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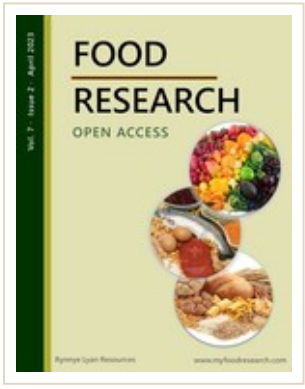

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FOOD RESEARCH

FULL PAPER

Sauerkraut inoculated with *Lactobacillus casei* as a potent immunomodulator in *Escherichia coli* infected mice

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Abstract

The aim of this study was to evaluate the immunomodulatory property of sauerkraut (in the presence and absence of *Lactobacillus casei* culture) on *Escherichia coli* infected Balb -C mice. Fermentation of freshly washed, shredded cabbage was fulfilled by adding 2% salt to prepare 'sauerkraut control', whereas 'inoculated sauerkraut' was prepared by adding 2% salt, 2% sugar and 20% *Lactobacillus casei* culture. After incubation for five days at room temperature, fermented sauerkrauts were tested *in vivo* on mice and the stimulation process was carried out orally for 19 days. Both 'sauerkraut control' and 'inoculated sauerkraut' have shown an increased number of T cell populations namely: $CD4^+$ $CD8^+$ IFN-γ^+, TNF-α^+. These findings were associated with enhanced performance of macrophages and reduction in a number of $CD68^+$ IL-6^+ cell populations [a pro-inflammatory cytokine]. The impact of the immunomodulatory property of inoculated sauerkraut was higher compared to control sauerkraut.

1. Introduction

The immune system in the body is naive if not trained or exposed to microorganisms and or foreign objects (Baratawidjaja, 2004). The foremost function of the immunomodulatory system is to repair the immune system by stimulation (immunostimulant) and normalize abnormal immune reactions (immunosuppressants) (Spelman *et al*., 2006). Therefore, immunomodulators play a pivotal role in the restoration of the imbalance of the immune system whose function is impaired.

Sauerkraut is a product of fermented cabbage that occurs spontaneously with the addition of salt of approximately 2.5%. *Leuconostoc, Lactobacillus* and *Pedococcus* are types of bacteria that play a key role in the fermentation of sauerkraut (Farnworth, 2008). At the beginning of fermentation, *Leuconostoc mesenteroides* dominate the bacterial medium, however, with the gradual reduction of pH more acid-resistant bacteria, *Lactobacillus plantarum* and *Lactobacillus brevis* will dominate until the end of fermentation when the pH reaches ± 3 within 12 days (Plengvidhya *et al*., 2007).

The addition of lactic acid bacteria can accelerate the fermentation process and increase the content of bioactive compounds in sauerkraut. *Lactobacillus casei* is an antibacterial and can increase Th1 cells associated with the release of cytokines such as interleukin (IL-12), interferon (IFN-γ^+) and tumor necrosis factor (TNF-α^+). Lactic acid bacteria will degrade glucosinolate metabolites into their derivative compounds such as isothiocyanate (ITC) and indole-3-carbinol (I3C) (Das *et al*., 2000) which have immunomodulatory effects and can inhibit the enzyme phase I which is an indole-3-acetonitrile (I3ACN) and ascorbigen (ABG) carcinogen (Martinez-Villaluenga *et al*., 2009).

Therefore, this study aimed to evaluate the immunomodulatory property of sauerkrauts [control versus inoculated with *L. casei* culture] on *E. coli* infected Balb-C mice. The specific objective was to determine the key bioactive compounds in sauerkrauts by ascertaining their physicochemical, microbiological and antioxidant characteristics.

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2. Materials and methods

2.1 Materials procurement

White fresh cabbages (*Brassica olerace* L.) were obtained from markets in Batu City, Malang, East Java, Indonesia. *Lactobacillus casei* FNCC 0023 lactic acid bacterial culture was sourced from Gajah Mada University, Yogyakarta. *Escherichia coli* was obtained from the Microbiology Laboratory, Faculty of Medicine, Brawijaya University, East Java, Indonesia.

