Daily intake of *Lactobacillus casei* Shirota increases natural killer cell activity in smokers

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Abstract

Dietary probiotics supplementation exerts beneficial health effects. Since cigarette smoking reduces natural killer (NK) activity, we evaluated the effect of *Lactobacillus casei* Shirota (LcS) intake on NK cytotoxic activity in male smokers. The double-blind, placebo-controlled, randomised study was conducted on seventy-two healthy Italian blue-collar male smokers randomly divided for daily intake of LcS powder or placebo. Before and after 3 weeks of intake, peripheral blood mononuclear cells were isolated and NK activity and CD16+ cells' number were assessed. Daily LcS intake for 3 weeks significantly increased NK activity ($P<0·001$). The increase in NK activity was paralleled by an increase in CD16+ cells ($P<0·001$). Before intake, NK cytotoxic activity inversely correlated with the number of cigarettes smoked ($R-0·064$). LcS intake prevented the smoke-dependent expected NK activity reduction. The analysis of the distribution of changes in smoke-adjusted NK activity demonstrated that the positive variations were significantly associated with LcS intake, while the negative variations were associated with placebo intake (median value of distributions of differences, 20·98 lytic unit (LU)/10^7 cells for LcS v. $-4·38$ LU/10^7 cells for placebo, $P=0·039$). In conclusion, 3 weeks of daily LcS intake in Italian male smokers was associated with a higher increase in cytotoxic activity and CD16+ cells' number in comparison to the placebo intake group.

Key words: Probiotics: *Lactobacillus casei* Shirota: Natural killer cells: Smoking habit

Epidemiological studies indicate that dietary probiotics supplementation may exert a wide range of beneficial health effects on humans: from reducing infections and seasonal illness, to decreasing symptoms' perception or duration[1–3]. However, the precise mechanisms influencing probiotic–host crosstalk are still largely unclear. Probiotics appear to exert a pleiotropic effect resulting chiefly in a protective role on the intestinal tract where they exert direct antimicrobial effects, by competing with enteric pathogens and indirectly by enhancing the intestinal barrier functions[4]. Most interesting of these appears to be the probiotics' capability to modulate host's defences, both local and systemic mucosal immune systems[5]. It has been shown that probiotics can shape the mucosal immune system towards a non-inflammatory, tolerogenic pattern by increasing IL-10 levels[6], the induction of CD4+Foxp3+ regulatory T cells and by inhibiting the production of pro-inflammatory cytokines, but also may skew T cells towards T helper 1 polarisation[7–10]. The predominant cytokine profile depends on the nature and strength of the stimulus and strain of probiotic bacteria[11–13].

Dietary probiotic supplementation could represent an effective instrument to improve natural killer (NK) activity particularly in the elderly[2,3,14,15]. In fact, increased risk of diseases and higher mortality rates correlate with the age-related progressive decline of lymphoid cell activity[16,17]. However, senescence does not represent the only factor of immune function impairment, but also particular lifestyles may negatively influence the immune functions[2,18]. Reduced NK or lymphokine-activated killer activities were detected in the young with depressive disorders[19], or in subjects after acute stress (i.e. victims of an earthquake, or women after breast biopsy)[20,21] and in individuals with occupational stress, increased job demand (JD), repetitive or day/night work shifts or in conditions of job insecurity[22–24]. In contrast,

higher NK activities were associated with healthy lifestyle and stable mental conditions[25–27]. Physical activity was shown to improve NK activity, to increase the number of CD16$^+$ cells and of anticancer-protein expressing cells as well as to induce a general reduction of anxiety and depression[25,28,29]. However, in healthy smokers, NK activity is reduced and inversely correlated with the number of cigarettes smoked[25,30]. Recently, additional evidence indicated that cigarette smoking impairs NK cell-dependent tumour immune surveillance[31]. Thus, the means of enhancing NK-mediated immunity are most desirable.

In the present study, we sought to evaluate the effect of *Lactobacillus casei* Shirota (LcS) intake on NK-mediated activity in a population of Italian males with different smoking habits.

