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# Stimulation of murine cell-mediated immunity by dietary administration of a cell preparation of *Enterococcus faecalis* strain KH-2 and its possible activity against tumour development in mice

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**It is well known that dietary lactic acid bacteria (LAB) stimulate cell-mediated immunity such as natural killer (NK) activity in mice. Here, we aimed to assay the immunomodulatory effects of a cell preparation of *Enterococcus faecalis* strain KH-2 (CPEF). We further evaluated the possibility of antitumour activity caused by CPEF administration, because NK cells actively participate in the prevention of tumour formation. NK cell activity and gene expression of IFN-γ and Perforin 1, which were induced most likely by a synergetic action of their cytotoxic activity, were higher in splenocytes of CPEF-administered mice than they were in control mice. Moreover, unlike those of control mice, the splenocytes of CPEF-administered mice had significantly higher $CD28^+CD69^+/CD4^+$ and $CD28^+CD69^+/CD8^+$ ratios that resulted in a survival rate with a tendency toward improvement after 47 days of CPEF administration (p=0.1) in Meth-A fibrosarcoma-bearing mice. In conclusion, we showed that CPEF might be effective in treating Meth-A fibrosarcoma in mice, as it helped increase their survival rate via stimulation of an immune response in splenocytes, which involved systemic cellular immunity processes such as cytotoxic activity, and active T cells.**

**Key words: *Enterococcus faecalis*, activation of cell-mediated immunity, murine model, natural killer activity**

## INTRODUCTION

Bacterial strains with probiotic properties including lactic acid bacteria (LAB) can induce protective immune responses by constantly stimulating mucosal surfaces [1], which activates and matures immune cells [2], including natural killer (NK) cells and T cells [3]. For example, it has been shown dietary LAB stimulate the NK activity of splenocytes in both young [4] and elderly mice [5], which results in suppression of tumour growth [6]. More recently, we demonstrated several times in our premises the immunostimulating effects of a cell preparation of *Enterococcus faecalis* (a species of LAB) not only on lymphoid tissue associated with the gut [7, 8] but also on lymphatic systemic tissue [9, 10].

The immune system plays a critical role in the control of progression of tumours [11]. In an immune response, innate immune cells such as macrophages and NK cells, as well as adaptive immune cells such as B and T cells, can be found within the tumour microenvironment [12, 13]. Previous studies reported that NK cells alone actively participate in the prevention of tumour formation [14] and that cytotoxic T lymphocytes (CTLs) are capable of killing tumours or blocking their growth following a stimulus by a metabolite [15, 16] or other immune cells [17, 18]. This close crosstalk between CTLs and NK cells in the tumour microenvironment [19] seems to enhance the immune response and prolong patient survival, which can result in complete tumour suppression [20]. However, if CTLs and NK cells lose functionality or their activity is not properly orchestrated, immune cell mobilisation can result in induction of growth dormancy [21, 22] or generation of more tumour cells even in immunocompetent individuals [21, 23], as it has been experimentally demonstrated in mice [24, 25].

Sarcomas are rare and heterogeneous malignancies found in bone, muscle, fibrous, or similar tissues and, like epithelial tumours, exhibit formidable acquired and adaptive resistance to drugs [26]. Because of this, aggressive chemotherapy and surgical resection remain the most effective therapeutic approaches to date [26]. Nonetheless, previous studies

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reporting information on the immunosuppressing effects of beneficial microorganisms on sarcoma have provided valuable insight and warranted further investigation [27, 28].

In the present study, we evaluated whether or not oral administration of a cell preparation of *E. faecalis* strain KH-2 (CPEF) stimulated cell-mediated immunity such as NK activity in a mouse model. We further aimed to test whether or not oral administration of CPEF was effective for preventing tumour development using Meth-A fibrosarcoma-bearing mice, which represent an *in vivo* screening model commonly used to evaluate the antitumour activity of functional foods or alternative medicines [29–31].

## MATERIALS AND METHODS

### *Cell preparation of heat-treated CPEF*

*Enterococcus faecalis* strain KH-2 was retrieved from a bacterial library deposited in our premises. *E. faecalis* strain KH-2 was then inoculated into a culture medium (1 l) containing 50 g of glucose, 20 g of yeast extract, 10 g of peptone, 2 g of monopotassium phosphate, 1 g of magnesium sulfate, 1 g of trisodium citrate, and 0.5 g of glycerin-fatty acid ester, at pH 6.5, and incubated at 37°C for 24 hr. Bacterial cells were harvested with a filter and subsequently pasteurised. The *E. faecalis* cell density was $5 \times 10^{12}$ cells/g. CPEF was concocted by suspending the cell fraction in sterile saline and was used for CPEF treatment in all experiments.

