Probiotic *Lactobacillus casei* Shirota Supplementation Does Not Modulate Immunity in Healthy Men with Reduced Natural Killer Cell Activity\(^1\)\(^2\)\(^3\)

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Abstract

Oral intake of probiotic bacteria may beneficially modulate functions of NK cells. In healthy individuals, contradictory results exist as to whether NK cell functions can be modulated by probiotic bacteria. Therefore, the primary objective of our randomized, double-blind, placebo-controlled trial was to determine the effects of the probiotic strain *Lactobacillus casei* Shirota (LcS) on the activity of NK cells in healthy men who had been preselected for a reduced lytic function of their NK cells. Study participants (\(n = 68\)) were supplemented for 4 wk with a probiotic drink providing \(1.95 \times 10^{10}\) CFU LcS/d or with a similar milk drink without probiotic additive. A run-in period of 2 wk preceded the probiotic supplementation followed by a 2-wk follow-up phase without the probiotic or control drink. Changes in the relative proportions of NK cells and other leukocytes as well as multiple functional measurements were determined longitudinally at baseline, after the 4-wk supplementation, and at the end of the follow-up. The probiotic supplementation had no significant effect on NK cell numbers and function or on phagocytosis, respiratory burst, or cytokine secretion of peripheral blood mononuclear cells. In conclusion, 4 wk of supplementation with LcS does not increase NK cell activity in healthy men with a reduced NK cell lytic activity. However, other doses of LcS, time of intervention, or differences, e.g. in the background diet, may result in a different outcome.  J. Nutr. 141: 978–984, 2011.

Introduction

A probiotic is defined as a “live microbial food ingredient that is beneficial to health” (1). A functional, health-promoting aspect of foods that contain probiotics is the modulation of the immune system. Data from previous human intervention studies suggest that in healthy humans, functions of the innate immune system, in particular phagocytosis, oxidative burst, and the activity of NK cells, may be stimulated by the ingestion of probiotic bacteria (2–5).

NK cells are characterized by their innate ability to rapidly lyse virus-infected cells and tumor cells without prior sensitization. NK cells are therefore of particular importance to immune defense in the early phases of infection and in combating tumors (6,7). The lytic potential of NK cells is a measure of the defense against virally infected or malignant cells. It also serves as a prognostic factor by which to assess a predisposition for the development of cancers and a general susceptibility to infections. It has been shown in prospective studies that humans with low NK activity are at increased risk of developing cancer and infections (8–10). In the case of patients who already have cancer, if their preoperative NK activity is low, there is a high risk of recurrence and development of tumors (e.g. colon cancer), whereas patients with high NK cell activity often remain tumor free for a longer time (11–13).

At the time the study was designed, 2 human intervention studies had investigated whether the activity of NK cells can be stimulated in healthy individuals by the consumption of a fermented milk drink containing *Lactobacillus casei* Shirota (LcS)\(^6\) (4,14). Nagao et al. (4) showed in a small group of 9 Japanese participants that the activity of the NK cells in the blood was increased by supplementation with the probiotic bacterium, whereas the NK cell count remained the same. In contrast to the study by Nagao et al. (4), an intervention study with LcS by Spanhaak et al. (14) did not result in modulation of the cytotoxic killer cell activity. The 2 studies are very comparable in terms of the period of intervention (3 and 4 wk, respectively).

\(^6\) Abbreviations used: AS, autologous serum; BrdU, 5-bromo-deoxy-uridine; CRP, C-reactive protein; LcS, Lactobacillus casei Shirota; MRI, Max Rubner-Institut; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; T, time point.
respectively), the daily dose of the probiotic (4 × 10^{10} and 3 × 10^{11} CFU/d, respectively), and the vehicle (both fermented milk drinks). The discrepancy in the study results, however, is most probably attributable to the selection of the group of study participants, which, in the case of Nagao et al. (4), were selected according to the level of NK cell activity at the start of the intervention. Only persons with low NK cell activity, which was fixed at a maximum of 45%, were allowed to participate in the study. In fact, the rise in NK cell activity correlated inversely with the baseline NK cell activity. This suggests that healthy individuals with low NK cell activity particularly could benefit from the immune-stimulating property of LcS. It is possible that with a group of healthy persons with a normal level of NK cell activity, as in the study of Spanhaak et al. (14), immune effects expressed as an increase in NK cell activity cannot be achieved. Unfortunately, the results by Nagao et al. (4) were obtained from a very small group of participants. In addition, extrapolating their results to Western populations is limited due to differences in dietary habits between Asian and Western participants. The results of the study by Nagao et al. (4) therefore needed to be verified in a larger cohort consuming a Western diet. Thus, the aim of this study was to investigate whether the daily ingestion of a probiotic food containing LcS by healthy individuals with low NK cell activity (mean of ≤40%) can modulate NK cell functions and other immunological markers.