Materials and methods

Subjects

A total of seventy-two healthy Italian blue-collar male subjects with smoking habits (more than five cigarettes/d for at least 5 years) and age ranging between 40 and 60 years were recruited for the study. Table 1 summarises the main physical parameters of the individuals enrolled in the study as well as the number of cigarettes smoked (cigarettes/d average 19·95, range 5–40) with an average pack/year consumption of 364·1 (range 91·2–730·0). Exclusion criteria included any medical condition or the use of medications that could interfere with the probiotic intake in a period of 4 weeks before the start of or during the study. Among the seventy-two subjects enrolled, seven individuals were not included in the study owing to dropout/non-compliance/technical/procedural reasons.

The study was conducted at the University "G. d'Annunzio" of Chieti-Pescara, Italy and performed in accordance with the guidelines laid down in the Declaration of Helsinki and was approved by the Ethics Committee of the University "G. d'Annunzio". Written informed consent was obtained from all individuals enrolled in the study.

Experimental design

The placebo-controlled, double-blind study was performed by randomly dividing the seventy-two subjects into two groups: a group received LcS powder (from now referred to as 'LcS')

and a group received a powder without LcS (from now referred to as 'placebo') orally every day for 3 weeks. For allocation of the subjects, a computer-generated list of random numbers was used. Participants were assigned following equal randomisation (1:1) to the LcS or placebo intake group.

The *L. casei* powder (LcS powder) preparation contained about 1×10^{10} lyophilised viable LcS strain per gram per sachet (four sachets/d for 3 weeks). The placebo preparation contained only sterile maize starch powder. LcS and placebo-containing sachets were reconstituted using cold water just before intake. LcS and placebo preparations were provided by Yakult Honsha Company Limited, Tokyo, Japan. All individuals were supplied with the total number of sachets to be used for the 3-week intake. Compliance was based both on self-reporting to the monitoring medical staff and by verification of the number of sachets left during and at the end of the study. Randomisation codes were disclosed after experimental data collection and statistical analyses.

Determination of natural killer activity

NK activity was measured using peripheral blood mononuclear cells (PBMC) isolated from 8 ml of heparinised blood using BD Vacutainer CPT (Becton Dickinson, Franklin Laker, NJ, USA). PBMC were resuspended in Roswell Park Memorial Institute (RPMI) 1640 with 10 % fetal bovine serum (Sigma-Aldrich Co., St Louis, MO, USA) and used as effector cells. The K562 human erythroleukaemia cell line was used as the target for determining NK cell cytotoxicity[29]. K562 cells at 1×10^{7}/ml were labelled with 100 µCi of ^{51}Cr for 60 min at 37°C (^{51}Cr; Perkin Elmer, Waltham, MA, USA), plated into round-bottomed ninety-six-well plates at 1×10^{4}/well, mixed with effector cells at different effector target cell ratios (E:T 40:1, 20:1 and 10:1) and incubated at 37°C for 4 h. Supernatants from each well were collected after centrifugation and measured in a gamma counter. All experiments were performed in triplicate. The percentage of specific lysis was determined according to the following formula: specific lysis = (mean counts per min (cpm) experimental release − mean cpm spontaneous release)/(mean cpm maximal release − mean cpm spontaneous release) × 100. One lytic unit (LU) was defined as the cytotoxic activity giving 33·3 % of maximal release.

Flow cytometry

The numbers of leucocytes, neutrophils, monocytes, eosinophils and lymphocytes were determined by haemocytometric analysis. CD16$^+$ lymphocytes were determined using phycoerythrin (PE)-conjugated anti-CD16 antibodies (Becton Dickinson, San Jose, CA, USA) by flow cytometry performed using the FACScalibur platform (BD Biosciences, Franklin Lakes, NJ, USA).