### *Tumour cell line culture*

RPMI-1640 medium (Sigma, Tokyo, Japan) containing 10% fetal bovine serum and 1% Penicillin-Streptomycin solution (10,000 U/ml; Gibco, Tokyo, Japan) was used as the cell culture medium in all experiments. Meth-A, a fibrosarcoma cell line, was kindly donated by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer of Tohoku University (Sendai, Miyagi, Japan). Meth-A cells were cultured in 75 $cm^2$ culture flasks containing 10 ml of RPMI-1640 medium for 3 days at 37°C in a humidified atmosphere (5% $CO_2$). A murine lymph node lymphoma cell line (YAC-1) was purchased from DS Pharma Biomedical (Osaka, Japan) and used as the target cells. YAC-1 cells were cultured and incubated as described above for Meth-A cells. Afterwards, YAC-1 cells ($1.0 \times 10^6$ cells/ml) were labelled with 10 μM 3,3′-dioctadecyloxacarbocyanine perchlorate (Dioc18; Life Technologies Japan, Tokyo, Japan) and further incubated for 15 min at 37°C [32]. YAC-1 cells were then washed three times with Hank's Balanced Salts Solution (HBSS, Sigma-Aldrich Japan, Tokyo, Japan), resuspended in RPMI-1640 medium, and counted (TC10 automated cell counter; Bio-Rad, Richmond, CA, USA).

### *Animals*

Five-week-old female BALB/c mice were used as the animal model in all experiments. All animals in this study were purchased from Japan SLC (Shizuoka, Japan) and fed a commercial AIN-93G diet (LSG, Tokyo, Japan) throughout the study. All animals were maintained in an air-conditioned room (25°C) with a 12-hr light and dark cycle at the Kyoto Institute of Nutrition and Pathology (Kyoto, Japan). Food and water were provided *ad libitum* in all experiments. Handling of mice and experimentation humane endpoints in Experiments 1 and 2 were carried out in accordance with the guidelines for animal studies of the Kyoto Institute of Nutrition and Pathology.

### *NK activity in ex vivo splenocytes from CPEF-administered mice (Experiment 1)*

After 7-days of acclimatisation, 50 mice were randomly and equally allocated into five groups as follows: CPEF free, sterile saline (group $T_1$0, control group); 0.2 mg of CPEF/kg BW/day (group $T_1$0.2); 1 mg of CPEF/kg BW/day (group $T_1$1); 2 mg of CPEF/kg BW/day (group $T_1$2); and 5 mg of CPEF/kg BW/day (group $T_1$5). Mouse groups were housed in separate plastic cages throughout the study. CPEF was suspended in sterile saline and given orally to mice with a commercial disposable feeding needle (Fuchigami, Kyoto, Japan) every day at 10 a.m. for 7 consecutive days (day 0–day 6). Upon completion of the CPEF administration period, all animals were euthanised by exsanguination under overdose anaesthesia with an intraperitoneal injection of sodium pentobarbital (Somnopentyl, Kyoritsu, Tokyo, Japan) and had their spleen aseptically collected. Splenocytes were prepared as previously described [9] with some modifications. Briefly, a single-cell suspension was prepared with HBSS, filtered through a 40-μm cell strainer (BD Biosciences, Bedford, MA, USA), and centrifuged at 800 × *g* for 3 min at room temperature (RT). After removal of the supernatant, cells were resuspended in ACK lysing buffer (0.5 mol/l $NH_4Cl$, 10 mmol/l $KHCO_3$ and 0.1 nmol/l $Na_2EDTA$; pH 7.2) and incubated at RT for 10 min to remove erythrocytes. The remaining cells, i.e., splenocytes, were further washed twice with HBSS and resuspended in RPMI-1640 medium. Splenocytes were then stained with trypan blue and counted with the automated cell counter (TC10).

As an NK activity assay (described below) is a labour-intensive activity, the start of CPEF administration to mice was carried out in stages as follows: day 1, three mice of each group (total: 15 mice); day 2, three mice of each group (total: 15 mice); and day 3, four mice of each group (total: 20 mice).