2.2 Sauerkraut production

Fresh cabbages were washed and shredded before the addition of salt at a concentration of 2% to prepare 'sauerkraut control', whereas 'inoculated sauerkraut' was prepared by adding 2% salt, 2% sugar, and 20% *L. casei* culture (Zubaidah *et al*., 2020). The fermentation process was fulfilled at 28°C (room temperature) for 5 days. The sauerkraut was then analyzed for its physicochemical, microbiological, and antioxidant characteristics.

2.3 Total lactic acid bacteria of sauerkraut

Total lactic acid bacteria (LAB) analysis was carried out according to Peñas *et al*. (2010). The sauerkraut (5 g) was prepared aseptically and diluted with buffer peptone water into serial dilution. The sample suspension was poured on De Man, Rogosa and Sharpe (MRS) Agar and then incubated at 37°C for 48 hrs.

2.4 Total acidity and pH of sauerkraut

Total acidity analysis was carried out according to the procedure by Ranggana (1997). Direct titration of sauerkraut solution was done with NaOH solution (0.1 N) and phenolphthalein indicator. Total acidity was expressed as lactic acid percentage. pH was measured with a pH meter.

2.5 Total phenolic content of sauerkraut

Sauerkraut of 1 g was extracted in 10 mL of methanol and centrifuged at 6000 rpm for 20 mins. The extract (0.5 mL) was put into a test tube, added with 10% Folin Ciocalteau reagent (2.5 mL) and 7.5% Na_2CO_3 (2.5 mL), and incubated at room temperature for 90 mins. Absorbance was measured with a spectrophotometer at 750 nm and gallic acid as the standard (Yang *et al*., 2007). Total phenolic content of sauerkraut was expressed as mg GAE/g.

2.6 DPPH scavenging activity of sauerkraut

The sauerkraut extract was prepared with the same procedure as the total phenolic content analysis. The extract was diluted into 10, 20, 30, 40, and 50 rpm. The diluted sample (4 mL) was added to 1 mL of 0.2 mM diphenyl-1-picrylhydrazyl (DPPH) radical. The mixture was incubated in a dark room for 30 mins and the absorbance was measured at 517 nm. DPPH scavenging activity was expressed as IC_{50}.

2.7 In vivo research design and analysis

Female Balb-C mice weighing 18-20 g were purchased from Malang Murine Farm. Trial animals were kept in cages and fed *ad libitum*. Prior to starting the experiment a 7-day adaptation period was allowed for all mice, afterward, randomization and dividing them into four groups (P0, P1, P2, and P3) with five [5] mice in each. The treatment group P0 (negative control), group P1 (positive control), group P2 (sauerkraut without culture inoculation), and P3 (sauerkraut inoculated with culture). Sauerkraut was administered orally at a dose of 0.15 mL/kg/BW/day for 14 days. On the 15th day, the mice were injected with *E. coli* 1.3×10^8 CFU/mL and then incubated for 5 days. The mice were fed according to treatment. The surgical procedure was carried out on the 19th day to extract and analyze the $CD4^+$ $CD8^+$ INF-γ^+, TNF-α^+, and $CD68^+$ IL-6^+ with flow cytometry.

2.8 Total Escherichia coli count

Intraperitoneal fluids from the mice were retrieved using peptone solution. Violet red bile agar (VRBA) plates were incubated aerobically at 37°C for 24 hrs. Formed colonies were calculated as CFU/mL according to Hosseinzadeh *et al*. (2007).

2.9 Statistical analysis

Data were analyzed by completely randomized design along with analysis of variance (ANOVA) and further tested by Tukey at a 95% confidence interval. The experimental protocols and procedures of care and use of animals used in the present study were approved by the Ethics Committee (ethical clearance No. 15-KEP-UB-202). The National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) was followed in this experiment.

