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Research article

Protein Produced by *Lactobacillus plantarum* ATCC 8014 during Stress

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Abstract

Previous studies have established that subinhibitory concentrations of antibiotics or antimicrobials are potent modulators of transcription process in bacterial cells. Hence, the bacteria might be introduced new proteins in mild stress environments like in the presence of antimicrobial agents at low concentrations. Although, there are still limited studies on the potential of antimicrobials at low doses play as a signaling agent that capable to modulate biological functions in bacteria. Therefore, this study aims to explore proteins production by *Lactobacillus plantarum* ATCC 8014 during stress which is in the presence of ethyl pentanoate at sub-minimal inhibitory concentration (sub-MIC). The Minimum Inhibition Concentration (MIC) of ethyl pentanoate against *L. plantarum* is 14.29% and was performed by microdilution assay. *L. plantarum* cells were treated with ethyl pentanoate at sub-MIC (0.05 x MIC) in the fermentation process. Two new protein bands (approximate size of 46.51 kD and 6.91 kD) were detected for the treated bacteria showed by SDS-PAGE profile. Of the two bands, eight possible proteins were identified by LC-MS/MS analysis. Thus, *L. plantarum* ATCC 8014 capable to produce new proteins in mild stress condition with the presence of 0.05 x MIC ethyl pentanoate. Futhermore, the isolated microbial proteins exhibit antimicrobial activity against several Gram-positive and Gram-negative bacteria.

Keywords: antimicrobial, transcription, mild stress, sub-MIC, proteins, *Lactobacillus plantarum* ATCC 8014, ethyl pentanoate

Introduction

Lactobacillus plantarum, a facultative heterofermentative lactic acid bacterium (LAB), is one of the most widespread in the environment. A natural inhabitant of the human gastrointestinal tract and it is also encountered in a variety of food and feed fermentations for which stress conditions such as heat, cold, and acidity are common [1-2]. The survival of *L. plantarum* in this stressful environment indicates that it has to respond to numerous changing conditions, and the cellular processes involved in these responses are frequently regulated at the transcriptional level [3]. Therefore, *L. plantarum* have developed several strategies including the production of proteins with antimicrobial properties such as bacteriocins. Bacteriocins have extensive potential for food preservation due to an increasing demand for natural and microbiologically safe food products, as well as for human therapy as potential supplements or replacements for currently used antibiotics [4].

Antimicrobials are the broad classes of substances acting against microorganisms and considered as one of the stresses that bacteria need to deal with. The antimicrobial agents or antibiotics have unexpected ability to modulate global transcription processes in target cells. This activity is detected at much lower concentrations than that required for inhibitory activity. The characteristic of possessing contrasting effects at low and high concentrations has been referred as hormesis [5]. Ethyl pentanoate is one type of flavour ester and antimicrobial agent that could act as a weapon at high concentration and perform as a signaling compound when used in low doses. The biological activity of ester was determined by their structure-function relation for saturated and unsaturated compound. Ethyl pentanoate exhibited antimicrobial activity towards several strains of bacteria including *Bacillus subtilis*, *Escherichia coli*, *Salmonella thyphimurium* and *Staphylococcus aureus* [6].

Apart from studies conducted on the bacteriocin production by *L. plantarum*, little is known about the production of proteins by *L. plantarum* cells when they encounter mild stress environment. Currently, there is still limited study on the effect of antimicrobial agent as signaling molecule at sub-inhibitory concentration showing ability to trigger transcription changes by modulating specific proteins in bacteria. Hence, this present study aims to explore the production of proteins by *L. plantarum* ATCC 8014 under mild stress condition which is in the presence of 0.05 x MIC ethyl pentanoate.

Materials and Method

Ethyl Pentanoate, Bacterial Strains and Culture Conditions

Commercial ethyl pentanoate was purchased from MERCK-Schuchardt. A serial dilution of standard ethyl pentanoate in de Man, Rogosa, Sharpe Broth (MRSB, Oxoid, USA) was performed in order to obtain a series of concentration ranging from 1% to 100%. *Lactobacillus plantarum* ATCC 8014 was obtained from American Type Culture Collection (ATCC) and cultured for an overnight in MRSB at 37°C with gentle agitation. Test microorganisms used for antimicrobial screening were grown in Mueller-Hinton Broth (MHB; Oxoid, USA) include:

(a) Gram-positive bacteria: *Bacillus cereus* ATCC 13061, *Bacillus subtilis* ATCC 11774 and *Enterobacter faecalis* ATCC 29212.

(b) Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 13883 and *Salmonella thyphimurium* ATCC 13331.

Minimum Inhibition Concentration (MIC) Determination

The MIC of ethyl pentanoate against *L. plantarum* ATCC 8014 was determined by Microdilution assay [7] with slightly modification by Hanina *et al*. (2002) [8]. Serial dilutions of ethyl pentanoate was added to 10^7 cells/ml *L. plantarum* ATCC 8014 culture in microtire plate. The plate was incubated at 37°C for 24 h.The bacterial viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., USA) solution. The MIC value was described as the lowest concentration of ethyl pentanoate could inhibit the growth of bacteria.

