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In Vitro Inhibition of *Lactobacillus Plantarum* 1 from Fermented Coconut Toddy against Human 5-Lipoxygenase

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Abstract

Background and objectives: An acute inflammation, when left untreated will result in the development of persistent chronic inflammation and may lead to tissue destruction and tissue injury. Consequently, medical practitioners rely on steroids to suppress inflammation. Yet, these widely used medications impose harmful effects on the individuals who rely on them. Thus, this study was formulated; to be able to find a new potential source of 5-lipoxygenase inhibitors from probiotics such as lactic acid bacteria.

Materials and Methods: The researchers isolated a pure colony of the organism from the coconut toddy with the use of de Man Ragosa Sharpe (MRS) agar. From this, a sub-culture was made in an MRS broth which was then subjected to gram-stain with a reaction of gram-positive and appears as non-spore forming cocci. The colony growth in the MRS broth, which was later identified as *Lactobacillus plantarum* 1, was centrifuged and was filtered to produce the Cell-Free Supernatant and Crude Bacteriocin. These were then used to evaluate the anti-inflammatory activity of the bacteria using Enzyme-Linked Immunosorbent Assay (ELISA).

Results & conclusion: The results clearly indicate that the isolated *Lactobacillus plantarum* 1 has the ability to inhibit the action of the anti-inflammatory enzyme, 5-lipoxygenase. The Cell-Free Supernatant (CFS) has a notable mean percent inhibition of 61% which is comparable to the positive control with 73% inhibition ($p>0.09$). Moreover, the biosurfactant activity of the *Lactobacillus plantarum* 1 showed a positive result. The biosurfactant activity and the *in vitro* inhibition of *Lactobacillus plantarum* 1 against 5-lipoxygenase may become a platform in pharmaceutical research and development of new anti-inflammatory agents from a natural, harmless, lactic acid bacteria source.

Keywords: Medical science; Coconut toddy; 5-lipoxygenase; Cell Free Supernatant; Crude Lactobacillus plantarum 1

Introduction

Humans have evolved highly complex immune systems that are composed of specialized immune cells that protect the host from infection and damage. But an uncontrolled immune response such as chronic inflammation leading to autoimmunity can itself inflict damage. In chronic and autoimmune diseases such as rheumatoid arthritis, lupus, ulcerative colitis, ankylosing spondylitis, osteoarthritis, and others, the immune system turns against the bodies' organs. These painful and, in some cases, progressively debilitating conditions can take a toll on people's quality of life and create both societal and economic burdens [1]. Chronic inflammation is a condition resultant to uncontrolled immune reaction of host immune system when there is tissue injury. Basically, the cells will release phospholipase A2 that will directly act to phospholipids breaking to two by products – fatty acids and arachidonic acid. Moreover, arachidonic acid as a potent substrate will be acted upon two enzyme pathway – Cyclooxygenase and 5-lipoxygenase pathway that produces different inflammatory mediators [2]. Remarkably, among all inflammatory mediator, leukotrienes play a significant role in the progression of acute to chronic inflammation since leukotrienes allows immune cells to migrate continuously to site of inflammation leading to chronic state [3].

In line with this, medical practitioners relied on steroids to suppress uncontrolled-immune responses. Although an effective measure, steroids come with common side effects such as weight gain and with potentially harmful side effects such as enlargement of the heart and liver and gastrointestinal cancer. Further, other medications such as 5-lipoxygenase inhibitors which are non-

steroidal anti-inflammatory drugs (NSAIDs) are available in the market; these drugs' mechanism attacks on the synthesis of potent pro-inflammatory Leukotrienes by the inhibition of 5-lipoxygenase [4]. However, these medications could have underlying adverse effects on the individuals taking them. The effect may result in inflammation or reaction which can cause a more chronic debilitating disease that is hard to treat. Today, as science has advanced, a new potential source of 5-lipoxygenase inhibitors from probiotics have been developed such as lactic acid bacteria that are transforming the management of inflammatory diseases.

Interestingly, coconut toddy is a drink common in the Philippines which is usually called "tuba". This fermented product is proven to be a good source of Lactic Acid Bacteria. Moreover, lactic acid bacteria have various potential biologic functions. Several studies have provided evidence about lactic acid bacteria being used as an antioxidant against various chemicals. According to [5], lactic acid bacteria possess antioxidant activity via different mechanisms such as radical scavenging, metal iron chelation, and inhibition of lipid peroxidation. Moreover, Lactic acid bacteria (LAB) has been identified to produce soluble factors such as peptides and proteins that could possibly regulate inflammatory mediators and provide biosurfactant properties that provide antibacterial activity [6].

