulgaricus* AV31 and *S. thermophilus* AV4, strains with no intrinsic immunomodulatory activity) for five consecutive days and then challenged nasally with 10^6 *S. pneumoniae* cells. Control mice were infected without previous treatment. Shown are mean $\pm$SD ($n = 6$ mice/group). *Significant differences compared to the control group ($P < 0.05$). IgA+ cells in BALT of control mice (B) and yogurt P group (C) on day 10 post-infection. Original magnification $\times 100$.

CRL 423 and *S. thermophilus* CRL 412 was more effective to protect against the respiratory challenge. Mice in the YP group showed higher pneumococcal clearance rates in lung and blood and reduced lung injuries than YC mice.

It is known that the clearance of *S. pneumoniae* from the lungs takes place through the phagocytosis of the bacterium by neutrophils and alveolar macrophages. This process is facilitated by the presence of serotype-specific immunoglobulins (IgG1, IgG2, IgM and IgA) and active complement (Catterall, 1999; Twigg, 2005). This opsonisation initiated by antibodies that activate the classic pathway of the complement is considered to be the most important immune mechanism in the protection of the host against infection by this pathogen (Bogaert, Hermans, Adrian, Rümke, & Groot, 2004). Thus, in order to attain full protection against the neumococcus both innate and specific immune mechanisms are required.

Alveolar macrophages play a prominent role in lung immunity by initiating inflammatory and immune responses. In the event that the invading pathogens are too virulent or represent too large a load to be contained by alveolar macrophages alone, these cells are capable of generating mediators that recruit large numbers of neutrophils into the alveolar space. These recruited neutrophils provide auxiliary phagocytic capacities that are critical for the effective eradication of offending pathogens (Zhang, Summer, Bagby, & Nelson, 2000). On the first day after pneumococcal infection, we observed a higher activation of macrophages and neutrophils in the groups that consumed yogurts, evidenced by a significant increase

in blood and lung peroxidase activity and in the percentage of BAL NBT+ cells. However, there were no significant differences when comparing YP and YC. These results would indicate that the increase of the innate immune response against the pneumococcal infection, achieved by the administration of YP and YC is independent of the LAB present in them. *In vitro* studies have demonstrated that bioactive peptides from yogurt can stimulate phagocytosis in mice peritoneal macrophages (Parker et al., 1984). In addition, it was described that proteolytically hydrolysed milk induced an increased stimulation of phagocytosis on the pulmonary alveolar macrophages in mice (Moineau & Goulet, 1991). It is probable that the stimulation of innate immunity depend not only on the LAB, but also on non-bacterial component of yogurt, as bioactive peptides.

The increase in the number of IgA-producing cells is the most remarkable property induced by probiotic microorganisms (Galdeano, de Moreno de LeBlanc, Vinderola, Bonet, & Perdigón, 2007). It has been demonstrated that the IgA+ cells in the lamina propria of the small intestine can be increased with orally administered LAB (Perdigón, Alvarez, Medina, Vintiñi, & Roux, 1999; Perdigón et al., 1998). It has also been demonstrated that this effect of LAB on gut immunity can help to increase resistance to infections due to intestinal pathogens significantly. A common mucosal immune system exists whereby immune cells stimulated in one mucosal tissue spread and relocate to various mucosal sites. This concept implies that oral immune stimulation can induce immunity in distal intestinal mucosal sites. Our lab and others have demonstrated that the oral administration of certain LAB strains induced the IgA cycle and an increase in IgA+ cell population at the bronchial level (Agüero et al., 2006; Perdigón et al., 1998; Racedo et al., 2006; Villena et al., 2008). When studying the capacity of YP and YC to stimulate the IgA cycle, we found that only YP was capable of increasing the number of IgA+ cells in bronchus. Thus, the stimulation of the IgA cycle induced by the LAB strains present in YP could explain at least partly the greater resistance of the treated mice to the challenge with *S. pneumoniae*. When studying the levels of pathogen specific IgA, we found that YP treatment improved the production of anti-pneumococcal IgA in the airways which correlates with the increase in the number of BALT IgA+ cells.

In the deep lung, differentiation and expantion of IgG+ antigen-specific B-cells and production of opsonising IgG antibodies are important for complement fixation and for enhancing the efficiency of macrophage killing. This immune activation also induces the production at the systemic level of antibodies responsible for preventing the passage of pathogens to the blood and their subsequent dissemination (Moore, Moore, & Toews, 2001). Our experiments demonstrated that treatment with YP improved the production of anti-pneumococcal IgG in BAL and serum. This fact could be related to the stimulation of antigen presenting cells in the lung which induces T cell activation and B cell clonal expansion and differentiation into IgG+ antibody-secreting plasma cells. The most important antigen presenting cells for the specific lung response are pulmonary dendritic cells (Lambretch, Prins, & Hoogsteden, 2001). During the generation of an efficient effector immune response, dendritic cells have to overcome suppression by Treg cells, the main way through which they seem to do this is through IL-6 production, which releases suppression by naturally occurring Tregs (Pasare & Medzhitov, 2003). It has also been demonstrated that other cytokines are able to trigger dendritic cells activation (maturation), among them proinflammatory cytokines such as TNF-α (Blanco, Palucka, Pascual, &

Banchereau, 2008). It has been demonstrated that *L. bulgaricus* CRL 423 is able to increase the number of TNF-α+, INF-γ+ and IL-6+ cells in the small intestine (Dogi et al., 2008) and our previous results showed that preventive treatments with LAB were able to increase the levels of IL-6 y TNF-α in the respiratory tract after challenge with *S. pneumoniae* (Agüero et al., 2006; Racedo et al., 2006; Villena et al., 2008). Thus, it seems possible that the oral treatment with YP would be capable of improving antigen presentation mediated by pulmonary dendritic cells. Our laboratory is working to demonstrate this effect.

Although yogurt has long been believed to be beneficial for host-defence mechanisms, the components responsible for these effects or the way in which these components exert their immunologic modifications are not completely understood. In addition, the effect of yogurt on distal mucosal sites has not been studied extensively.

In this study, we can conclude that: (1) the administration of yogurt is able to improve immunity in the respiratory tract. Thus, yogurt is a good immune stimulatory food that could be added to the average daily diet and contribute to improve the defence mechanisms against respiratory infections. (2) The immunenhancing effect of yogurt could be improved if yogurt is prepared with starter strains of *L. bulgaricus* and *S. thermophils* that have intrinsic immunomodulatory activity.

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