Microbial Proteins Production

A colony of *L. plantarum* ATCC 8014 was cultivated in 10 ml of MRSB and harvested at 37 ºC with gentle agitation. Ethyl pentanoate at sub-MIC (0.5 x MIC) was added to the bacteria culture during log (7 h of incubation) phase of bacterial growth. The culture not treated with ethyl pentanoate served as a control.

Microbial Proteins Extraction

Microbial proteins were extracted based on method by Lash *et al*. (2005) [9]. An overnight bacteria culture was harvested and centrifuged at 7000 x *g* for 6 min at 4°C.The supernatant was separated from pellet and followed by filter sterilization process by using 0.2 µm syringe filter (Millex®-GV, Millipore, Bedford, Massachusetts, USA) due to produce sterile cell-free supernatant. Next, the supernatants were concentrated with 60% (w/v) of ammonium sulphate (Sigma, St. Louis, Missouri, USA) and left for 1 h at 4°C in order to precipitate the proteins. The precipitated proteins were pelleted by centrifugation at 15,000 x *g* for 20 min at 4°C and suspended into 200 µl phosphate-buffered saline (PBS, pH 6.8, Cambrex Bioscience, Verviers, Belgium) before centrifuged for another 10 min at 15,000 x *g* at 4°C. The pellets were dissolved in deionised distilled and mixed with Laemmli sample buffer (Biorad, Singapore) and β-mercaptoethanol (Biorad, Singapore) in 1:1 ratio. The diluted protein samples were then heated at 90-95°C for 5 min and ready for SDS-PAGE analysis.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Microbial Proteins Identification

Proteins were analyzed by electrophoresis on Any kD™ Mini-PROTEAN® TGX™ Precast Gel in Protean III electrophoresis system (Bio-Rad, Hercules, CA) with Precision Plus Protein™ Dual Xtra Standards (Biorad, USA). The protein bands were visualized by using Biosafe Coomassie blue staining (Bio-Rad, USA) and the bands of interest were identified by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) based peptide sequencing. Spectra were analysed to identify proteins of interest using Mascot sequence matching software (Matrix Science) with SwissProt database and taxonomy set to Bacteria.

Antimicrobial Activity Screening

Screening of antimicrobial activity *via* agar well diffusion test was done according to the method by Milette *et al*. (2007) [10]. Each bacterial culture at concentration of 10^7 cell/ml was spread onto Mueller-Hinton Agar (MHA; Oxoid, USA) plates. Wells with 6 mm diameter were made on agar by using sterile cork borer. 50 µl of each isolated protein and control samples was loaded into wells separately. The plates were incubated at 37 °C and the diameter of inhibition zones were measured after 24 h of incubation.

Results and Discussion

L. plantarum ATCC 8014 cells were cultured in fermentation medium with the presence of ethyl pentanoate at concentrations ranging from 1% to 100%. The effects of different concentration of antimicrobial on bacteria cell growth were evaluated in order to determine the MIC value. MIC is defined as the lowest concentration of antimicrobial that could inhibit the growth of microorganism after an overnight incubation. Therefore, MIC is considered the "gold standard" for determining the susceptibility of *L. plantarum* ATCC 8014 towards ethyl pentanoate. The bacteria viability was detected by using MTT solution as an indicator. As shown in Figure 1, the blue formazan was formed due to the bacterial growth. Meanwhile, the remaining yellow MTT colour indicates no bacterial growth. The results showed that the MIC value of ethyl pentanoate against *L. plantarum* ATCC 8014 was 14.29%. As indicated previously, this study aims to investigate proteins production by *L. plantarum* ATCC 8014 during mild stress environment which referred to the presence of ethyl pentanoate at sub-MIC value (0.05 x MIC). Sub-MIC of antimicrobial agents is the concentration below the MIC and it is subject of interest since sub-MIC has numerous effects on bacterial cells. It is supported by the previous study stated that the antimicrobials at sub-MIC can act as signaling molecule or inducer in the bacteria metabolite

process by modulating their transcriptional machinery process [11]. This phenomenon presents some adaptation capacity of *L. plantarum* in order to survive under multiple stress conditions in the ecosystems.

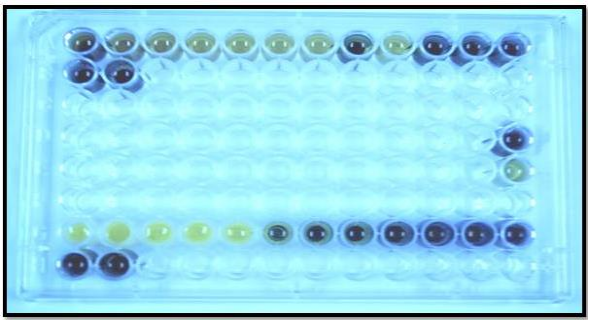

Figure 1: Determination of MIC value by microdilution assay.

