I. INTRODUCTION

1.1 Background

Foodborne diseases have been a common issue among both consumers and the food industry. This is mainly due to contamination of foodborne pathogens including *Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, and Bacillus cereus.* Moreover, fresh foods such as meat and vegetables have very short shelf life. In order to prevent microbial contamination as well as lengthen food shelf life, chemical preservatives have been used. However, it soon became apparent that the chemical preservatives are causing health problems including, neurological, heart, or liver difficulties (Teshome at al., 2022) as well as headache, asthma, and allergies (Bondi et al., 2017). It has been reported that nitrates and nitrites, the most commonly used chemical preservatives, when ingested will react with other compounds and become carcinogenic (Trasande et al., 2018; Karwowska & Kononiuk, 2020).

As a result, consumers are demanding a natural approach that will allow them to safely extend the shelf life of food. Natural food preservatives are safe to use as they are commonly derived from animal, plant, or microbial origins. Among many categories of natural food preservative is the antimicrobial agent which focuses on controlling the growth of pathogens present in food products. Recently, lactic acid bacteria (LAB) have been investigated as a potential natural food preservative (Shi & Maktabdar, 2022; Zapasnik et al., 2022). LAB are a group of gram-positive bacteria known for their ability to produce bacteriocin and are commonly isolated from fermented foods.

Bacteriocins are ribosomally synthesized peptides produced by bacteria which is able to inhibit bacterial growth. Bacteriocins produced by Gram-positive LABs is especially known to have antimicrobial activity and has been used in the food industry (Juturu & Wu, 2018). It is also known to be both heat and pH tolerance as well as very effective towards foodborne pathogens including *L. monocytogenes, S. aureus, E. coli, Salmonella typhi, Pseudomonas aeruginosa*, and *P. fluorescens* (Darbandi et al., 2022). These characteristics made bacteriocin a suitable natural food preservative.

Among many types of bacteriocin, pediocin produced by *Pediococcus acidilactici* has been gaining interest. According to Khorshidian et al., (2021), pediocin produced by *P. acidilactici* shows a wide range of antimicrobial activity against gram-positive bacteria, particularly *L. monocytogenes*, by forming pores in its cytoplasmic membrane. Pediocin is also known to not only be heat stable, but is also stable in wide pH range. Papagianni & Anastasiadou, (2009), stated that pediocin is stable at 121°C for 15 minutes as well as pH 2-4. These characteristics of pediocin has made it very suitable as a natural food preservative.

In addition to pediocin, the antimicrobial activity of *P. acidilactici* can be attributed to other metabolites it produces, mainly organic acid. Organic acid is known to decrease the pH of its surrounding, causing osmotic stress in the pathogen bacteria cells which eventually results in death (Kovanda et al., 2019). A study by Kumar et al., (2020), stated that organic acid produced by *P. acidilactici* is found to exhibit inhibitory activity against *E. coli*. Another study by Castellano et al., (2018), stated that organic acid exhibits inhibitory activity as well as increases the antimicrobial activity of bacteriocin against *L. monocytogenes*.

Metabolites responsible for antimicrobial activity can be found in the cell-free supernatant (CFS) of *P. acidilactici* (Mani- López et al., 2022). According to Wang et al., (2015), pediocin in *P. acidilactici* CFS shows high antimicrobial activity against *L. monocytogenes*. Another study by Hufalar et al., (2022), stated that the CFS of *P. acidilactici* shows high antagonistic activity against S. aureus. Khalkhali et al., (2017), also reported a similar finding of *P. acidilactici* CFS showing antimicrobial activity against various foodborne pathogens including *L. monocytogenes, S. aureus, E. coli,* and *S. typhi*. CFS is also found to be more effective in inhibiting pathogenic growth compare to whole cells (Bussaman et al., 2012). Moreover, using CFS as a natural food preservative eliminates the risks that comes with utilizing whole cells, including infection and allergies (Witkowski et al., 2022).

The antimicrobial activity of *P. acidilactici* CFS, however, is affected by the incubation period of the bacteria as the production of metabolites will vary with time. Danial et al., (2016), reported that at 37°C, *P. acidilactici* produces the highest amount of bacteriocin at approximately 14-16 hours of incubation. On the other hand, Mani- López et al., (2022) stated that the optimum incubation period for *P. acidilactici* to produce the highest amount of organic acid is 8 hours. The study also mentioned that the antimicrobial activity of CFS might be attributed to more than one metabolite and maximum amount of metabolites does not necessarily results in the highest antimicrobial activity. Another paper by Chen et al., (2022), stated that antimicrobial activity is normally associated to both organic acid and bacteriocin. Due to this, is it still unclear on what incubation period will result in CFS exhibiting the highest antimicrobial activity. This information is crucial in determining the most appropriate harvest time of *P. acidilactici* and thus, further research needs to be conducted.

As the concentration of metabolites varies with time, antimicrobial characterization on *P. acidilactici* CFS can be conducted in order to determine which metabolite is responsible for the antimicrobial activity. pH sensitivity test as well as heat and enzyme sensitivity test is commonly performed to determine if the antimicrobial activity is due to acidic and bacteriocin like compounds respectively (Kaya & Simsek, 2020). In these tests, acidic like compounds will be deactivated by adjusting the pH of the CFS and bacteriocin like compounds will be deactivated by adding proteinase

K to the CFS. Therefore, this study aims to determine the appropriate harvest time of *P. acidilactici* as well as identifying the metabolites responsible for the antimicrobial activity.

1.2 Research Question

Based on the background, several research questions were formulated:

1. What is the appropriate harvest time of *Pediococcus acidilactici* to produce cell-free supernatant that exhibits the highest antimicrobial activity against the pathogens tested?
2. Which metabolite(s) are responsible for the antimicrobial activity of *Pediococcus acidilactici* cell-free supernatant?

1.3 Hypothesis

These are the hypothesis related to the research questions:

1. H0: *Pediococcus acidilactici* cell-free supernatant did not exhibit any antimicrobial activity against the pathogens tested.

   H1: The appropriate harvest time of *Pediococcus acidilactici* to produce cell-free supernatant that exhibits the highest antimicrobial activity against the pathogens tested is during the beginning of the stationary phase.
2. H0: No metabolites in *Pediococcus acidilactici* cell-free supernatant are responsible for the antimicrobial activity.

   H1: Protein and organic acids are responsible for the antimicrobial activity exhibited by *Pediococcus acidilactici* cell-free supernatant.

1.4 Research Aim and Scope

The aim of this research is to determine the optimum incubation period of *Pediococcus acidilactici* that produces cell-free supernatant exhibiting the highest antimicrobial activity, hence determining the appropriate harvest time. This research also aims to identify the metabolites which are responsible for the antimicrobial activity of *Pediococcus acidilactic*i cell-free supernatant.

The scope of this study will focus on *Pediococcus acidilactici* as the chosen LAB. The antimicrobial activity and characterization will only be determined through the cell-free supernatant of *P. acidilactici.* Based on these, the scope of this study includes:

1. Cell-free supernatant tested will be obtained by culturing *Pediococcus acidilactici* in De Man, Rogosa, and Sharpe (MRS) broth in batch culture.

2. Antimicrobial activity testing using agar-well diffusion and time-kill assay of *Pediococcus acidilactici* cell-free supernatant against *Listeria monocytogenes* and *Staphylococcus aureus*.
3. Antimicrobial characterization of bacteriocin-like compounds and organic acids in *Pediococcus acidilactici* cell-free supernatant in order to identify the metabolites responsible for the antimicrobial activity.