Evaluation of the Antimicrobial Activity of Probiotic *Lactobacillus casei* Cell-Free Supernatant Extract against *Escherichia coli* ATCC 25922

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ABSTRACT

The antimicrobial activity of the members of genus *Lactobacillus* has been investigated over the years due to their ability to regulate the gastrointestinal tract microflora. The present study was conducted to evaluate the antimicrobial activity of this commercially utilized *Lactobacillus* strain. Toward this end, the study aimed to evaluate the antimicrobial activity of *Lactobacillus casei* against *Escherichia coli* ATCC 25922. Specifically, the effects of the cell-free supernatant extract to the hydrophobicity, growth and viability of *E. coli* in culture broth after exposure to different concentrations of the extract were investigated to determine whether the extract is bactericidal or bacteriostatic. The hydrogen peroxide and protein content was also measured to characterize and explain the antimicrobial activity of the cell-free extract.

The cell-free supernatant extract was obtained from *L. casei* Shirota strain in MRS broth using saturated ammonium sulphate precipitation and dialysis using cold phosphate buffer solution to obtain heat-stable peptides and proteins. The extract was prepared into different concentrations and mixed to a culture broth to evaluate the antimicrobial activity against *E. coli* using turbidimetric method with subsequent inoculation to agar media to check for microorganism viability. Protein determination and H_2O_2 quantification were performed using spectrophotometric methods. The change in hydrophobicity was determined using Microbial Adhesion to Hydrocarbon Assay (MATH assay). One way Analysis of Variance with post hoc Tukey HSD test at α=0.01 was utilized for statistical analysis.

The cell-free supernatant extract produced by ~ 5.62 x 10^{11} CFU*mL^{-1}contained 60.51µg*mL^{-1} heat-stable proteins and 0.014 ± 0.002 µmol*mL^{-1} H_2O_2. Generally, the addition of the extract to the culture broth inhibited the growth of *E. coli* in a dose-dependent fashion due to lower optical density but did not exhibit bactericidal effects, since the microorganisms are still viable after inoculation to a culture medium. A concentration of 100% (v/v) of the extract showed the highest reduction in the hydrophobicity and bacteriostatic activity against *E. coli.* Thus, the cell-free supernatant extract of *L. casei* Shirota strain exhibits bacteriostatic, not bactericidal activity against *E. coli* ATCC 25922 *in vitro*. The extract also reduces the hydrophobicity of *E. coli* in a dose-dependent manner, possibly through non-specific cell surface interactions. Hydrogen peroxide is unlikely to be a cause of the antimicrobial property of the cell-free supernatant extract due to its very low concentration in the extract.

KEYWORDS: Bacteriostatic, Cell-free supernatant, *Escherichia coli* ATCC 25922, *Lactobacillus casei*

1. INTRODUCTION

The antimicrobial activity of *Lactobacillus casei* has been utilized extensively in the food industry during the past decades. *Lactobacilli* produce organic acids, diacetyl, acetoin, hydrogen peroxides and bacteriocins[1] making them suitable as probiotics against other microorganisms[2]. An earlier study recognized that *Lactobacilli* species help in maintaining the balance of microflora in human and animal gut[3], and possess anti-inflammatory properties[4].

The determination of antimicrobial activity of *Lactobacilli* in fermented foods and dairy products focused on evaluating the ability of the microorganisms to produce bacteriocin[5,6,7,8,9], which is usually found in the supernatant. Other studies reported that probiotic *Lactobacilli* cells in preventing urinary tract infection[10,11] and in lowering cholesterol levels [12]. *Lactobacillus casei*, a probiotic strain was also reported to exhibit moderate immunomodulation *in vivo*[13,14].

While some studies reported high antimicrobial properties of both cell-free supernatant of *L. plantarum*[15,16] *L. acidophilus*[17], *L. bulgaricus*[18] consistently, only a few number of studies investigated the antimicrobial properties of the cell-free supernatant of the probiotic *Lactobacillus casei in vitro*. *Lactobacillus casei* Shirota strain has been documented to exhibit antimicrobial properties[19] if co-incubated with other microorganisms.