Analysis of anxiety, occupational stress and perception of symptoms

The questionnaires for determining the perception of anxiety, occupational stress and subjective symptoms were

Table 1. Characteristics of subjects enrolled in the randomised study

(Mean values and standard deviations)

Variables	Placebo group		LcS group		
	Mean	SD	Mean	SD	P*
Age (years)	49·2	6·5	51·4	6·2	0·150
Height (cm)	174·5	6·0	174·5	6·9	0·879
Weight (kg)	79·7	10·1	81·4	11·5	0·535
BMI (kg/m^2)	26·1	2·8	26·7	3·5	0·604
No. of cigarettes/d	19·2	8·9	20·7	9·7	0·386

LcS, *Lactobacillus casei* Shirota.
* Mann–Whitney: placebo v. LcS.

administered before and after intake. State-trait-anxiety inventory (STAI) was used in Scale I to measure state-anxiety as a temporary and varying condition, and in Scale II to monitor trait-anxiety, as a relatively fixed personality tendency[32,33]. Occupational stress was determined by the Karasek test including nine items for decision latitude (DL, skill decision + decision authority), twenty for JD, eight for social support (SS) and nine for job insecurity[34–36]. Job strain (JS) was determined by the ratio JD:DL. Perception of subjective symptoms (e.g. headache, nausea, stomach-ache) was determined by the Somatisation scale of Symptom Checklist SCL-90 evaluated on a Likert scale ranging from 0 = not at all to 5 = very much.

Statistical analysis

A total of sixty subjects (thirty for the LcS group and thirty for the placebo group) were required to provide 80 % power to detect, at the 0·05 two-sided level, a difference between the group proportions of NK cell activity of 30 %. Assuming a 20 % dropout rate, seventy-two subjects were enrolled and randomised for the two groups of the study.

Quantitative variables were summarised as mean and standard deviation, while the qualitative variables were expressed as frequency and percentage. Statistical significance of differences between groups for qualitative variables was assessed using the χ^2 test or Fisher's exact test. Non-parametric Mann–Whitney U test was applied for assessing the comparison of the quantitative variables between the placebo and LcS groups, while the Wilcoxon rank sum test was applied for within-group comparison. Spearman's ρ correlation coefficient was applied to evaluate the correlation between quantitative variables. Linear regression analysis was applied to evaluate the relationship between NK cell activity, expressed as LU and smoking habits, expressed as linear combination $(0·5X_1 + X_2)$ of number of cigarettes daily smoked (X_1) and number of smoked cigarettes before blood bleed (X_2). The linear relationship estimated was used to adjust NK cell activity measured before and after the intake. The differences between the adjusted values before and after intake were summarised as median and interquartile range. Data were analysed by SPSS® Advanced Statistical™ version 13.0, 2004 (SPSS Inc., Chicago, IL, USA).

Results

Subject characteristics

The baseline physical characteristics, age, weight and BMI as well as smoking habits, were similar among all seventy-two healthy males enrolled in the randomised, double-blind, placebo-controlled study to evaluate the effect of LcS intake (Table 1). Similarly, leucocyte distribution of all individuals did not change after placebo or LcS intake (Table 2).

Effect of Lactobacillus casei Shirota intake on natural killer cell activity

NK activity was assessed before and after LcS or placebo intake using PBMC isolated immediately after blood sampling. Table 3 shows NK-mediated cytotoxic activities at different E:T ratios before and after LcS and placebo intake. Different starting NK activity levels were detected in LcS and placebo intake groups, although these differences were not statistically significant. Overall, significant NK activity increases were detected after LcS intake at all E:T ratios (range 16·9–26·0 %) as compared to placebo intake (range 8·4–10·5 %). These observations were confirmed by calculating the LU for each individual and for each group. While the increase in LU for the placebo was not statistically significant, a highly significant increase in LU (127·0 %, $P<0·001$) was associated with LcS intake. However, due to the apparently lower NK basal levels in the LcS group, the LcS-dependent increase in NK activity not necessarily represents an improved NK cell activity over the placebo group.

Although LcS and placebo intake did not modify the leucocyte distribution (Table 2), significant increases in CD16+ cells' numbers in the LcS (50 %, $P<0·001$) and placebo (20 %, $P=0·007$) intake groups were observed (Table 3). The CD16+ cell number increase after LcS intake significantly and directly correlated with NK cytotoxicity ($\rho = 0·496$, $P<0·01$) as compared to placebo intake ($\rho = 0·057$, NS).