### *Evaluation of natural killer cell activity*

The NK activity assay was carried out as previously described [33] with slight modifications. Briefly, splenocytes identified as NK effector cells were adjusted to $5 \times 10^6$ cells/ml of RPMI-1640 medium. A 100-μl aliquot of the splenocytes cell solution was inoculated into a 96-well flat-bottom culture plate (AGC Techno Glass, Shizuoka, Japan) and mixed with 100 μl of YAC-1 cells (T) as target cells to achieve NK effector cell/YAC-1 cell (E/T) ratios of 2:1 and 10:1. The mixture was incubated for 4 hr at 37°C in a humidified atmosphere (5% $CO_2$). Two microliters of propidium iodide (PI; 2 mg/ml; Sigma-Aldrich Japan) was then added to each well to stain

Table 1. Primers and probes used in this study

| Gene name | Primers or probes 5′-3′ | Probe number | GenBank accession number |
|---|---|---|---|
| Granzyme A | F ggccatctcttgctactctcc | 75* | NM_010370.2 |
| | R cgtgtctcctccaatgattct | | |
| Granzyme B | F gctgctcactgtgaaggaagt | 2* | NM_013542.2 |
| | R tggggaatgcattttaccat | | |
| Perforin 1 | F gaagaagaaacagcacaaaatgg | 31* | NM_011073.3 |
| | R gacgtgacgctcacggtag | | |
| Interferon-γ | F cagcaacagcaaggcgaa | - | NM_008337.1 |
| | R cggatgagctcattgaatgct | | |
| | P ttgccaagtttgaggtcaacaaccca | | |
| Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) | F ggtgtcttcaccaccatgga | - | NM_008084.2 |
| | R cagaaggggcggagatgat | | |
| | P aaggccggggcccacttgaa | | |

*Listed probe numbers indicate the product number of the Universal ProbeLibrary Set (Human and Extension Set) sold by Roche Applied Science. Primers and probes of IFN-g and GAPDH were designed and synthesized by Bioresearch Technologies Japan (Tokyo, Japan).

the dead cells. NK (or cytotoxic activity against Meth-A cells) activity was calculated using the following formula:

$$\text{NK activity (\%)} = \frac{[(\text{killer T cells}) - (\text{natural dead T cells})]}{[(\text{maximum dead T cells}) - (\text{natural dead T cells})]} \times 100$$

Evaluation and confirmation of NK activity of splenocytes were carried out using a BD Accuri™ C6 flow cytometer (BD Bioscience Japan, Tokyo, Japan) following a previously reported method [4]. Experiments using the E/T ratios described above were carried out in duplicate. "Maximum dead T cells" were performed treating 3% saponin solution as described previously [4]. In Experiment 1, the NK activity assay was carried out using only six mice from each group because of YAC-1 cells were not in good condition of the final slaughter day.

*Measurement of gene expression*

To determine the mRNA expression associated with NK activity, the remaining splenocytes were immersed in RNA-*later* (Sigma). Immersed splenocytes were first stored overnight at 4°C and then were stored at −80°C until subsequent RNA extraction. Total RNA extraction and cDNA synthesis were carried out as previously described [34]. Real-time PCR was conducted using a Rotor-Gene 6200 (Qiagen, Tokyo, Japan). The primers and TaqMan probes associated with NK activity and used in this study are listed in Table 1. Optimal primers and probes were designed using freely available online tools (https://www.roche-applied-science.com/) or selected from Bioresearch Technologies Japan (Tokyo, Japan). PCR analysis methods were conducted as previously described [34]. The relative expression levels of the mRNAs were calculated by the comparative Ct method [35]. In addition, the amount of target cells relative to housekeeping mRNA (glyceraldehyde 3-phosphate dehydrogenase; GAPDH) for each sample was calculated and treated as a *ΔΔ* Ct value.

*Survival rate of CPEF-administered mice after Meth-A inoculation (Experiment 2)*

Thirty mice were randomly and equally allocated into three groups and housed as described for Experiment 1. CPEF was given orally to mice every day at 10 a.m. for 47 consecutive days (day 0–day 46). Mice were orally given CPEF as follows: CPEF free, saline inoculation (group $T_20$); 5 mg/kg BW/day (group $T_25$); and 10 mg/kg BW/day (group $T_210$). CPEF was administered in a manner similar to that in Experiment 1. On day 7, Meth-A was intraperitoneally injected into all mice ($2.0 \times 10^5$ cells/head) to induce fibrosarcoma [36]. Clinical observations and measurement of body weight were carried out on mice twice daily. The humane endpoint was established to be a 20% body weight loss following Meth-A inoculation. The experiment was ended on day 46.