In the Philippines, it has been reported that this *Lactobacillus* strain in exhibits broad spectrum antimicrobial properties, even to microorganisms with multiple drug resistance[20] when incubated together with other micro-organisms as well.

Conflicting data exists as to how *Lactobacillus casei* exhibits antimicrobial properties since reports do not agree whether the cell-free supernatants exhibit bacteriostatic or bactericidal effects. In one study, cell suspensions and cell-free supernatants of *L. casei* did not exhibit antibacterial properties against *H. pylori*[21] although another study reported an antibacterial activity against *E. coli*[19], although the antimicrobial effects are only seen if *L. casei* is co-incubated with *E. coli*. Generally, antimicrobial studies which utilize cell-free supernatant of *L. casei* are still few.

Hence, this study investigated the *in vitro* antimicrobial activity of *Lactobacillus casei* strain. The parameters enumerated in other studies by [13,22,23] were used to investigate the activity of the crude supernatant extract. The parameters include: production of hydrogen peroxide, reduction of hydrophobicity and reduction of bacterial density in bacterial suspensions. Hydrogen peroxide is responsible in causing oxidative stress to microorganisms while hydrophobicity promotes cell to cell adhesion of microorganisms.

The results of the study could be utilized to provide additional data on the possible mechanism of the antimicrobial properties of cell-free supernatant of *L. casei* in *in vitro* bacterial suspension models using a hydrophobic microorganism, *Escherichia coli* ATCC 25922.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

American Type Culture Collection 25922 *Escherichia coli* was obtained from the Department of Science and Technology-Cordillera Administrative Region (DOST-CAR) while *Lactobacillus casei* (Shirota strain) was obtained from Yakult, based from a previous method[20].

2.2 Preparation of Bacterial Suspensions

A volume of 10µL of Yakult was aseptically transferred to 200mL of sterile MRS broth (Sigma Aldrich, Germany) and incubated at 37°C for 24 hours [16]. The pH of the media was obtained before and after the incubation period. After incubation, the bacterial density was obtained. The bacterial cells in this stage are in the logarithmic phase. Subcultures were prepared by inoculating 200mL of sterile MRS with 10µL of the previous culture to maintain the log phase of the bacterial cells. For *E. coli* ATCC 25922, Luria Bertani (LB) Broth was used to obtain bacterial cells in the logarithmic phase. The pH of LB broth was adjusted to 7.1 to 7.3 using diluted 2.5N NaOH solution. The number of viable *E. coli* cells was estimated by determining the colony forming units (CFU) after spread plating in nutrient agar from 10^1 to 10^{10} serial dilutions in phosphate buffered (PBS), then incubated for 24 hours at 37 °C. For *L. casei*, MRS-agar was used to determine the number of colony forming units*mL^{-1} coupled with spread plating.

2.3 Collection and Purification of Crude Cell-free Supernatant Extract

The *Lactobacillus* strain was incubated in de Man Rogosa Sharpe (MRS) broth at 37°C for 24 hours based from a described method[16]. The bacterial suspensions were centrifuged at 2500g for 15 minutes with the pH of the supernatant adjusted to 6.5. The supernatant was filtered through a 0.22µm pore size nylon filter paper and heat-treated at 80 °C for 10 min to inactivate proteases present in the filtrates. In order to precipitate peptides and proteins, the supernatant was mixed with sterile 80%ammonium sulfate solution (w/v), stirred gently, and allowed to precipitate at 4°C for two days[2].