Fig. 1 illustrates NK activity relative variations in the placebo and in the LcS intake groups. It is to be noted that minimal changes occurred within the placebo group (median 0·25), while appreciable variations were observed after LcS-intake (median 1·26, LcS *v.* placebo; $P=0·059$).

Table 2. Leucocyte subpopulations in the placebo and *Lactobacillus casei* Shirota (LcS) intake groups
(Mean values and standard deviations)

	Placebo group				LcS group			
	Before intake ($n \times 10^{-3}$/mm³)		After intake ($n \times 10^{-3}$/mm³)		Before intake ($n \times 10^{-3}$/mm³)		After intake ($n \times 10^{-3}$/mm³)	
Cell types	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Leucocytes	7·0	1·7	7·0	2·0	7·1	1·8	6·9	1·8
Neutrophils	4·0	1·6	4·1	1·9	4·4	1·5	4·2	1·4
Monocytes	0·4	0·2	0·4	0·1	0·5	1·1	0·4	0·1
Eosinophils	0·2	0·2	0·2	0·2	0·2	0·1	0·2	0·1
Lymphocytes	2·3	0·6	2·2	0·5	2·0	0·6	2·0	0·6

Table 3. Natural killer (NK) cytotoxic activity, lytic units (LU) and CD16$^+$ in the *Lactobacillus casei* Shirota (LcS) and placebo intake groups
(Mean values and standard deviations)*

	Placebo group						LcS group					
	Before intake		After intake				Before intake		After intake			
NK cytotoxicity	Mean	SD	Mean	SD	P†	Δ%‡	Mean	SD	Mean	SD	P†	Δ%‡
E:T ratio 40:1	50·8	18·2	55·3	15·1	0·150	8·8	46·8	19·0	54·7	16·1	0·017	16·9
E:T ratio 20:1	39·2	17·4	42·5	15·3	0·242	8·4	34·1	16·1	41·9	15·5	0·001	22·9
E:T ratio 10:1	27·5	15·2	30·4	12·8	0·153	10·5	23·4	12·3	29·6	14·3	0·002	26·5
LU	104·2	144·7	151·1	228·6	0·140	45·0	70·1	61·3	159·3	290·6	<0·001	127·2
CD16$^+$ cell no.	0·5	0·2	0·6	0·2	0·007	20·0	0·4	0·2	0·6	0·2	<0·001	50·0

* NK activity was expressed as mean and standard deviation of % cytotoxicity at indicated E:T ratios and as LU representing the number of effector cells required to lyse 33 % of target cells.
† Wilcoxon signed ranks test: before intake *v.* after intake.
‡ The mean of relative differences of all subjects.

Smoking habits and natural killer cell activity: effect of *Lactobacillus casei* Shirota or placebo intake

The number of cigarettes daily smoked by all the enrolled subjects was carefully monitored to ensure that this did not change during the 3-week treatment. The smoking habits were calculated by the linear combination of the number of cigarettes smoked the day before (X_1) and the same day just before blood bleed (X_2), as previously indicated by Morimoto *et al.*[2]. The smoking habit of each subject was calculated on a daily scale only to represent the long-term smoking habits of all individuals, and not to indicate that the negative effects of smoke would be over in 1 d. The effect of cigarette smoking on NK activity (expressed as LU) in all subjects is represented in Fig. 2(a). It appears evident that there is an inverse correlation between smoking habits and NK lytic activities in all individuals before LcS or placebo intake. In fact, heavier smokers displayed lower NK activity, while higher NK activities were associated with light smoking habits.

The relative variations of NK activity in relation to smoking habits after LcS or placebo intake, represented in Fig. 2(b), revealed that the inverse relationship between smoking habits and NK activity was maintained in the placebo group, whereas in the LcS group the NK relative variations did not change in relation to the number of cigarettes smoked. Although the R values of the two regression lines are very similar, the slopes of the two lines are different (regression coefficient $b = -0.071$ for placebo and $b = -0.012$ for LcS). These results indicate that LcS intake increased the relative variations of NK cell activity in smokers.