Study Participants, Study Design, and Methods

Study participants and design

In the current study, we tested the hypothesis that the daily ingestion of a probiotic food containing LcS by healthy individuals with low NK cell activity can affect NK cell functions. The primary endpoint with respect to efficacy of LcS treatment was the modulation of NK cell activity in the peripheral blood. On the basis of the primary endpoint, a sample size of 35 persons/group was calculated by statistical power estimation (power 0.80, significance level P < 0.05) to be able to detect a 25% increase or decrease of an assumed mean NK cell activity of 40% (i.e. 40 ± 10%), taking into account the method variability and the intervention effects with foods from earlier studies. The modulation of other specific immune functions and changes in hematology parameters, body mass, and BMI were investigated as secondary endpoints.

The study was a randomized, double-blind, single-centered, and placebo-controlled trial of parallel approach. It complied with the Helsinki Declaration as revised in 1983 and was approved by the Medical Ethical Committee of the Landesärztekammer Baden-Württemberg. The trial was conducted between January and March 2007 at the Max Rubner-Institut (MRI) in Karlsruhe, Germany. Healthy male Caucasian nonsmokers between 18 and 60 y of age were candidates for inclusion in this study. They were not admitted to the study if any of the following criteria applied: serious gastrointestinal or lipid metabolism disorders, a history of cancer, chronic inflammatory or immunological disorders, infectious diseases, alcohol or drug abuse, a history of food allergies or regular intake of dietary supplements, antibiotic treatment 6 mo prior to the onset of the study, and excessive physical activity during the study period. For the recruitment of study participants, men with low NK cell activity (<50%) known from former intervention trials at the MRI were informed about the trial. Additional volunteers were recruited by placards displayed at the MRI, at various enterprises in the vicinity of the MRI, and at the local university. A preselection was made based on the outcome of a short telephone interview. Those persons meeting our eligibility criteria were invited to a preliminary medical examination at the human nutrition unit of the MRI where their medical history was documented by the leading physician. In addition, a blood count was carried out, markers of clinical chemistry were measured, and the NK cell activity in the peripheral blood was determined. Individuals with a NK cell activity < 50% (effector:target ratio of 25:1) were invited for a second blood donation 3–4 wk later to confirm the low NK cell activity level measured at the first visit. All participants gave their consent in writing and 116 volunteers in total were recruited between October 2006 and January 2007. Of 72 allocated participants, only 4 did not complete the trial, which lasted from January 2007 until March 2007, due to conflicting antibiotic treatment or schedule difficulties; thus, data of 34 participants receiving LcS and data of 34 men under control treatment were available for the statistical analysis. The flow of participants through each stage of the study is depicted in Supplemental Figure 1.

Fasting blood samples were collected at time point T0 (start of the 2-wk run-in-phase), after 2 wk of run-in (baseline, T1), at the end of the 4-wk supplementation period (T2), and after a 2-wk follow-up phase (T3). An overview of baseline characteristics at T1 is given in Table 1. Participants did not differ between control and LcS groups with regard to age, body mass, BMI, and NK cell activity levels. Although prestudy measurements had been done to confirm NK cell activity levels < 50% (for effector:target ratio 25:1), NK cell activity levels of 6 individuals were >50% at T1. Nevertheless, our primary goal to achieve an arithmetic mean of ≤40% with the study collective was achieved.

The intervention was split into 2 parallel courses in weekly intervals. Participants with low NK cell activity in the peripheral blood were randomized to receive either a fermented milk drink with LcS, which was identical to the commercially available Yakult product, or an equally composed milk drink without the probiotic additive (control) daily for 4 wk between T1 and T2. After completion of recruitment, the study participants were randomly allocated to the LcS or the control group by the Center for Biostatistics of the Utrecht University, The Netherlands, to avoid any bias for the researchers at the MRI. Randomization was done without blocking and stratification. The randomization code was not revealed to the researchers until completion of data collection and laboratory analyses.