Though many pharmacological benefits of lactic acid bacteria have been postulated such as maintaining the intestinal microbial system and improving immune function, still, little work has been done to explore the anti-inflammatory effects specifically Human lipoxygenase of lactic acid bacteria to date. Hence, this study was conducted. This study aimed to determine the *in vitro* inhibition activity of isolated lactic acid bacteria on commercially available coconut toddy against Human lipoxygenase and to screen its biosurfactant activity. The findings of this study may help pharmaceutical industries to provide a solution for the increasing mortality rates of autoimmune and chronic diseases by providing them ideas as their bases to generate new and more effective drug to regulate the uncontrolled immune response.

Materials and Methods

Sample Collection

The samples were taken only from the retailer in Bankerohan. The coconut toddy that was used is a Bahal (60 days old), a type of coconut toddy wherein the fermentation process was 2-3 months [7]. To ensure the days of fermentation, the researchers procured the study material from the retailer in Bankerohan who sourced his coconut toddy from a producer in Santo Tomas, Davao Del Norte.

Lactic Acid Bacterial strains

Lactic acid bacteria strains were collected from coconut toddy (tuba) fermented food product. For the actual experiment, all bacteria were anaerobically grown (using Anaerogram) in de Man Ragosa and Sharpe (MRS) broth to stationary phase at 37ºC for 72 hours, pelleted by centrifugation and re-suspended in 0.1M PBS buffer (pH 7.4). Briefly, 10 ml of the fermented coconut toddy was added to 90 ml of sterile saline solution (0.85% NSS), with a serial dilution of 10-1 to 10-6. From each dilution, duplicate 0.1 ml samples were inoculated in de Man Ragosa and Sharpe (MRS) agar and incubated anaerobically at 37ºC for 72 hours [8]. Colonies were subjected for identification by Analytical Profile Index (API) 50 CH.

Cell-free supernatant Lactic Acid Bacterial strains

Cell-free supernatant was prepared based on methods by Schillinger and colleagues. The culture extract of the lactic acid bacteria was obtained from 18 hours culture grown in MRS broth. The cultures were then centrifuged at 6000 rpm for 10 minutes. The supernatant was adjusted to pH 6.5 using 10N NaOH and was filtered through 0.22 µm Millipore. The resulting supernatant was used immediately for experimentation.

Determining the sources of inhibitory activity

Lactic acid bacteria are able to produce several potential anti-inflammatory compounds, and this includes organic acids. Therefore, it was important to eliminate the inhibition by these compounds in order to ensure that the inhibition is caused by the bacteriocin only. Inhibitory activity by acids can be reduced by using MRS-0.2% glucose. Furthermore, preparation of cell-free supernatant at pH 6.5 can eliminate the effect of acids produced by lactic acid bacteria.

Lactic Acid Bacteria anti-inflammatory activity against Human 5-Lipoxygenase

The 5-lipoxygenase inhibitory activity was used as an indicator of the anti-inflammatory activity. The assay was done using arachidonic acid as substrate and 5-lipoxygenase as an enzyme. A total volume of 200 µL assay mixture containing 160 µL sodium phosphate buffer (100 mM, pH 8.0), 10 µL neutralized cell-free lactic acid bacteria supernatant and 20 µL of lipoxygenase enzyme was used. The contents were incubated at 25 °C for 10 min. The reaction was then initiated by the addition of 10 µL linoleic acid solution. The change in absorbance was observed after 6 min at 234 nm. All reactions were performed in triplicates in 96-well microplates (Positive control, Negative control, Test samples). Aceclofenac was used as a positive control drug.

The percentage inhibition was calculated by using this formula:

$$\text{Inhibition (\%)} = [\text{AControl} - \text{ATest}/\text{AControl}] \times 100$$

Biosurfactant Activity

The drop collapse test was carried out as described by [9]. Lactic Acid Bacteria (LAB) was cultivated in MRS at 37°C for 24 h,

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centrifuged at 12,000 × g for 5 min and 100 µl of the supernatants were added to each well of 96-well microplates and then 5 µl of crude motor oil was added to the surface. A result was considered positive for biosurfactant production when the drop diameter was at least 1 mm larger than that produced by deionized water (negative control). All tests were done in triplicate.

Data Analysis

The researchers utilized T-test to determine the significant difference of anti-inflammatory activity against Human 5-Lipoxygenase between the known anti-inflammatory drug and Cell-free supernatant Lactic Acid Bacterial strains from coconut today.

Results

Isolation of Lactic Acid Bacteria

The isolated lactic acid bacteria from the coconut toddy were identified using Analytical Profile Index (API) 50 CH. Data in Table 1 shows the colony characterization of the lactic acid bacteria. There were pinpoint, milky white colonies that were isolated. Gram staining results showed gram-positive bacilli which are then subjected for Analytical Profile Index identification. Lactic Acid Bacteria anti-inflammatory activity against Human 5-lipoxygenase.

Table 1: Lactic acid bacteria characteristics and identification.