In order to compare the LcS effect on NK activity in individuals with different smoking habits, it was necessary to compensate the individual differences in smoking habits, and thereby the effect of smoke. Thus, the NK activity of each subject was adjusted, using the regression line, to the value that would have been obtained assuming that the subject did not smoke at all. Fig. 3 illustrates the relative variations of NK activity in the LcS and placebo intake groups adjusted for
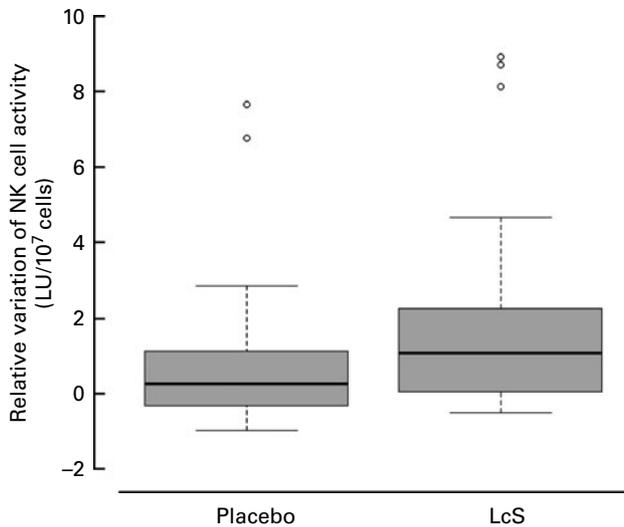

Fig. 1. Relative variation of natural killer (NK) cell activity in the placebo and *Lactobacillus casei* Shirota (LcS) intake groups. Box-whisker plots show the 25th and 75th percentile range (box) and median values (transverse line in the box). Relative variation was evaluated as: (lytic unit (LU) after intake − LU before intake)/LU before intake. Statistical significance of the differences between groups was evaluated by the Mann–Whitney test (P=0.059). ○, Values associated with single individuals.
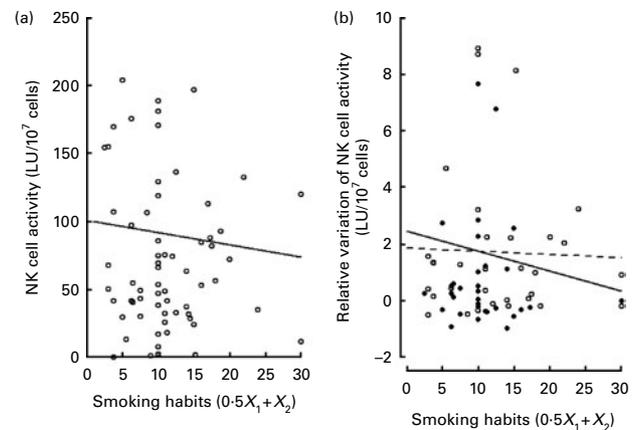

Fig. 2. Relationship between smoking habits and natural killer (NK) cell activity. To represent the long-term smoking habits of each individual, smoking habits were quantified on the basis of number of cigarettes daily smoked (X_1) and the number of smoked cigarettes until giving blood (X_2). (a) NK cell activity before intake in all subjects. The solid line is the estimated regression line, R −0.064. (b) Relative variation of NK cell activity in the *Lactobacillus casei* Shirota (LcS) group (dotted regression line, R −0.032, regression coefficient $b = -0.012$) and the placebo group (solid regression line, R −0.028, regression coefficient $b = -0.071$). ○, Values associated with single individuals.

the smoke-related effects. Indeed, negative variations were frequent in the placebo group, while positive variations were observed more frequently in the LcS group. The resulting median values of distribution of differences were -4.38 for the placebo group and 20.98 for the LcS group. The difference between these medians was statistically significant ($P = 0.039$).