All participants were asked to completely abstain from the consumption of fermented milk products throughout the study period from T0 to T3, in accordance with a list of prohibited products (including probiotic products, fermented milk products such as yogurt, kefir, and soured milk; participants were allowed to consume milk, cheese, curd cheese, and buttermilk). Both probiotic and control drinks were provided, blinded, and coded by the manufacturer Yakult Honshu but handed out by the examiners. The composition and the nutritional value of the probiotic and of the placebo products are given in Supplemental Table 1.

The products were delivered twice by the manufacturer to the MRI in a 2-wk interval, where the samples were kept at +4°C until distribution. To keep contact to the participants and to ensure compliance, packages of the second charge were handed out to the participants after the first one-half of the intervention period. Participants of both groups were asked to consume 3 bottles daily, evenly distributed across the day, with each bottle containing 0.065 L of the milk drink (total amount per day: 0.195 L). According to the manufacturer, in the case of the LcS group, 1 bottle contained 1 × 10^{11} CFU LcS (daily dosage: 1.95 × 10^{10} CFU LcS). This daily dosage was chosen to achieve comparability with former intervention trials conducted with probiotic products. Both milk drinks were distributed in bundles sealed with the outer packaging, which is normally used for the commercially available product, so that the LcS and placebo drinks were identical in appearance. Both drinks were consumed well within the shelf-life so that in case of the probiotic drink, the stability of the product and the viability of the probiotic additive were guaranteed.

### TABLE 1 Baseline characteristics of study participants at T1

<table>
<thead>
<tr>
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<th>LcS</th>
<th>Control</th>
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<tr>
<td>n</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td>Age, y</td>
<td>32 ± 11</td>
<td>31 ± 10</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>82 ± 12</td>
<td>81 ± 14</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.2 ± 3.2</td>
<td>24.9 ± 4.1</td>
</tr>
<tr>
<td>NK cell activity, %</td>
<td>32 ± 14</td>
<td>32 ± 14</td>
</tr>
<tr>
<td>Range</td>
<td>12–69</td>
<td>11–70</td>
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1 Values are means ± SD.
**Methods**

Isolation of peripheral blood mononuclear cells and preparation of serum, autologous serum, and plasma. The isolation of peripheral blood mononuclear cells (PBMC) and the preparation of serum, autologous serum, and plasma were conducted according to the methods of Warzl et al. (15).

**Lymphocyte proliferation.** The proliferation of PBMC after 72-h stimulation with concanavalin A (Sigma-Aldrich) in medium containing AS was measured as previously described (16). Briefly, cells were pulse-labeled with 5-bromo-deoxy-uridine (BrdU; 100 μmol/L; Roche Diagnostics) during the final 3 h of incubation, which is incorporated into newly generated cellular DNA and detected by a commercial ELISA kit (Cell Proliferation ELISA BrdU, colorimetric; Roche Diagnostics). The amount of incorporated BrdU is quantified by measuring the absorbance of the samples at 450 nm (reference wavelength 650 nm). As background control, absorbance was measured in samples that were not pulse-labeled with BrdU. Proliferative responses were expressed as the net absorbance values (absorbance_{CA,450 nm-650 nm} - absorbance of pulse-labeled cells - absorbance_{CA,450 nm-650 nm} of unlabeled cells).

**Quantification of cytokine secretion.** PBMC were cultured at 1 × 10^6 cells/L in RPMI medium containing 10% AS and were stimulated with a mixture of 2.5 mg concanavalin A/L and 1 μg LPS (from E. coli 0111: B4)/L (Sigma-Aldrich) for 24 h at 37°C to measure ex vivo cytokine production. Cell-free supernatant fluid was collected and stored at −80°C until analyzed. The following cytokines were measured with Fluorokine multiplex assay kits (R&D Systems) according to the manufacturer’s instructions: IFNγ, IL-1β, IL-2, IL-10, and TNFα. Measurements with high- and low-sensitivity settings and data analyses included as the main effect, changes in immune variables over time (T1 at the end of the run-in phase compared with both T2 at the end of the 4-wk intervention period and T3 after a 2-wk follow-up) were the dependent variables. P-values (time × treatment) < 0.05 were considered significant.