Code	LAB characteristics	API CH50 Identification
LAB001	Pinpoint, translucent, milky white colonies. The microscopic result showed gram-positive bacilli, non-spore forming.	Lactobacillus plantarum 1

The results presented in figure 1 show the mean percent inhibition of the *Lactobacillus plantarum* 1 and the positive control – Aceclofenac. The results clearly indicate that the isolated *Lactobacillus plantarum* 1 has the ability to inhibit the action of the inflammatory enzyme, 5-lipoxygenase. The Cell-Free Supernatant (CFS) has a notable mean percent inhibition of 61% which is comparable to the positive control with 73% inhibition ($p>0.05$).

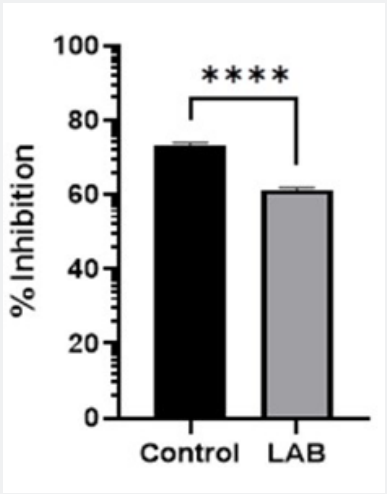

Figure 1: Mean Percent Inhibition of *Lactobacillus plantarum* 1 (LAB) and Aceclofenac (Positive control) against Human 5-lipoxygenase activity.

Biosurfactant Activity

Drop collapse test is used to screen the initial identification of biosurfactant-producing lactic acid bacteria. Based on the results, the isolated lactic acid bacteria – *Lactobacillus plantarum* 1 showed a complete spreading in the drop collapse test and it showed that it has the ability to produce biosurfactants.

Discussion

The isolated lactic acid bacteria from the coconut toddy were identified using Analytical Profile Index (API) 50 CH. The culture characteristics were pinpoint, milky white colonies that were consitent to typical lactic acid bacteria colonies. Biochemical profile of the isolated lactic acid bacteria showed consistency with that of the *Lactobacillus plantarum* 1. *Lactobacillus plantarum* 1 was also observed in the study of Ali (2011) wherein they isolated *Lactobacillus plantarum* 1 in a fermented product such as drinking yogurt. This confirms that the identified organism using API system is not an environmental contamination but a pure isolate from the coconut toddy.

Lactobacillus plantarum 1 human 5-lipoxygenase inhibition activity was evaluated using ELISA method; the results clearly indicate that the isolated *Lactobacillus plantarum* 1 has the ability to inhibit the action of the inflammatory enzyme, 5-lipoxygenase. The Cell-Free Supernatant (CFS) has a notable mean percent

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inhibition of 61% which is comparable to the positive control with 73% inhibition (p>.05). This can be supported with the previous studies where they suggested that the *Lactobacillus plantarum* 1 was effective in the prevention of inflammatory reactions by intermediating the key role biological functions of inflammation such as pro-inflammatory cytokines and enzymes such as 5-lipoxygenase and cyclooxygenase pathways (Versalovic et. al., 2009 and Harazallah et. al., 2013). This may serve as a good foundation for pharmaceutical industries to elucidate the active components of the Cell-Free Supernatant (CFS) of the isolated *Lactobacillus plantarum* 1 to provide a new theraputic agent for acute and chronic inflammation. Moreover, the result also showed that *Lactobacillus plantarum* 1 have a biosurfactant activity. This can be supported with the study of [10]. where lactic acid bacteria are known to produce biosurfactants to protect the environment from the harmful effect of chemically synthesized surfactants. Production of biosurfactants indicates the capacity of the organism to increase surface area thereby increasing the rate of bacterial penetration. The biosurfactant activity of *Lactobacillus plantarum* 1 can be used as a good candidate for biofilm-forming activity as it possess biosurfactant activity [11-14].

Conclusion

The results showed a significant inhibition action of *Lactobacillus plantarum* 1 which is comparable to Aceclofenac, a known anti-inflammatory agent. These preliminary results may become a platform in pharmaceutical research and development of new anti-inflammatory agents from a natural, harmless, lactic acid bacteria source. Moreover, the biosurfactant activity of the isolated *Lactobacillus plantarum* 1 indicates the capacity of the organism to increase surface area thereby increasing the rate of bacterial penetration.

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Authors Contribution

Conceptualization, Alfredo Jr., Hinay. And April Joy Parilla.; methodology, Alfredo Jr., Hinay, April Joy Parilla, Jive Java; software, Alfredo Jr., Hinay; validation, Alfredo Jr., Hinay and April Joy Parilla.; formal analysis, Alfredo Jr., Hinay.; investigation, Alfredo Jr., Hinay, April Joy Parilla, Jive Java; resources, Alfredo Jr., Hinay; data curation, Alfredo Jr., Hinay; writing—original draft preparation, Alfredo Jr. Hinay and April Joy Parilla; writing—review and editing, Alfredo Jr., Hinay.; visualization, Alfredo Jr., Hinay; supervision, Alfredo Jr., Hinay; project administration, Alfredo Jr., Hinay; funding acquisition, Alfredo Jr. Hinay.

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