The salted out precipitate was centrifuged at 6000g for 15 minutes and redissolved in an equal volume of cold sterile phosphate buffered solution (pH 6.5). The precipitate was concentrated to 3 mL and loaded to dialysis tubes (Sigma Aldrich, Singapore). Dialysis was performed for 24 hours at 4°C using sterile PBS as the dialyzing gradient. Spectrophotometric quantification of protein was performed at 260nm and 280nm. Sterile PBS was used as the blank solution. The formula used was:

$$Protein\ Concentration\ (mg*mL^{-1}) = 1.55*A_{280} - 0.76*A_{260}$$

2.4 Measurement of H_2O_2 production using o-Dianisidine Assay

Briefly, a volume of 100µL of the MRS supernatant was mixed with 0.5 mL of horseradish peroxidase and 0.5 mL of *o*-dianisidine. The mixture was mixed in 2mL of PBS and incubated for 30 minutes at 37°C[24]. A volume of sterile MRS mixed with the reagents stated above to remove the interference caused by the colour of the culture broth. The absorbance was measured at 460 nm. The concentration of reduced *o*-dianisidine was obtained by using the molar coefficient of reduced *o*-dianisidine.

2.5 Microbial Adhesion to Hydrocarbon Assay (MATH Assay)

Hydrophobicity assay was based from an earlier study[25]. After incubating *E. coli* cells in Luria Bertani Broth supplemented with increasing concentrations of crude cell-free supernatant extracts, bacteria pellets were obtained from 4 mL of the bacterial suspensions, washed three times with cold sterile PBS then resuspended in 4mL of sterile phosphate buffer solution (pH = 7.1 to 7.3). The initial optical density was measured spectrophotometrically at 400nm using sterile PBS as blank. An equal volume of n-hexadecane was mixed to the bacterial suspension and vortexed for one minute, then kept still for thirty minutes to allow the polar and non-polar phases to separate. The hydrophilic phase was removed using a micropipette and transferred to a clean quartz cuvette. Absorbance was measured using sterile PBS as blank.

The optical density of *E. coli* suspended in PBS before and after the assay was compared. The change in hydrophobicity was expressed in % using the following formula:

$$\%Decrease\ in\ Hydrophobicity = \frac{(OD_a - OD_b)}{OD_b} * 100$$

2.6 Determination of Antimicrobial Activity of Crude Cell-Free Supernatant Extracts

The crude supernatant extract of *Lactobacillus casei* Shirota strain was diluted into 25%, 50% and 75% (v/v) using PBS (pH=7.1 to 7.3). In a sterile test tube, a volume of 2mL of *E. coli* ATCC 25922 suspended in Luria Bertani Broth (OD_{600} = 0.05, pH = 7.2±1) was mixed with 2mL of crude cell-free supernatant extracts at different concentrations. The culture broths were incubated for 24 hours at 37°C.

Three replicates were prepared per concentration. A negative control was also prepared by mixing 2mL of sterile PBS with 2mL of the bacterial suspension. After incubation, the bacterial suspensions were centrifuged at 2500g for 5 minutes. The bacterial pellets were washed with cold, sterile PBS then centrifuged to isolate bacterial pellets. The procedure was performed twice. Finally, the bacterial pellets were suspended in 4mL of PBS. The optical density of samples was measured against PBS at 400 nm. The procedure was done in triplicates per concentration.

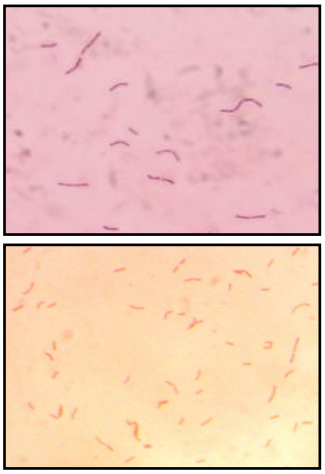
2.7 Statistical Test

Data on bacterial densities and mean % decrease in hydrophobicity and mean optical densities of the microorganism were presented using bar graphs. One way analysis of variance (ANOVA) with post hoc Tukey HSD test (α=0.01) was used to determine the significant differences in antimicrobial activity in terms of bacterial density differences and changes in hydrophobicity of *E. coli* ATCC 25922 in different concentrations *L. casei* crude bacteriocin extracts.

