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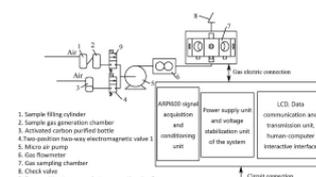
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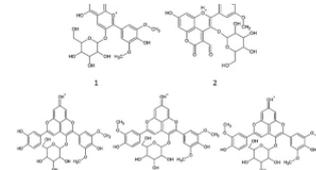
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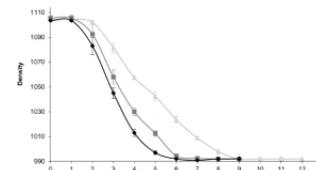
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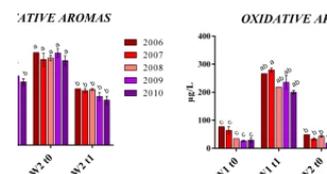
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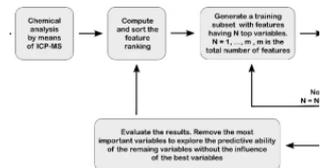
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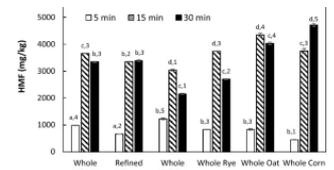
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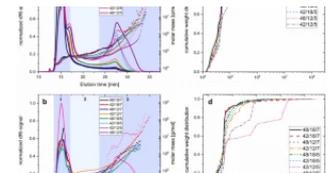
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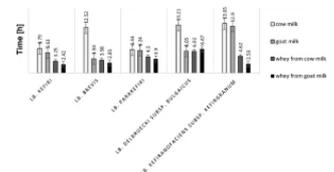
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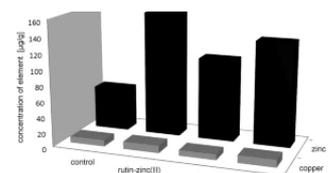
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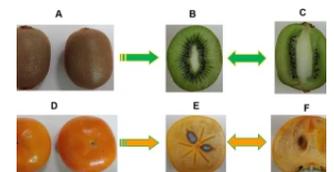
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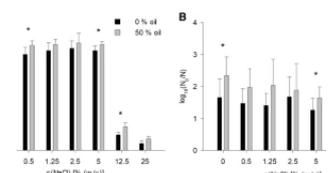
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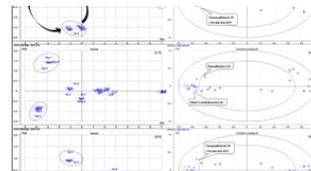
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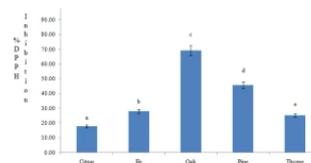
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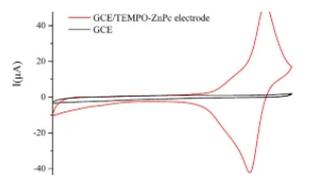
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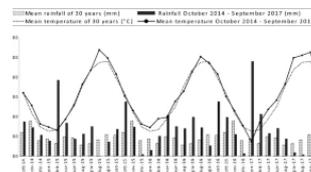
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The combined impact of sauerkraut with *Leuconostoc mesenteroides* to enhance immunomodulatory activity in *Escherichia coli*-infected mice

Zubaidah, E., Susanti, I., Yuwono, S.S., Rahayu, A.P., Srinta, I. and Tewfik, I.

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immunomodulatory activity of sauerkraut combined with *Leuconostoc mesenteroides* was higher than that showed in sauerkraut control solely.

Keywords: immunomodulatory activity; sauerkraut; *Leuconostoc mesenteroides*; mice

25

26

27 **Introduction**

28 The immune system plays a pivotal role in maintaining the body-integrity against foreign objects and
29 pathogens. Bacterial infection poses negative impact on immune system by reducing its capacity and may
30 cause disease. Immunomodulator is defined as compound that enhances the immune system capacity [1-2].
31 Sauerkraut has been reported as effective, potent immunomodulatory. Sauerkraut is a cabbage vegetable
32 produced by the fermentation of lactic acid bacteria (LAB) which occurs spontaneously with the addition
33 of salt. *Leuconostoc mesenteroides* is a heterofermentative Gram positive bacterium that plays key roles in
34 fermentation of foods such as: kimchi, sauerkraut, and milk, leading to the production of various organic
35 acids and aromatic compounds. Additional, bacteria species that have role in fermentation process are:
36 *Leuconostoc mesenteroides*, *Lactobacillus cucumeris*, *Lactobacillus plantarum* and *Lactobacillus*
37 *pentoacetius* [3-4].