Effect of Lactobacillus casei Shirota on perception of anxiety, job stress and physical symptoms

Table 4 shows the results of the questionnaire administered to all enrolled subjects, before and after LcS or placebo intake, to determine their perception of anxiety (STAI I and STAI II), job stress (JD, DL, SS, job insecurity) and subjective perception of physical symptoms. The examined blue-collar workers showed anxiety and occupational stress within the normal range. No significant differences for these parameters were demonstrated after LcS or placebo intake. Interestingly, JD and JS directly correlated with CD16$^+$ cell number (JD, $\rho = 0.358$, $P < 0.05$) (JS, $\rho = 0.389$, $P < 0.05$), while an inverse correlation was found between SS and CD16$^+$ cell number only after LcS intake (SS, $\rho = -0.395$, $P < 0.05$). Indeed, NK activity is normally reduced in stressful conditions; however, LcS intake may counteract NK activity reduction by mobilisation of an increased CD16$^+$ cell number.

The simultaneous perception of one or more symptoms decreased after both LcS and placebo intake. Since this is a subjective assessment, it is not surprising that there were no obvious differences between the two groups of intake. However, it is to be noted that the percentage of individuals not reporting any specific symptom increased from 11 to 16 % after LcS intake, while this percentage did not change

in the placebo group. Table 4 illustrates the percentages of individuals referring representative specific symptoms. It is to be noted that perception of 'nausea and stomach-ache' decreased after LcS intake (from 25.7 to 8.9 %), and not in the placebo group. Therefore, this result suggests a possible role of LcS intake in reducing gastrointestinal discomfort.

Discussion

This double-blind, randomised, placebo-controlled study evaluated the effect of 3-week intake of a LcS powder preparation on NK cytotoxic activity in seventy-two Italian male blue-collar volunteers with different smoking habits. The physical and demographic characteristics of the enrolled individuals were similar between the LcS and placebo groups and did not change during the study. LcS intake did not influence leucocyte total number or the relative percentages of neutrophils, eosinophils, monocytes and lymphocytes. On the contrary, the LcS intake significantly increased blood CD16$^+$ lymphocytes. CD16$^+$ cell number was paralleled by increased NK-mediated cell cytotoxicity. Some studies indicated that LcS improved NK activity without modification of the cytotoxic cell number[12,27,37]. In our study, instead, we observed a significant positive correlation between NK activity and CD16$^+$ cell number. However, since CD16$^+$ cells include both NK cells, and cells, such as monocytes, capable of enhancing cytotoxic activity[13], we cannot in this study discriminate whether NK activity increase was due directly to an enhancement of the cytotoxic activity of the individual NK cells or to their relative increased number.

The analyses of distribution of changes in NK activity induced by LcS or placebo daily intake revealed that the positive variations of NK activity were more frequent in the LcS group, as compared to the placebo group ($P = 0.039$). This is in agreement with the previous study on Japanese smokers (blue-collar workers)[2]. Inverse correlations between the smoked cigarettes and NK cells' activity were demonstrated in all individuals. A smoke-related decrease in NK activity was appreciable in the placebo group, but not in the LcS intake group, indicating that LcS restored NK activity in these smokers. It has been reported that the blue-collar workers have an increased JD and JS in comparison to office employees or schools teachers[24,38]. However, the blue-collar workers enrolled in our study resulted in normal psychological conditions, in contrast to their relative poor lifestyle standard. Thus, in these individuals, the LcS treatment did not improve the parameters of anxiety and occupational stress. However, a significantly positive correlation was found between JD and JS and the number of CD16$^+$ cells in the group treated with LcS. In addition, a significant inverse correlation was demonstrated between SS and CD16$^+$ cell number, independently from blood cytotoxic activity. This is in agreement with the results of previous studies demonstrating that short, acute psychological stress affects the immune responses by mobilising NK cells[39,40].