*Immunostimulation activity of CPEF under Meth-A inoculation conditions (Experiment 3)*

Twenty mice were randomly and equally divided into two groups and housed as described for Experiment 1. CPEF administration was carried out every day at 10 a.m. for 17 consecutive days (day 0–day 16). CPEF was given orally to mice as follows: CPEF free, saline inoculation (group $T_30$), and 10 mg/kg BW/day (group $T_310$). CPEF was administered in a manner similar to that in Experiment 1. On day 7, Meth-A cells were subcutaneously injected into all mice in the same concentrations as described above. Clinical observations and measurement of body weight were carried out on mice twice daily until study completion on day 17. Subsequently, all mice were euthanised by exsanguination under overdose anaesthesia with an intraperitoneal injection of sodium pentobarbital (Somnopentyl) and their spleen aseptically collected.

The NK activity of splenocytes was determined as described for Experiment 1, except that the E/T ratios were 5:1 and 25:1. The cytotoxic activity of splenocytes against the Meth-A cells was also evaluated. The cytotoxic activity

of splenocytes against Meth-A cells was determined as for the NK activity described for Experiment 1, except that Meth-A cells were used as the target cells.

The CTL subpopulation was measured using fluorescently conjugated antibodies. The splenocytes were stained in triplicate with FITC-labelled anti-Mouse CD3 (TONBO Biosciences, San Diego, CA, USA), PerCP-Cy5.5-labelled anti-Mouse CD4 (TONBO Biosciences) and APC-labelled anti-Mouse CD8a (TONBO Biosciences) antibodies. They were also stained with four other antibodies, namely, PerCP-Cy5.5-labelled anti-Mouse CD4, APC-labelled anti-Mouse CD8a, PE-labelled anti-Mouse CD28 (Abcam, Cambridge, MA, USA), and FITC-labelled anti-Mouse CD69 (Abcam). After incubation with the antibodies at 4°C for 30 min, the cells were washed with PBS and fixed with 1% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) in PBS for 30 min. After fixation, the samples were washed with 0.5% bovine serum albumin (Nacalai Tesque, Kyoto, Japan) in PBS and incubated overnight at 4°C. Afterwards, the samples were analysed using a flow cytometer.

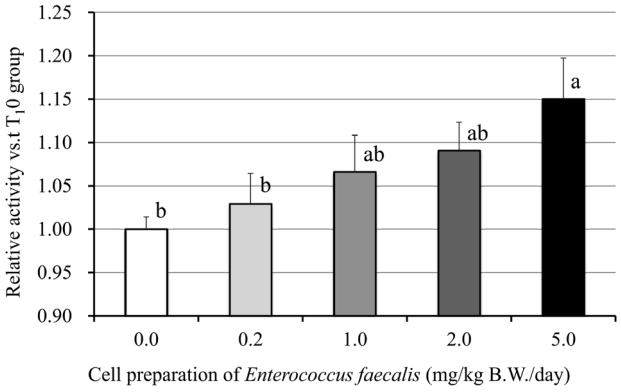


Fig. 1. Relative values of splenocyte NK activity with or without CPEF administration in Experiment 1.

The results shown are representative of an E/T ratio=2:1. Bars with different letters indicate significant differences at $p<0.05$. Values are means ± SE (n=6).

*Statistical analyses*

The randomised block design 1-way ANOVA was used to analyse the differences between means in the parameters of Experiment 1. Tukey-Kramer post hoc comparison was used for multiple comparisons when needed. Survival curves were drawn using the Kaplan-Meier method, and the differences compared with group $T_20$ were analysed by a log-rank test in Experiment 2. Depending on the results of the *F*-test, either the Student's or Welch's t-test was used to analyse the differences between means in the parameters of Experiment 3. A difference between means was considered significant at $p<0.05$, and a tendency was considered to be significant at $p\leq0.1$ in all statistical analyses. All calculations were made using Statcel3 (OMS, Tokyo, Japan) or StatLight 2000 (Yukms Co., Ltd., Tokyo, Japan), as add-in applications for Microsoft Excel® (Microsoft, Seattle, WA, USA). All values are given as the means ± SE.

## RESULTS

*NK activation in ex vivo splenocytes from CPEF-administered mice (Experiment 1)*

The results of dose-dependent stimulation of NK activity by CPEF administration are shown in Fig. 1. NK activity in splenocytes of mice given 5 mg/kg BW/day of CPEF was significantly ($p<0.05$) higher than it was in those of control mice and mice given only 0.2 mg/kg BW/day of CPEF (Fig. 1). Regarding the gene expression associated with NK activity, there was no difference in gene expression of granzyme B between groups (Fig. 2D). However, there was a tendency ($p<0.1$) for the gene expression of granzyme A to be higher in mice given 2 mg/kg/day of CPEF when compared with control mice (Fig. 2B). Furthermore, the gene expression of Perforin 1 and gamma interferon (IFN-γ) was significantly ($p<0.05$) higher in mice given 5 mg/kg/day of CPEF, when compared with control mice (Fig. 2A, 2C), and control mice and mice given only 0.2 mg/kg BW/day of CPEF (Fig. 2C), respectively.