3. Results and discussion

3.1 Physicochemical characteristics of sauerkraut

Sauerkraut is a lactic acid fermented cabbage that occurs spontaneously with the addition of salt. The fermentation process will produce lactic acid and acetic acid which causes a reduction in pH. Table 1 shows the characteristics of sauerkraut and the differences before and after the fermentation process. The addition of *L. casei* culture affects the characteristics of soluble solids, according to Zubaidah *et al*. (2020). The inoculation of *L. casei* increases both: total lactic acid bacteria and total acid, which speeds up the fermentation process. Such an

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Table 1. Characteristics of sauerkraut without culture and sauerkraut with culture addition

Parameter	Sauerkraut type			
	Sauerkraut + culture		Sauerkraut without culture	
	Day - 0	Day - 5	Day - 0	Day - 5
Total Lactic Acid Bacteria (CFU/mL)	2.93×10^7	3.89×10^9	1.2×10^7	1.2×10^9
Total Acid (%)	0.59±0.03	1.78±0.02	0.59±0.05	1.47±0.05
pH	6.27±0.06	3.03±0.06	6.23±0.06	4.51±0.10
Total Phenol (mg GAE/g)	0.46 ±0.11	7.55±0.74	0.41±0.05	4.04±0.02
Antioxidant/IC_{50} (pp)	120.00±7.63	62.09±14.65	152.09±34.02	101.79±1.01

Values are presented as mean±standard deviation (n = 4). Significant differences between data on days 0 and 5 for each observation parameter $p<0.05$. GAE: Gallic acid equivalent, IC_{50} (Inhibitory concentration).

increase in total acid is followed by a faster decrease in pH. The cabbage fermented with the addition of *L. casei* reaches a pH value of ± 3 on the 5th day.

The addition of salt and sugar enhances the antioxidant activity, the greater the decrease in IC_{50} the higher the antioxidant activity value of sauerkraut. The antioxidant activity in sauerkraut is also influenced by the total acids, total phenols, and other bioactive compounds present in sauerkraut. For instance, the presence of sugar as a source of carbon for the metabolism of bacteria enhances the degradation of bioactive compounds in cabbage the total phenol has remarkably increased and resulted in increased antioxidant activity (Huang *et al*., 2005). As a result of the metabolism of microorganisms during fermentation there is an increase in phenol compounds due to biotransformation which produces phenolic compounds related to the associated group and the number of hydroxyl groups, for example, aromatic OH is a determinant of hydrogen donation and the capture of free radicals by phenol compounds (Dufresne and Farnworth, 2000). While the fermentation process is taking place a sugar reshuffle by bacteria resulted in the formation of organic acids which cause acidic conditions. Acidic conditions result in the formation of phenol compounds through hydroxy acid and ferulic acid. This degradation can increase phenol levels followed by an increase in antioxidant activity. The presence of a sugar overhaul by bacteria causes a high level of antioxidant activity because it is synergistic by giving H^+ ions to free radicals thereby increasing antioxidant activity primary (Peñas *et al*., 2010).

3.2 Sauerkraut as potential 'immunomodulator'

The immune response in this study was carried out on the T cell's adaptive immune response with cytokines $CD4^+$, $CD8^+$, IFN-γ^+, and TNF-α^+ cytokines. In addition, the immune response testing process was also carried out on innate immune responses through $CD68^+$ and IL-6^+ macrophages. Identification of immune responses in macrophages and T cells was confirmed using flowcytometry.