38 At the beginning of fermentation process, *Leuconostoc mesenteroides* dominate to produce lactic and
39 acetic acids that decrease pH. The fermentation process is then sustained by the bacteria *Lactobacillus*
40 *plantarum* and *Lactobacillus brevis* until the pH reaches 3 [5-6]. The addition of *L. mesenteroides* and *L.*
41 *plantarum* cultures accelerate the fermentation process and reduce the amount of added salt [7-8].

42 In the literature it was noted that lactic acid bacteria increases vitamins, phenolic and glucosinolate
43 compounds of Sauerkraut [insert reference, please]. Meanwhile, phenolic compounds are famous with their
44 antioxidant activity and the ability to scavenge free radicals, [10-11]. Sulforaphan which is an
45 isothiocyanate derivative, has the ability to prevent cancer through DNA protection by modulating enzymes
46 and inhibiting gene mutations [9-13]. Notwithstanding, a study on the addition of *Leuconostoc*
47 *mesenteroides* as immunomodulator has never been reported, hence, this study aimed to investigate the
48 immunomodulatory activity of sauerkraut combined with *L. mesenteroides* culture.

49 **Materials and Methods**

50 **Materials**

51 White cabbage (*Brassica olerace* L. var) were obtained from local markets. *Leuconostoc*
52 *mesenteroides* [FNCC 0023] was obtained from Food and Nutrition Culture Collection [give details]. All
53 chemical used were analytical grade purchased from local distributors.

54 **Sauerkraut Production**

55 Fresh cabbage was washed, shredded, before the addition of salt at a concentration of 0.5%, then
56 inoculated with 20% culture [give details is it *Leuconostoc mesenteroides* ?], and incubated at room
57 temperature (28°C) for 5 days. A control sauerkraut was prepared with salt concentration at 2% without
58 culture addition. Subsequently, the prepared sauerkrauts were subjected to quality analysis and
59 immunomodulatory activity assay.

60 **Sauerkraut quality analysis**

61 Total lactic acid bacteria was determined according to Penas et al. [9] with counting the colonies grow on
62 MRS Agar after incubation at 37°C for 48 hours. Titratable acidity was measured according to Rangana
63 [13] using direct titration with NaOH solution of 0.1N and expressed as % lactic acid. pH was measured by
64 using pH meter (Manual pH meter Micro Bench TI 2100). Total phenolic content was determined according
65 to Yang *et al.* [14] with measurement of complex compound formed, after the reaction with Folin
66 Ciocalteu reagent and Na₂CO₃ solution, spectrophotometrically at 750 nm, and the content was expressed
67 as mg GAE/g. DPPH scavenging activity was determined by measuring the absorbance at 517 nm, and was
68 expressed as IC₅₀. Sulforaphane content analysis was carried out using *Liquid Chromatography-Mass*
69 *Spectrometry* according to Kim *et al.* [15] under the below conditions:

70 HPLC system was equipped with API 400 Q TRAP mass spectrometry system, electrospray ionization
71 mass (ESI) on positive ions ([M + H] +) mode, ion spray voltage (5.5 kV), gas (20 psi), nebulisation gas
72 (50 psi), heater gas (50 psi), nitrogen purity (N₂), heater gas temperature (550°C), de-clustering potential
73 (100 V), entrance potential (10 V), and spectrum range (m/z 100-1000) in 4.8 seconds.

74 **Immunomodulatory activity assay**

75 The immunomodulatory activity assays of the sauerkrauts were performed in vivo with 20 female six-week-
76 old Balb/c mice, 18-20 grams weight. The experimental protocols and procedures of care and use of animals
77 used in the present work were approved (ethical clearance No. KEP-751-UB) by the Ethics Committee.
78 The National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No.
79 8023, revised 1978) was followed in the this experiment. After 7 days adaptation, mice were divided into

80 four groups: P0 (negative control), P1 (positive control), P2 (sauerkraut without culture) and P3 (sauerkraut
 81 with culture). Sauerkrauts were administered orally at a dose of 0.15ml / kg / BW / day for 14 days. *E. coli*
 82 of 1.3×10^8 CFU / ml was injected into the mice on the 15th day and then incubated for 5 days, and then
 83 CD4⁺ CD8⁺ INF- γ , TNF- α ⁺ and CD68⁺ IL-6 were analyzed with flowcytometry. Total *E. coli* in
 84 intraperitoneal fluid was determined on violet red bile agar (VRBA) with incubation at 37°C for 24 hours
 85 [16].