*Survival curves of CPEF-administered mice after Meth-A fibrosarcoma inoculation (Experiment 2)*

Figure 3 shows the survival curves of Meth-A-inoculated mice with or without CPEF administration. Mice given orally 10 mg/kg/day of CPEF tended ($p=0.10$) to show improved survival (6 mice after 47 days) compared with control mice (2 mice after 47 days).

*Immunostimulation activity of CPEF under Meth-A inoculation conditions (Experiment 3)*

Administration of 10 mg/kg/day of CPEF triggered a higher NK activity in Meth-A-inoculated mice than it did in those mice not given CPEF (Fig. 4A). In addition, cytotoxic activity against Meth-A cells in Meth-A-inoculated mice administered 10 mg/kg/day of CPEF was higher than it was in those not given CPEF (Fig. 4B). Separately, immunostaining of splenocytes showed that the $CD4^+/CD3^+$ and $CD8^+/CD3^+$ ratios in the $T_30$ and $T_310$ groups were not different (Fig. 5). However, when looking at the activity of $CD4^+$ and $CD8^+$ cells in the presence of $CD28^+$ and/or $CD69^+$, although the $CD28^+/CD4^+$ ratio showed a tendency to be higher in the $T_310$ group than it did in the $T_30$ group (Fig. 6C), other $CD4^+$ or $CD8^+$ ratios did not differ significantly between groups (Fig. 6D, 6F, and 6G). Nonetheless, the double positive $CD28^+CD69^+/CD4^+$ and $CD28^+CD69^+/CD8^+$ ratios were found to be remarkably higher in the $T_310$ group than in the $T_30$ group (Fig. 6E and 6H).

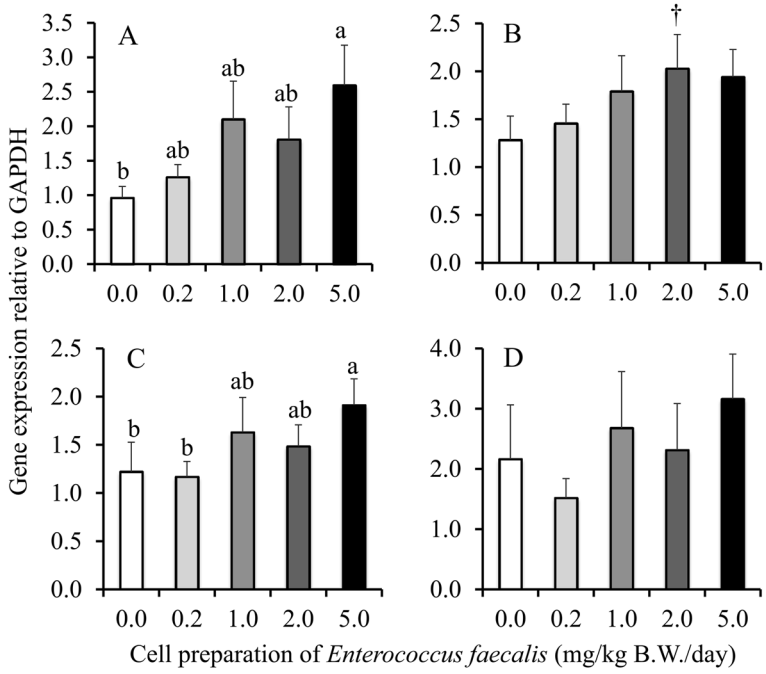


Fig. 2. Relative gene expression associated with NK activity in splenocytes with or without CPEF administration in Experiment 1.

(A) Gene expression of IFN-γ. (B) Gene expression of granzyme A. (C) Gene expression of Perforin 1. (D) Gene expression of granzyme B. Bars with different letters indicate significant differences ($p<0.05$). A dagger indicates a tendency to be different compared with the $T_10$ group ($p<0.1$). Values are means ± SE (n=10).