**Statistical calculations** were performed with the StatView program version 5.0 (SAS Institute).

**Results**

Phenotype of PBMC and NK cell activity. The percentage of total CD3+ CD56+ NK cells as well as the percentage of NK cell subsets expressing CD56<sup>dim</sup>/bright and NKp46<sup>+</sup> did not differ at baseline (T1) and were not affected by the supplementation with LcS (Table 2). Further, the percentage of NK cells and the NK cell cytotoxicity (for both effector:target ratios) were comparable in both groups throughout the study period (Table 2). Both control and LcS groups had high correlation coefficients between numbers of NK cells (CD3<sup>+</sup> CD56<sup>+</sup>) and NK cell cytotoxicity (control r = 0.64, P < 0.0001; LcS r = 0.48, P < 0.0001 for effector:target ratio 25:1; control r = 0.46, P < 0.0001; LcS r = 0.65, P < 0.0001 for effector:target ratio 12.5:1).

**Blood count, T lymphocytes, and lymphocyte proliferation.** Total leukocyte and lymphocyte numbers, the percentage of lymphocytes, and specifically CD3<sup>+</sup> T lymphocytes as well as mitogen-activated proliferation of lymphocytes were not affected by the probiotic intervention (Supplemental Table 2). Other hematological data, i.e., characteristics of RBC (erythrocyte reaction was stopped by adding 100 μL ice-cold quenching solution (10% Trypan blue in PBS). After washing with PBS (without Ca and Mg), whole blood cells were fixed with 100 μL FACS lysing solution. DNA was stained with 300 μL propidium iodide solution (0.05 mmol/L PBS) and fluorescence was directly measured.

For the determination of the respiratory burst, 100 μL chilled whole blood was incubated with 20 μL PBS (control) or 20 μL phorbol myristate 13-acetate (0.6 μmol/L; Sigma-Aldrich) as stimulus for 10 min at 37°C. Dihydrorhodamine 123 (1.7 μL; 29 mmol/L DMSO; Molecular Probes) was added and samples were incubated for 10 min at 37°C. Cells were fixed with 2 mL of FACs lysing solution. After washing with PBS (without Ca and Mg), DNA was stained with 300 μL propidium iodide solution (0.05 mmol/L PBS) and the samples were incubated for 10 min on ice. Burst activity (percentage of burst-active cells) and burst intensity (generation of reactive oxygen species, indirectly assessed by the oxidation of dihydorhodamine 123 to fluorescent rhodamine and expressed as mean fluorescence) were determined in neutrophils and monocytes by flow cytometry (FACSCalibur; BectonDickinson).

**TNFα and C-reactive protein in blood sera.** TNFα and C-reactive protein (CRP) were determined with ELISA (TNFα, Immunotech; CRP, DSL) in blood sera according to the manufacturers’ instructions.

**Statistical evaluation.** As the initial 2-wk run-in phase was performed to level potential intergroup variability, data at T0 were routinely acquired and considered in the statistical evaluation but are not depicted in the results section (except for NK cell activity levels). Data acquired at T1 serve as baseline values. To test time, treatment, and their interaction, i.e., time × treatment effects, a 2-factor ANOVA for repeated measures containing 4 time points and 2 groups and the Tukey-Kramer post hoc test were used to analyze the data after verifying normal distribution of the data set. The intake of the milk drink with or without LcS (LcS, control) was included as the main effect, changes in immune variables over time (T1 at the end of the run-in phase compared with both T2 at the end of the 4-wk intervention period and T3 after a 2-wk follow-up) were the dependent variables. P-values (time × treatment) < 0.05 were considered significant.

Correlation coefficients r between the number and the lytic activity of NK cells were computed by a parametric test to quantify their strength of association.

Statistical calculations, including the calculation of correlation coefficients, were performed with the StatView program version 5.0 (SAS Institute).
numbers, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and hemoglobin concentration) and platelets (platelet count, mean thrombocyte volume), were comparable at baseline (T1) and did not differ between groups at the end of probiotic supplementation (T2, T3; data not shown).

**Neutrophil and monocyte counts, phagocytosis, and respiratory burst.** The total numbers and percentages of neutrophils and monocytes as well as the phagocytic and respiratory burst activity were not modulated by LcS (Table 3).