Furthermore, LcS treatment appeared to reduce the perception of nausea and stomach-ache symptoms, reflecting an improvement of gastrointestinal functions possibly resulting
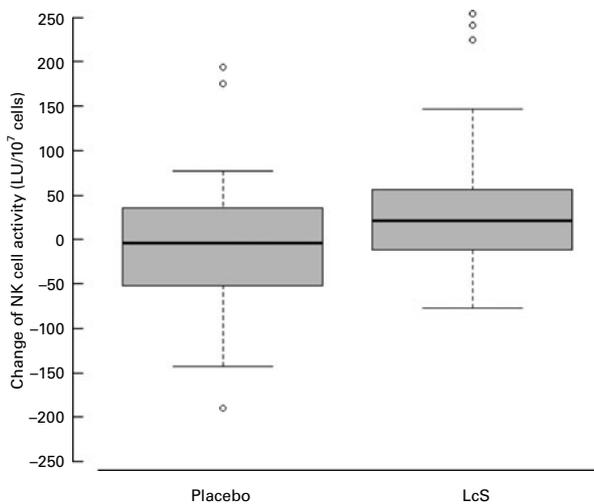
Fig. 3. Changes in natural killer (NK) cell activity before and after intake adjusted for smoking habits. Variations in NK cell activity, measured before and after intake, were adjusted for each individual using the regression line to eliminate the effect of the different smoking habits. Box-whisker plots show the 25th and 75th percentile range (box) and median values (transverse line in the box, -4.38 and 20.98 for placebo and *Lactobacillus casei* Shirota (LcS), respectively). The difference, calculated by the Mann–Whitney test, between the median values of the two groups was significant, $P = 0.039$. ○, Values associated with single individuals.

Table 4. Parameters of anxiety, occupational stress and perception of symptoms in the placebo and *Lactobacillus casei* Shirota (LcS) intake groups (Medians and ranges)

	Placebo group				LcS group			
	Before intake		After intake		Before intake		After intake	
	Median	Range	Median	Range	Median	Range	Median	Range
Psychological parameters*								
STAI I	34	20–55	34·5	22–55	35	22–51	33·5	23–49
STAI II	29	20–57	30	20–54	30	21–42	30·5	21–44
JD	32	24–42	32	19–38	31	23–42	31·5	23–43
DL	68	48–92	68	48–92	66	52–90	67	52–90
JD:DL	0·50	0·3–0·7	0·50	0·3–0·7	0·48	0·26–0·67	0·47	0·30–0·69
Social support	24	18–35	24	19–36	24	19–36	24	19–36
Job insecurity	10	6–20	10	6–19	10	6–19	9	6–19
Subjective symptoms perception†								
Nausea and stomach-ache (%)	17·6		17·6		25·7		8·9	
Back pain (%)	64·7		56·9		60·0		55·9	
Headache (%)	50·0		47·9		42·8		35·3	
Body weakness (%)	14·7		14·5		14·3		15·0	

STAI, state-trait-anxiety inventory; JD, job demand; DL, decision latitude.
* Median values obtained in the state-trait-anxiety inventory and Karasek questionnaires.
† Percentages of individuals reporting the specific subjective symptom.

from a restored microbiota balance. Although it is well established that ingested LcS transits alive through the stomach and survives in the intestine[41], it is still unclear as to how LcS may improve the upper gastrointestinal tract symptoms, such as nausea or stomach-ache. Direct interaction of LcS with the stomach or indirect action through hormonal or nervous regulation may be assumed and may represent future research subjects.

In conclusion, this study demonstrated that daily LcS intake significantly increased NK cytotoxic activity and CD16$^+$ cell number. Moreover, a reduced symptom perception in healthy Italian habitual smokers was reported. Since smokers exhibit higher susceptibility to infections and inflammatory diseases and NK cells play an important role in controlling infections and eliminating aberrant cells, dietary supplementation with LcS could contribute to increased NK cytotoxic activity with significant health advantages for individuals with smoking habits. However, these results should not be interpreted as an attempt to overcome smoke-related damages, but on the contrary they strengthen the notion of tobacco interference with the immune system. Smoking habit and alcohol consumption are the two most important preventable causes of disease and premature death. Despite increasing knowledge of their hazard, prevalence is declining very slowly and their decrease appears to be limited to the higher socio-economic class among populations. Smoking threats are not limited to the immunosuppressive effect, but have a major health impact. Thus, despite the LcS increase of NK activity, a concerted effort to quit smoking is the only way to real prevention of cancer and CVD.

Acknowledgements

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