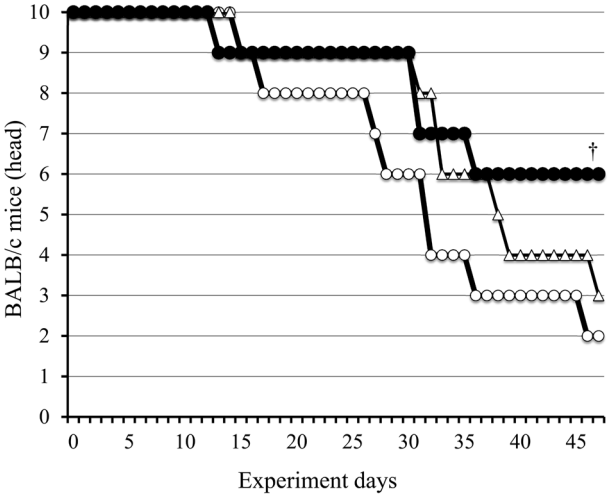


Fig. 3. Survival curves of mice after Meth-A inoculation, with or without CPEF administration in Experiment 2.

Open circles indicate $T_20$ group mice. Open triangles indicate $T_25$ group mice. Closed circles indicate $T_210$ group mice. A dagger indicates a tendency to be different compared with the $T_20$ group ($p=0.1$).

## DISCUSSION

In the present study, we observed that an immune response identified by a strong NK activity (Figs. 1 and 4A) was triggered in mice orally given CPEF (5 or 10 mg/kg BW/day) compared with those that were not. This result is in accordance with previous studies at these premises, where we demonstrated that a different *E. faecalis* strain induced an immune response in gut-associated [7, 8] and systemic [9, 10] lymphoid tissues. Elsewhere, researchers have also shown the immunostimulatory properties of *E. faecalis* strains, including activation of dendritic cells and interferon-γ production [37] and cytokine production in human peripheral blood mononuclear cells [38]. In addition, we found that although gene expression of granzyme A and B in splenocytes was not affected significantly by CPEF administration, gene expression of IFN-γ and Perforin 1 was stimulated only in splenocytes of mice in the $T_15$ group in Experiment 1 (Fig. 2A and 2C). Furthermore, we observed that CPEF administration (10 mg/kg BW/day) stimulated cytotoxic activity including NK and CTL activity against Meth-A in Experiment 3 (Fig. 4). CTL releases Perforin 1, which facilitates the entry of granzyme B into the cytoplasma of target cells and cause apoptosis [39]. On the other hands, granzyme B expression was not stimulated in this study, so

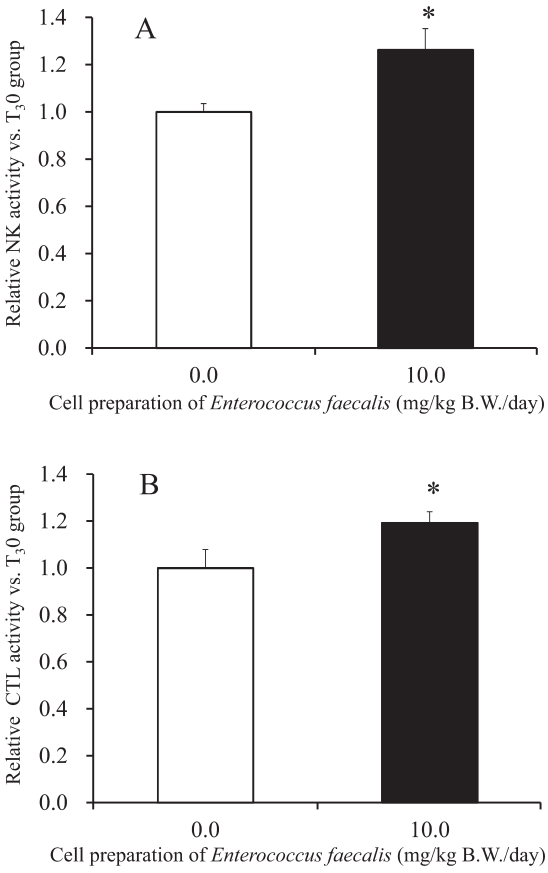


Fig. 4. Relative values of splenocyte NK and CTL activity with or without CPEF administration in Experiment 3.

(A) Splenocyte NK activity. (B) Splenocyte CTL activity. The results shown are representative of an E/T ratio=25:1. Asterisks indicate significant differences at $p<0.05$. Values are means ± SE (n=10).