**Mitogen-activated cytokine secretion and serum concentrations of biomarkers of inflammation.** The capacity to secrete a number of cytokines ex vivo by mitogen-activated PBMC including IFNγ, IL-1β, IL-2, IL-10, and TNFα was not affected by the probiotic treatment (Supplemental Table 3). Further, serum concentrations of TNFα and CRP did not differ between groups (Supplemental Table 4).

**Discussion**

In the present study, the effects of the daily consumption of a milk drink containing the probiotic LcS (daily dose: $1.95 \times 10^{10}$ CFU) on the immune status of 68 male healthy nonsmokers with low NK cell activity were determined. Immune markers of adaptive and innate immunity were measured at the beginning of the study, at the beginning and the end of the 4-wk intervention phase, and at the end of the follow-up phase. The primary study criterion was the modulation of NK cell function, because it was hypothesized that healthy individuals with low
NK cell activity could benefit from immunostimulating effects of the probiotic bacterium LcS. The results of the study clearly show that under these study conditions, LcS supplementation had no effect on any of the immune markers studied, although it is assumed that LcS survives the passage through the gastrointestinal tract, which is based on findings from other LcS intervention trials. Spanhaak et al. (14) and Matsumoto et al. (18) demonstrated an increase in LcS and concomitantly total Lactobacilli counts as well as an increase in Bifidobacteria counts in stool samples after uptake of LcS, clearly pointing at a modulation of the intestinal microbiota.

Preselection of participants in our trial was based on a mean NK cell activity of <40% (for an experimental effector:target ratio of 25:1). Imai et al. (8) defined low NK cell activity as \( \leq 42\% \). Further stratification between high and low NK cell activity at baseline (T1) on the basis of NK cell activity levels (i.e., considering only persons with a NK cell activity \( \leq 40\% \) or even below this margin) and statistical analyses with these confined data regarding changes between T1 and T2 also showed no significant treatment effect for all variables studied.

Several mostly small-scale studies recruiting healthy Japanese report a modulation of NK cell activity by LcS intake (4,19–21). Nagao et al. (4) investigated the effects of LcS (4 \( \times 10^{10} \) CFU LcS/d) in 9 young males and females with a crossover design. A similar preselection of participants based on a NK cell activity \( <45\% \) (for effector:target ratio of 20:1) as in our study was performed. A significant time effect on NK cell activity was observed within the LcS group; however, it was not tested whether this change over time differed between LcS and placebo groups. Baseline data for NK cell activity significantly differed between groups. Our data clearly show that a run-in phase is crucial for leveling inter-group variation. Furthermore, it is not obvious whether the authors considered that the NK cell activity may be affected by the female menstrual cycle (22), so the variation of NK cell activity within a 3-wk intervention period may also be ascribed to hormonal fluctuations.

Cigarette smoking inversely affects NK cell activity (19,23,24). Morimoto et al. (19) reported that the mean NK cell activity after adjustment for the numbers of cigarettes smoked daily was elevated in individuals \( (n = 31) \) consuming LcS (daily dose: \( 4 \times 10^{10} \) CFU) for 3 wk compared with those receiving placebo \( (n = 30) \). Unfortunately, no baseline data for NK cell activity and no statistical comparison of changes over time between groups (time \( \times \) treatment effects) were reported, which impedes the interpretation of these results. Takeda et al. (20) extended their earlier study with young adults (4) by recruiting 10 elderly volunteers. Again, a crossover design was used. In contrast to the outcome of the study with the young adults, no significant stimulatory effect of LcS on NK cell activity was observed. Whether the reported tendency of a decline in NK cell activity in the placebo group was prevented by the LcS supplementation is not known, because treatment effects were not statistically analyzed. Results from both studies were recently published as a symposium paper (21).

The outcome of the present study is in line with findings from Spanhaak et al. (14), who investigated the impact of the daily consumption of fermented milk containing LcS (daily dose: \( 3 \times 10^{11} \) CFU) over 4 wk on a range of immune markers concerning the adaptive and innate immune system in a small group of 20 middle-aged and elderly European males. Both studies are comparable in terms of study design, duration of the intervention period, ethnicity, and most probably also in terms of general dietary habits of the participants. However, both studies differ in regard to the inclusion of test persons who were healthy and immunologically unchallenged in both trials but had normal blood NK cell activity levels in the study of Spanhaak et al. (14) and in regard to the administered daily LcS dose, which in the present trial was only 7% of the dose administered by Spanhaak et al. (14). Despite these discrepancies, no immunomodulatory effects could be detected in either study.