86 **Statistical analysis**

87 Data were analyzed by complete random design along with analysis of variance (ANOVA) and further
 88 analysis by Tukey at $\alpha=5\%$.

89 **Results and Discussion**

90 **Sauerkraut quality**

91 During the sauerkraut fermentation, lactic acid bacteria grew and created the sauerkraut
 92 characteristics. Table 1 presents the results of the sauerkraut quality analysis.

93 **Table 1** Sauerkraut quality with and without *L. mesenteroides* culture addition
 94

Parameter	Sauerkraut with culture	Sauerkraut without culture
Lactic acid bacteria (CFU/ml)	2.40×10^8	2.60×10^7
Titrateable acidity (%)	1.41 ± 0.01	0.80 ± 0.02
pH	3.67 ± 0.06	4.97 ± 0.09
Total Phenolic content (mg GAE/g)	72.24 ± 0.92	46.59 ± 0.42
Antioxidant activity (IC ₅₀ , ppm)	95.39 ± 2.37	135.12 ± 2.75
Sulforaphane content (ng/g)	848.65	776.47

95
 96 Total LAB in sauerkraut with culture addition was higher than that of control, which resulted in higher
 97 titrateable acidity and lower pH value. This can be explained on the basis that lactic acid bacteria synthesize
 98 various enzymes such as invertase, cellulase, and amylase which are capable of breaking the complex
 99 between phenol compounds and tissue or cell structures to release the phenolic compounds [17-18]. Lee *et*
 100 *al.* also reported that fermentation of mulberry leaves by *L. plantarum* increase the total phenol, due to
 101 duration of the fermentation [19]. These activities resulted an increasing in total phenol and DPPH

102 scavenging activity. Data of sulforaphane content reflected that during fermentation the lactic acid bacteria
 103 produce myrosinase, which is capable of transforming glucoraphanin into sulforaphane compounds.

104 **Immunomodulatory activity of the sauerkraut**

105 The immune response analysis in this study was carried out on the T cell adaptive immune response
 106 with cytokines CD4⁺, CD8⁺, IFN- γ ⁺, TNF- α ⁺. The results are presented in Table 2.

107 **Table 2** Immunomodulatory activity of sauerkraut

Group	CD4 ⁺ IFN γ ⁺ (%)	CD4 ⁺ TNF α ⁺ (%)	CD8 ⁺ IFN γ ⁺ (%)	CD8 ⁺ TNF α ⁺ (%)
Control negative (P0)	0.36±0.14 ^c	0.44±0.19 ^b	0.23±0.04 ^c	1.57±0.14 ^c
Control positive (P1)	0.51±0.11 ^c	0.78±0.30 ^b	0.43±0.17 ^{bc}	5.08±1.01 ^a
Sauerkraut without culture (P2)	1.50±0.27 ^b	1.17±0.38 ^{ab}	0.64±0.14 ^b	3.02±0.17 ^b
Sauerkraut + culture (P3)	2.07±0.67 ^a	1.98±1.30 ^a	1.30±0.20 ^a	2.28±0.54 ^c

108 Note: Values are means \pm standard deviations (n=5). Different letter in the same column mean significant
 109 different at $\alpha=5\%$ ($p < 0.05$).

110 The results of cytokines in spleen were significantly different ($p < 0.05$) between the sauerkraut with and
 111 without *L. mesenteroides* culture. It was reported that IFN- γ induces macrophages by improving their ability
 112 to kill bacteria and parasites, while TNF- α inhibits the replication of intracellular pathogenic bacteria and
 113 directly kill infected cells. Notably, CD4⁺ functions as a co-receptor that strengthens the transduction signal
 114 so that T cells are activated, whereas CD8⁺ is a transmembrane protein that functions as a co-receptor on
 115 killer T cells. Castillo *et al.* has reported that lactic acid bacteria in mice can increase TLR2, TLR4, and
 116 TLR9 expression and surge TNF- α , IFN- γ and IL-10 secretion in Peyer patche's [20].