3. RESULTS AND DISCUSSIONS

3.1 Characteristics of Organisms

Lactobacillus casei appears rod-like and Gram positive while *Escherichia coli* ATCC 25922 appeared Gram-negative. Previously, the strain of *L. casei* in Yakult was identified as *Lactobacillus casei* Shirota 610 [20].



3.2 Protein Concentration of Crude Cell-Free Supernatant Extract

Using a spectrophotometric method, the protein concentration produced by *Lactobacillus casei* (OD_{600}=0.340, ~ 5.62 x 10^{11} CFU*mL^{-1}) relative to the aromatic amino acid content (tryptophan, tyrosine and phenylalanine was 60.51µg*mL^{-1}This concentration represents the salted out peptides and proteins produced by *Lactobacillus casei*. However, since the protein extract was heat-treated, the antimicrobial actions, if any, could be attributed to the heat-stable proteins only.

3.3 Hydrogen peroxide production of L. casei

Based from the result of *o*-dianisidine method, ~ 5.62 x 10^{11}CFU*mL^{-1} of *L. casei* produced 0.014 ± 0.002 µmol*mL^{-1}. The concentration of H_2O_2 was low relative to the bacterial density. The mean value reported in this study almost similar to the mean hydrogen peroxide production of *Lactobacillus casei* in another study[16] although a higher bacterial density was reported in this study in order to produce a similar amount of H_2O_2. In addition, this study concluded that MRS is a better culture broth to promote hydrogen peroxide production. Since the concentration of hydrogen peroxide was low, this was not considered to be relevant contributor to the antimicrobial activity of *L. casei*. Hence, oxidative stress may not be a mechanism of the antimicrobial property of cell- free supernatant extract of *L. casei*.

3.4 Effect of Crude Supernatant Extract to Growth of E. coli in LB Broth

In figure 2, the growth of *E. coli* in Luria Broth was significantly lower in LB broth supplemented with crude *L. casei* bacteriocin extract.

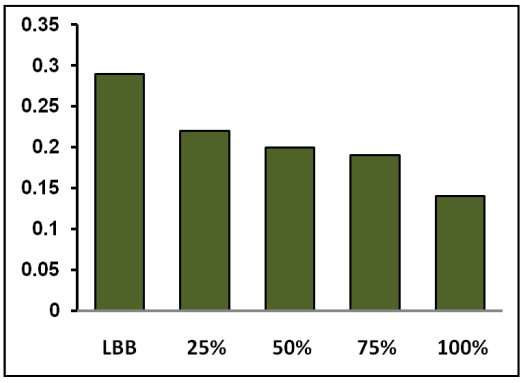


Figure 2. Mean Bacterial Density of *E. coli* treated with cell-free supernatant extract

A 100% crude extract significantly decreased the bacterial density by ~24.38%. Similarly, a 50% and 75% crude bacteriocin extract decreased the bacterial density by ~9.95% and ~8.79%, respectively.

Theoretically, the protein precipitated in the cell-free supernatant extract includes bacteriocins[26], although no confirmatory test was performed in this study. Since the collection of the supernatant involved heat treatment, all heat-sensitive proteases cannot be accounted for the result of the study.

The antimicrobial properties of lactobacilli bacteriocins are well documented. In one study, it was reported that proteins such as bacteriocins from *Lactobacillus casei* impair the swimming motility of *Helicobacter pylori* and *Salmonella enterica*[27,21]. Bacteriocins from LAB were previously reported to exhibit antibacterial properties against *Escherichia coli, Bacillus cereus, Pseudomonas fluorescens, Erwinia carotovora, and Leuconostoc mesenteroides subsp. Mesenteroides*. In addition *Lactobacillus casei* Shirota strain also exhibited antimicrobial properties against uropathogenic *E. coli*[10]. It was also previously reported that purified bacteriocins from *L. casei* did not inhibit *E. coli*[16].