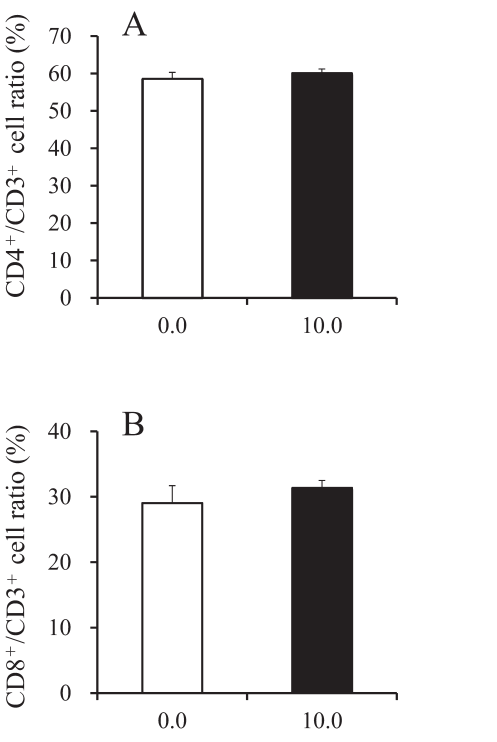


Fig. 5. Subpopulation ratios of $CD4^+$ or $CD8^+$ cells in T lymphocyte ($CD3^+$ cells) with or without CPEF administration in Experiment 3.

(A) The ratio of $CD4^+$ cells in $CD3^+$ cells in splenocytes. (B) The ratio of $CD8^+$ cells in $CD3^+$ cells in splenocytes. Values are means ± SE (n=10).

the Perforin/granzyme pathway might not be cascaded by CPEF administration. Another heat-killed LAB induced NK cytotoxicity without Perforin and granzymes upregulation [40], so CPEF might be also stimulated NK activity in a similar approach. CPEF administration (10 mg/kg BW/day) activated NK cells and CTLs in Meth-A bearing mice (Fig. 4), which in turn induced production of IFN-γ [41]. IFN-γ is a primary cytokine in the immune response to pathogens and malignancies [41]. We therefore theorised that an enhanced synergetic activity of IFN-γ and Perforin 1 was essential for CTLs to recognize infected cells following inoculation of Meth-A into mice, which tended to improve ($p=0.10$) twofold the survival of mice at 45 days and beyond (Fig. 3).

To confirm this, in the presence of $CD28^+CD69^+$ as an accessory cell, we measured the percentage of active T cells, which showed that administration of CPEF induced proliferation of $CD4^+$ and $CD8^+$ cells among lymphocytes in CPEF-administered mice but not in control mice (Figs. 5 and 6). Clearly, our work showed that administration of *E. faecalis* to mice induced a significant production of IFN-γ not only by CTLs but also by activation of NK cells, which are its predominant producers [42]. Induction of IFN-γ production by CTLs and NK cells may have been similar to that exerted by interleukin-12 [43]. It is well known that many *E. faecalis* strains stimulate IL-12 production in murine splenocytes *in vitro* [44]. The *E. faecalis* KH-2 used in this study also stimulates IL-12 production in murine splenocytes (Kan *et al.* unpublished data) and the mechanism of activation of CTLs and NK cells may have been stimulation of IL-12 production as a results of CPEF administration in the present study. Furthermore, Inoue *et al.* and Nishibayashi *et al.* [44, 45] suggested that the major active component of *E. faecalis* is RNA, and we also considered in the present study that the *E. faecalis* KH2 might have stimulated the immune function caused by bacterial RNA.

Interestingly, previous work found that upon stimulation by administration of *Lactobacillus johnsonii* and *L. sakei,* IFN-γ production by NK cells was induced in the presence of activated antigen $CD69^+$ but that a $CD8^+$ response was not activated [43], whilst others [46] showed that *L. casei*