117 The immune response analysis process was carried out on innate immune responses on CD68 and
 118 IL-6 macrophages (Figure 1). Statistical analysis results showed significant differences ($\alpha = 0.05$) between
 119 the sauerkraut without culture and that with culture. The reduction of CD68⁺ IL-6⁺ level is due to sauerkraut
 120 stimulation and enhancement of innate immune system when infected with *E. coli*, macrophage which can
 121 work against pathogens and phagocytosis and normalizing the infected immune system. Furthermore, lactic
 122 acid bacteria inhibits inflammation and activates the innate immune system that balances the Th1 and Th2
 123 responses so that they can fight off pathogenic bacterial infections. Lactic acid bacteria also modulates the
 124 expression of cytokines, maturation of immune cell surface markers, and increases lymphocyte
 125 proliferation. IL-6 is a multifunctional cytokine that regulates immune responses, acute phase responses,

126 hematopoiesis, and inflammation. This release of IL-6 stimulates macrophage cells to maturation stage so
 127 they are able to carry out phagocytosis more efficiently [21-22].

128 Lactic acid bacteria in sauerkraut plays a pivotal role in phagocytic pathogens. Lactic acid bacteria
 129 inhibits the growth of microorganisms by decreasing the pH of the environment. Total *E. coli* decreased
 130 after the treatment with sauerkrauts (Table 3). Bioactive compounds and BAL in sauerkraut improve the
 131 performance of the immune and antibacterial response. Furthermore, 2-phenylethyl isothiocyanate is one
 132 of the bioactive substances present in cabbage with antimicrobial ability [23-24].

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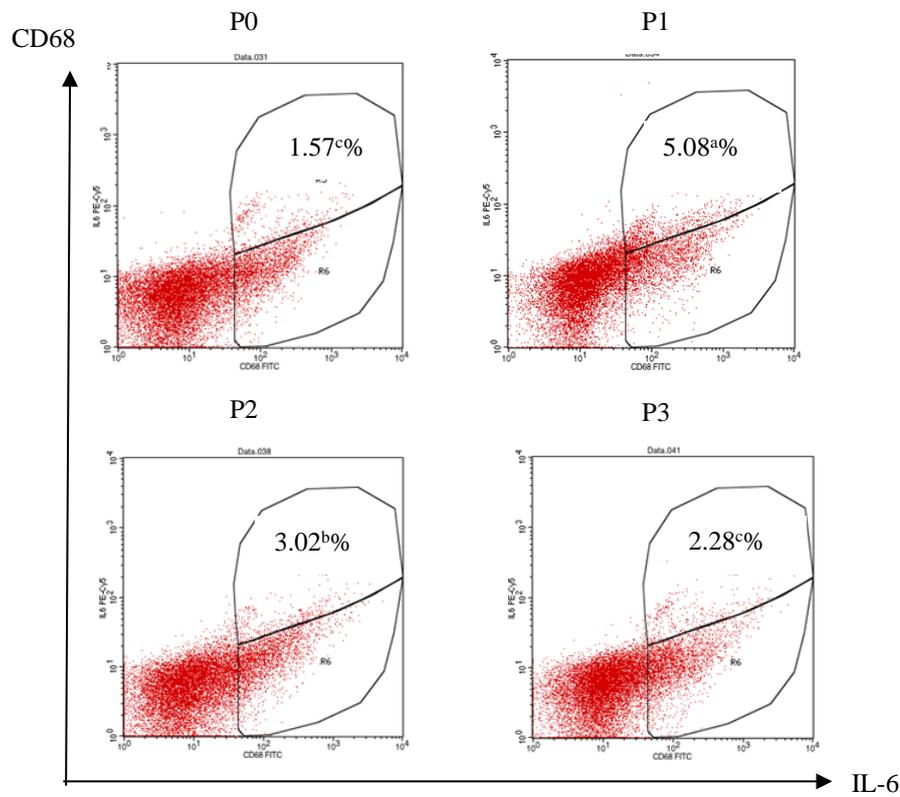
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156 **Fig. 1** CD68⁺IL-6⁺ macrophage cells at different treatments: (P0) control negative, (P1) control positive,
 157 (P2) sauerkraut without culture, (P3) sauerkraut + culture

158

159 **Table 3** Total *E. coli* in mice at different treatments

Group	Total <i>E. coli</i> (CFU/ml)
Control negative (P0)	-
Control positive (P1)	2.3 x 10 ⁷

Sauerkraut without culture (P2)	3.3 x 10 ³
Sauerkraut with culture (P3)	1.7 x 10 ²

160

161 **Conclusion**

162 Our findings highlighted that sauerkraut enhances the adaptive immune response [evidenced by an increase
 163 in CD4⁺ CD8⁺ IFN- γ , TNF α] and innate immune response [denoted by a decrease of CD68- IL-6].
 164 However, the in vivo immunomodulatory activity of sauerkraut combined with *Leconoctoc mesenteroides*
 165 was much higher than that showed in sauerkraut without fermenting bacteria.

166

167 **Acknowledgements**

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