Remarkably, all studies reported no effect of LcS supplementation on the absolute number of NK cells [present study and (14,19,21)], which restricts any potential effects of LcS on NK cells to changes in the cytotoxic activity per NK cell.

In 2 studies using mouse models to examine the effect of LcS on NK cells in aged and also neonatal and infant mice during influenza infection, the probiotic enhanced NK cell activity systemically in spleen (25) and also locally at the site of infection, in the lung (25,26). This may have been the cause of reduced viral titers in nasals washings, because NK cells are the main immune effectors in the early stages of viral infection due to their cytotoxic capacity against virally infected cells (27). During early stages of tumor development in mice, a decline in NK cell activity in the spleen as a result of carcinogenesis was counteracted by the oral uptake of LcS for 8 wk (28). In humans, the recurrence of colorectal and noninvasive bladder cancers might be favorably affected by daily LcS intake of \( 3 \times 10^{10} \) CFU for at least 1 y (29–32). However, none of these studies on tumor recurrence investigated the underlying mechanisms for protective effects. Therefore, we do not know if NK cell activity, a recognized prognostic factor for cancer recurrence (11–13), was enhanced due to LcS intervention.

Although studies in mice convincingly show that LcS stimulates NK cell activity, well-designed and controlled intervention trials with healthy humans, including the data presented here, do not unequivocally support the hypothesis that the intake of LcS by healthy adults confers immunostimulatory effects. Our data, which include a wide range of immunological markers, are impressively consistent. However, the size of our study was calculated to identify a 25% change in NK cell activity (mean NK cell activity: 32%) with a power of 80%. This means that smaller stimulatory effects of LcS on NK cell activity could not be detected by this study. Therefore, we cannot exclude the possibility that we have missed small changes in NK cell activity. The biological significance of such potential small changes is rather low. For other probiotics, there is also no evidence from well-controlled and appropriately and statistically analyzed human studies for an enhancement of NK cell activity (33,34). Because the turnover rate of NK cells in blood is \( \sim 2 \) wk (35), we think that the supplementation period of 4 wk was long enough to allow NK cell activation by the probiotic. However, our study design does not allow identifying effects that may have occurred after 2 wk, as reported by others (36). In addition, the daily LcS dosage chosen for our study reflects the upper range of habitual probiotic intake by European consumers. Because only 1 LcS dose was tested in the present trial, we cannot rule out that immunomodulatory effects might have been achievable by differing LcS loads.

Although participants with low NK cell activity were selected for the present trial, both studies with test persons from Western societies were negative. Several factors, such as the genetic background regarding immune cells and function as well as the composition and the fermentative capacity of the intestinal microbiota, might have impeded immunomodulatory effects by LcS intake in these collectives. Above that, European and Asian diets differ in type and quantity of nondigestible carbohydrates (37,38), which provide the substrates for probiotics like LcS in the large intestine. Besides the direct immunomodulatory effects,
LeS may exert indirect immunomodulatory effects by producing short-chain fatty acids and immunogenetic carbohydrate structures (39). Without appropriate substrates, LeS may not be capable of generating these immunomodulatory molecules, which must be investigated in future studies.

In conclusion, supplementation with LeS did not increase NK cell activity and modulate other immune functions in healthy men with reduced NK cell lytic activity. Other doses of LeS, time of intervention, or differences, e.g. in the background diet, may result in a different outcome.

Acknowledgments
We thank M. Brossart, S. Delong, M. Giorgi, E. Hoch, S. Merkel, G. Schultheiss, U. Stadler-Prayle, and L. Uhlmann for their excellent technical assistance. We thank L. Korn for his statistical consultation and N. Labetzsch for her help in measuring phagocytosis and respiratory burst. B.W., S.S., C.F., and A.B. designed research; S.S. and A.B. conducted research; S.S. analyzed data and performed statistical analyses, B.W. and S.S. wrote the paper; and B.W. had primary responsibility for final content. All coauthors participated in critically revising the manuscript. All authors read and approved the final manuscript.

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