Cell viability was assessed to determine the antibacterial property of the extract by inoculating the bacterial pellets to freshly prepared MRS. The results showed that the *E. coli* cells were still viable after exposure to crude supernatant extract.

The result of the study suggests that cell free supernatant extract from *L. casei* can inhibit the growth of *E. coli* in culture broths, but does mot exhibit bactericidal properties.

The antimicrobial property of *Lactobacillus casei* was generally related to their ability to produce lactic acid, hydrogen peroxide, short chain fatty acids and bacteriocins[19,27]. However, as discussed earlier, the low H_2O_2 obtained in the cell-free supernatant may not be accounted as a possible contributor to the antimicrobial activity of the cell-free supernatant extract.

3.5 Effect of Crude Cell-free Supernatant Extract to Hydrophobicity of E. coli ATCC 25922

Based from the result of the assay in Figure 3, there is a greater decrease in the hydrophobicity of *E. coli* treated with crude cell-free supernatant extract of

The significant difference lies between the mean hydrophobicity index of *E. coli* in LB broth supplemented with 100% crude bacteriocin extract*mL $^{-1}$ LB broth and the control group (non-treated). However, lower concentrations of the extract did not significantly decrease the growth of bacteria after 24 hours of incubation.

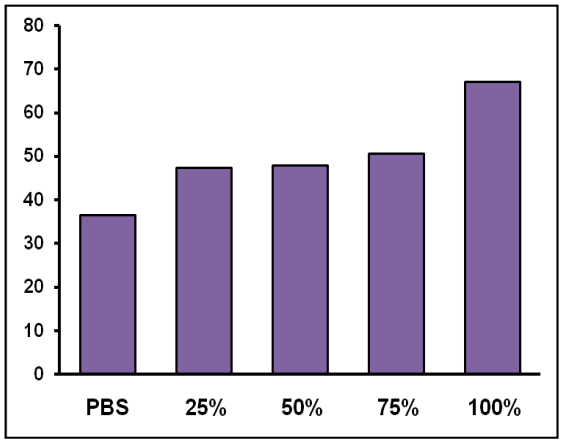


Figure 3. Mean % Hydrophobicity of *E. coli* treated with *L. casei* cell-free supernatant extract

The ability of the crude extract to reduce the hydrophobicity of *E. coli* has been explored though their application in preventing urinary tract infection [10]. Hydrophobicity is important in *E. coli* to establish infection. It has been suggested that *Lactobacillus* species competes with the adhesion of other microorganisms to cells[13] through a process known as competitive exclusion. The cell surface characteristics of L. casei have been extensively discussed elsewhere [28,29]

4. CONCLUSIONS

The results in the study suggest that the crude supernatant extract of *L. casei* has bacteriostatic, not bactericidal properties against *E. coli* ATCC 25922. There was low production of hydrogen peroxide from a high bacterial density, providing little or no contribution to the antimicrobial effects of *L. casei* in *in vitro* systems using culture broths. A pure cell-free supernatant extract significantly reduces hydrophobicity of *E. coli* ATCC 25922 and inhibits bacterial growth in culture broth compared to non-supplemented groups and lower concentrations. In addition, *E. coli* cells are still viable even after treatment with the cell-free supernatant exrreact. Still, it cannot be discounted that *L. casei* Shirota strain is a well-established probiotic microorganism *in vivo*.

5. RECOMMENDATIONS

Since the *in vitro* assays were performed in this study, it is suggested that *in vivo* experiments should be performed further to evaluate the bioactivity of *L. casei* cell suspensions or cell-free supernatants. In addition, hydrogen peroxide production was still detected albeit low levels. Controlling the experimental conditions such as effect of pH and presence of inhibitors should be further investigated in the cell-free supernatant extract.

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