3.2.1 $CD4^+$ IFN-γ^+ cell population

The population of T lymphocytes on $CD4^+$ cells that express IFN-γ^+ cytokines. $CD4^+$ is a transmembrane protein that functions as a co-receptor on helper T cells when helper T cell receptors recognize the antigen complex. $CD4^+$ functions as a co-receptor that strengthens the transduction signal so that T cells are activated. IFN-γ^+ is produced by $CD4^+$ which will be activated by the stimulation of antigens. IFN-γ^+ can induce macrophages to increase the ability to kill bacteria and parasites. IFN-γ^+ enhances target recognition in the early phase of non-specific immunity through protein regulation on the surface of macrophage cells (Yuan *et al*., 2017). Results of the analysis of variance showed significant differences in values (α = 0.05) between the treatment of sauerkraut without culture and sauerkraut inoculated with culture. This has resulted in an increase in the expression of $CD4^+$ IFN-γ^+ as shown in Table 2. Increase in $CD4^+$ that expresses IFN-γ^+ due to the presence of bioactive compounds in sauerkraut. Bioactive compounds have effects as an antitumor, antioxidant, immunostimulant, anti-inflammatory, analgesic, anti-viral, anti-fungal, and anti-bacterial. Flavonoid compounds have also been shown to increase IL-2 and lymphocyte proliferation (Nicholson *et al*., 2012). Lymphocyte proliferation will affect $CD4^+$ cells which will cause T helper (Th1) cells to activate and IFN-γ^+ will activate macrophages, so macrophages will experience phagocytic activity. According to Sulistiani *et al*. (2015), IFN-γ^+ is the main cytokine MAC (Macrophage Activated Cytokine) which will activate macrophages and increase phagocytic activity.

3.2.2 $CD4^+$ TNF-α^+ cell population

Tumor necrosis factor alpha (TNF-α^+) is the main cytokine in the acute inflammatory response to other pathogenic bacteria and microbes. Severe infection can trigger the production of TNF-α^+ in large numbers that cause systemic reactions. The main sources of TNF-α^+ are mononuclear phagocytes and antigen-activated T cells, and NK cells. Lipopolysaccharide (LPS) is a stimulus to macrophages to secrete TNF-α^+

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Table 2. Sauerkraut as potential immunomodulator

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}	0.41 ± 0.14^{c}
Control positive (P1)	0.51 ± 0.11^{c}	0.78 ± 0.30^{b}	0.43 ± 0.17^{bc}	0.53 ± 1.01^{a}
Sauerkraut without culture (P2)	1.50 ± 0.27^{b}	1.17 ± 0.38^{ab}	0.64 ± 0.14^{b}	0.91 ± 0.17^{b}
Sauerkraut + *L. casei (P3)*	2.5 ± 0.67^{a}	2.01 ± 1.30^{a}	1.65 ± 0.20^{a}	2.35 ± 0.54^{c}

Values are presented as mean±standard deviation. Values with different superscript within the same column are significantly different ($p<0.05$). P0: Negative control, P1: Positive control, P2: Sauerkraut without culture, P3: Sauerkraut + Culture.

(Baratawidjaya, 2004). TNF-α^+ is one of the cytokines that are essential for reducing infection, from pathogenic bacteria, during infection, TNF-α^+ will induce the production of pro-inflammatory cytokines. TNF-α^+ can inhibit the replication of intracellular pathogenic bacteria and can directly kill infected cells (Rahman and McFadden, 2006). Results of the analysis of variance showed significant differences in values ($\alpha = 0.05$) between the treatment of sauerkraut without culture and sauerkraut inoculated with culture. The results of the increase in $CD4^+$ TNF-α^+ is shown in Table 2. Increased the average number of $CD4^+$ TNF-α^+ expression due to the presence of phenol compounds and increased antioxidant activity in sauerkraut. Bioactive compounds have a function as immunostimulants which provide intracellular stimuli such as macrophages and T cells so that they can work well in eliminating incoming infections (Yuan *et al*., 2017). In addition, increased expression of TNF-α^+ is also caused by the presence of lactic acid bacteria in fermentation products. Given lactic acid bacteria in mice can increase TLR2, TLR4, and TLR9 expression and increase the secretion of TNF-α^+, IFN-γ^+, and IL-10 in Peyer patche's (Castillo *et al*., 2011). According to the research of Djunaedi (2006), lactic acid bacteria in yoghurt have the ability to increase the activity of TNF-α^+ cytokines, IFN-γ^+, and IL-1β.