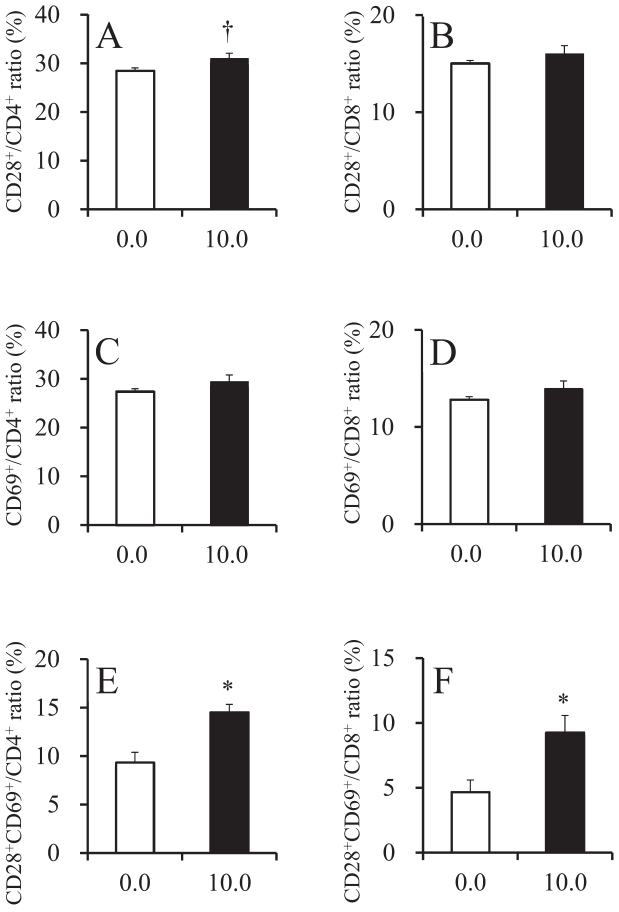


Fig. 6. Subpopulation ratios of active T lymphocytes with or without CPEF administration in Experiment 3.

(A) The ratio of $CD28^+$ cells in $CD4^+$ cells. (B) The ratio of $CD28^+$ cells in $CD8^+$ cells. (C) The ratio of $CD69^+$ cells in $CD4^+$ cells. (D) The ratio of $CD69^+$ cells in $CD8^+$ cells. (E) The ratio of $CD28^+CD69^+$ cells in $CD4^+$ cells. (F) The ratio of $CD28^+CD69^+$ cells in $CD8^+$ cells. Asterisks indicate significant differences ($p<0.05$). A dagger indicates a tendency to be different ($p<0.1$). Values are the means ± SE (n=10).

promoted IFN-γ production by $CD8^+$ CTLs but not NK cells. This apparent discrepancy between studies may be related to the dependence of immune cell activation on the presence of accessory cells such as $CD69^+$ [43, 47], which is in accordance with our results (Fig. 6). Moreover, LAB strains seem to respond differentially to the type of cells used as experimental models [3], and it is increasingly evident that the immune response level and identity of the immune cells involved depend on the strain itself [4–6, 9, 48–51] and cannot be extrapolated to other strains [52]. It is worth noting that in comparison with single positive ($CD28^+$ or $CD69^+$) cells, double positive ($CD28^+CD69^+$) cells developed remarkably as a result of CPEF administration in $CD4^+$ and $CD8^+$ cells in splenocytes of Meth-A bearing mice (Fig. 6). We found no significant differences in the $CD4^+$ and $CD8^+$ cell ratios (Figs. 5 and 6) upon administration of a probiotic such as CPEF, as reported previously by other researchers [53, 54]. Nonetheless, to the best of our knowledge, the present work is the first to report significant differences in double positive $CD28^+CD69^+$ cells after administration of CPEF to Meth-A fibrosarcoma-bearing mice (Fig. 6), although the precise role of these double positive cells remains unclear. We hypothesise that $CD28^+CD69^+$ cells may play a key role in suppression of tumour development, but further evidence is needed to establish their exact role within the immune response context.

Although immune cells such as NK cells and CTLs are capable of killing tumours or blocking their growth [14, 17, 18], certain tumours such as sarcomas show persistence due to a tremendous resistance to treatments [26]. As a result, these tumours recur in approximately 40–60% of patients, and 50% will die of metastases [55]. However, recent applications of bacterial strains with probiotic properties as immunotherapy for induction of antitumour activity, which improves the survival rate of experimental models, have shown very promising results [56, 57]. For example, a previous study reported that at least a fraction of the lipoteichoic acid in cell walls of *Enterococcus* sp. caused complete regression of Meth-A tumours in mice by inducing strong cytokine activity [51]. In this study, the survival rate under the Meth-A-inoculated condition tended to be improved by administration of 10 mg/kg BW/day of CPEF (Experiment 2), so a possible activity against tumour development might have been observed. This point needs further elucidation.

In the present study, we successfully demonstrated that administration of 10 mg/kg BW/day of CPEF as a probiotic treatment in mice induced strong activity in immune cells including CTLs and NK cells that resulted in the survival rate of these animals being twofold that of those not given the CPEF treatment. We also report that although the precise role of double positive cells remains unknown, significant differences in $CD28^+CD69^+/CD4^+$ and $CD28^+CD69^+/CD8^+$ ratios were remarkably high in CPEF-administered mice. Based on the present results, it is proposed that oral administration of CPEF might be used to help prevent tumour development in humans via systemic stimulation of NK and CTL activities.

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