3.2.3 $CD8^+$ IFN-γ^+ cell population

$CD8^+$ is a transmembrane protein that functions as a receptor on killer T cells. When killer T cell receptors recognize the antigen complex, MHC I, $CD8^+$ acts as a co-receptor that amplifies the transduced signal that the killer T cell is activated. $CD8^+$ is expressed by T cells and cells that are markers for cytotoxic T cells (killer T cells) (Rifa'i, 2011). $CD8^+$ expresses the receptor and destroys infected cells between the specific antigens that MHC I produces. Results of the analysis of variance showed significant differences in values ($\alpha = 0.05$) between the treatment of sauerkraut without culture and sauerkraut by culture addition. The results increase in $CD8^+$ IFN-γ^+ as shown in Table 2. Increased production of IFN-γ^+ by $CD8^+$ T cells can inhibit infection due to *E. coli* bacteria. IFN-γ can help cytotoxic cells more efficiently perform their role. IFN-γ^+ can direct the differentiation of naive T cells to Th1 which will help T cells to be more sensitive to mitogens or growth factors so IFN-γ^+ is said to be a modulator for the development and differentiation of T cells (Rifa'i, 2011).

3.2.4 $CD8^+$ TNF-α^+ cell population

The increase in the mean number of $CD8^+$ TNF-α^+ expression is due to the presence of bioactive compounds present in sauerkraut. Results of the analysis of variance showed significant differences in values ($\alpha = 0.05$) between the treatment of sauerkraut without culture and sauerkraut inoculated with culture. The resulting increase in $CD8^+$ TNF-α^+ is shown in Table 2. The increase in the mean on $CD8^+$ TNF-α^+ is also caused due to LAB. Given LAB to mice can increase the secretion of TNF-α^+, IFN-γ^+, and IL-10 in Peyer's patches (Castillo *et al*., 2011). LAB contained in yoghurt can increase the activity of TNF-α^+ IFN-γ^+ and IL-β cytokines (Djunaedi, 2006). LAB performance takes place through the mechanism of anti-microbial material production, stimulation of immunity, inducing IgA production, and increasing the role of Th1 response (Parvez *et al*., 2006). According to research by Lee *et al*. (2015) states that fermentation of mulberry leaves by *L. plantarum* can increase total phenol, this is caused during the fermentation process the content of bioactive compounds contained in mulberry leaves is hydrolyzed and removed from tissue or leaf cells by enzymes such as glucosidase amylase and cellulase.

3.2.5 $CD68^+$ IL-6^+cells population

Sauerkraut has various bioactive compounds such as phenol and sulforaphane derived from glucosinolate which can function as antibacterial and increase the immune response. The mechanism of bioactive compounds that can inhibit the adhesion of bacterial cells, both the attachment of bacteria to the surface of the substrate and the attachment between bacteria. Lactic acid bacteria have the ability to inhibit inflammation and activate innate immune systems (innate) that can balance the response of Th1 and Th2 to fight the resistance to pathogenic bacterial infections. Increased ability of macrophage cells or known as activated macrophages is morphological, metabolic, and functional abilities in

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eliminating infectious agents in the body. Results of the analysis of variance showed significant differences in values (α = 0.05) between the treatment of sauerkraut without culture and sauerkraut inoculated with culture. Treatment results are in Figure 1. This shows that with the treatment of sauerkraut, the number of $CD68^+$ $IL\text{-}6^+$ percentages decreased. Decreasing the number of $CD68^+$ $IL\text{-}6^+$ is suspected given sauerkraut can stimulate the innate immune system and when infected with *E. coli*, macrophages can work against pathogens and phagocytosis and can normalize the infected immune system thereby the treatment is not significantly different from the treatment of *E. coli*. The research of Penas *et al.* (2012) reported that the content of bioactive compounds derived from glucosinolate can inhibit nitric oxide (NO) which is the cause of inflammation due to lipopolysaccharide (LPS) infection. The same study was also carried out by Peñas *et al.* (2012) stating that sauerkraut added with the chemical compound selenium can increase the bioactive compounds present in sauerkraut and can prevent the increase in NO.

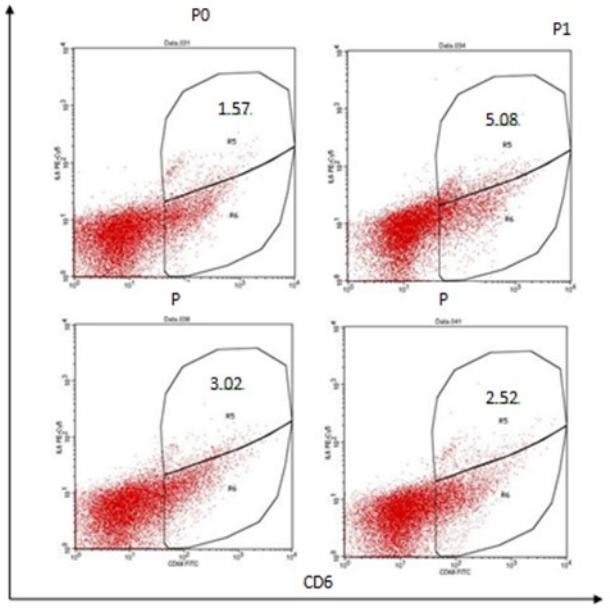

Figure 1. Macrophage Cell on $CD68^+$ $IL\text{-}6^+$. Values are presented as mean±standard deviation. P0: Negative control, P1: Positive control, P2: Sauerkraut without culture, P3: Sauerkraut + Culture.

3.3 Total changes in Escherichia coli in mice after sauerkraut administration for 19 days.

After the surgical process, *E. coli* is taken intraperitoneally. The results of observations showed that there was a decrease in total *E.coli* after the treatment process of giving sauerkraut. The total decrease in *E. coli* is shown in Table 3. This shows that the administration of sauerkraut can increase the immune response of mice and stabilize it in inhibiting the growth of *E. coli* and preventing infection. The content of bioactive compounds and LAB in sauerkraut can improve the performance of immune and antibacterial responses. 2-phenylethyl isothiocyanate is one of the bioactive substances present in cabbage with an antimicrobial ability (Hayes *et al.*, 2008; Aires *et al.*, 2009). Isothiocyanate hydrolyzed derivative products have the ability to inhibit the growth of pathogenic mycorrhoea activity. Fooks (2002) reported that the administration of probiotics can prevent the onset of colitis and can also reduce the production of bacterial toxins, in addition to the discovery that volatile fatty acids produced by LAB are able to control the colonization of *Shigella sonnei* and Entero Pathogenic *Escherichia coli* (EPEC).

Table 3. Decreased total *E. coli* bacteria

Group	Total *E. coli* (CFU/mL)
Negative control (P0)	-
Positive control (P1)	2.3×10^7
Sauerkraut without culture (P2)	3.3×10^3
Sauerkraut + culture (P3)	1.0×10^2

4. Conclusion

Inoculation with *Lactobacillus casei* culture can accelerate the fermentation process, and increase bioactive compounds in sauerkraut. Given sauerkraut without culture and addition of *Lactobacillus casei* culture can increase the average number of $CD4^+$ $IFN\text{-}\gamma^+$ T cells, $CD4^+$ $TNF\text{-}\alpha^+$, $CD8^+$ $IFN\text{-}\gamma^+$, $CD8^+$ $TNF\text{-}\alpha^+$ and decrease the average number of $CD68^+$ $IL\text{-}6^+$ T cells, $CD4^+$ $TNF\text{-}\alpha^+$, $CD8^+$ $IFN\text{-}\gamma^+$, $CD8^+$ $TNF\text{-}\alpha^+$ and decrease the average $CD68^+$ $IL\text{-}6^+$ T cells on macrophages infected with *E. coli*. The impact of the immunomodulatory property of inoculated sauerkraut was higher compared to control sauerkraut in *E. coli* infected mice.

Conflict of interest

The authors declare no conflict of interest

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