In this study, attempt was made to explore the effects of introducing 0.05 x MIC ethyl pentanoate during log phase of bacterial growth. As shown in Figure 2, two new proteins with approximate sizes of 46.51 kD and 6.91 kD were produced when *L. plantarum* ATCC 8014 was treated with ethyl pentanoate. Microbial secondary metabolites, including microbial proteins are usually not produced during the phase of rapid growth (log phase), but are synthesized during a subsequent production stage (stationary phase), which is when primary nutrient source is depleted [12]. A culture of *L. plantarum* cells that has exhausted one or more essential nutrients and experienced fluctuations in the surrounding will enter the stationary phase of growth. At this stage, regulons that function in the production of extracellular proteins and enzymes are induced. Hence, it demonstrated that *L. plantarum* ATCC 8014 cells were induced to introduce new proteins during stationary phase as an adaptation strategy in the sub-minimal stress condition caused by ethyl pentanoate.

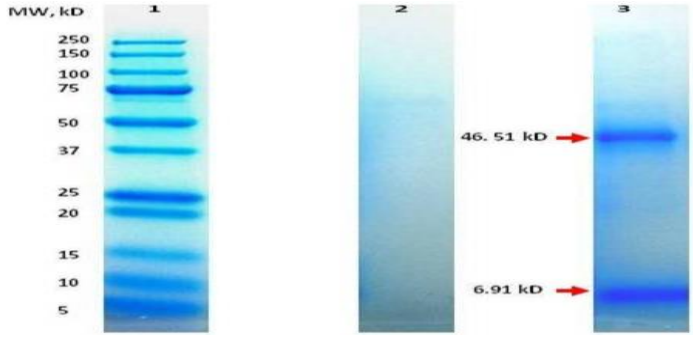

Figure 2: SDS-PAGE analysis: Lane (1) protein ladder (5-250 kD); Lane (2) in the absence of ethyl pentanoate (control); Lane (3) in the presence of ethyl pentanoate

There are three classes of bacteriocins produced by LAB based on their biochemical and genetic properties. Class I peptides are lantibiotics, which are less than 4 kD, post-translationally modified peptides that contain unusual amino acids such as lanthionine; Class II includes unmodified bacteriocins and are less than 10 kD; and Class III peptides are thermo-sensitive proteins and are larger than 30 kD [13]. Therefore, the two new proteins produced by *L. plantarum* ATCC 8014 might be categorized under Class II (6.91 kD) and Class III (46.51 kD) bacteriocins. In LAB, like in other Gram positive bacteria, secreted proteins are synthesized as a precursor containing an N-terminal extension called the signal peptide (SP) and the mature moiety of the protein. Precursors are recognized by the host secretion machinery and translocated across the cytoplasmic membrane (early step in protein secretion). The SP is then cleaved and degraded, and the mature protein is released in the culture supernatant (late steps in protein secretion) [14].

The identification of isolated and purified proteins was performed by high throughput analysis liquid chromatography-tandem mass spectrometry (LC-MS/MS). A configuration of peptide sequences to SwissProt database showed that different kind of proteins with various functional classes was produced by *L. plantarum* ATCC 8014 as shown in Table 1. The result indicated that six possible proteins with different functions were identified for the first protein sample (LP 1, 46.51 kD) and two different proteins presented by the second protein sample (LP 2, 6.91 kD). It seems that the identified proteins were include enzymes that are related to carbohydrate metabolism, fatty acid metabolism, energy metabolism, cofactors metabolism and translation process as well as proteins involved in signal transduction process. Metabolism alterations are examples of cellular responses to extracellular stimulation.

Table 1: Proteins identification by LC-MS/MS

Sample	Database Used	Proteins Significant Hits	Functional Classification
LP 1	SwissProt	D, D-heptose 1,7-bisphosphate phosphatase	Carbohydrate metabolism
		30S ribosomal protein S14 type Z	Translation
		Phospholipase A1	Fatty acid metabolism
		Phosphoglycerate kinase	Energy metabolism
		UPF0755 protein YrrL	Unknown function
		Phosphopantetheine adenylyltransferase	Cofactors metabolism
LP 2	SwissProt	Sulfurtransferase TusA	Translation
		Putative pyruvate, phosphate dikinase regulatory protein	Signal transduction

In this study, the *L. plantarum* ATCC 8014 cells were exposed to rapid changes in their environment after subjecting with ethyl pentanoate (stress). The fluctuations might induce the production of various proteins and enzymes related with metabolism. Notably, metabolism process is a vital process for microorganisms in order to obtain energy and nutrients it needs to live and reproduce. Metabolism alterations couple with gene activation leads to further cellular effects that require signal transduction. Hence, an initial stimulus can trigger the expression of a large number of genes which leading to physiological event likes the production of new proteins in microbial cells. Interestingly, this study showed that most of the proteins are related with the metabolism networks. Hence, the *L. plantarum* ATCC 8014 is developing adaptive strategies to cope with the environmental hardships. Furthermore, the bacterial survival depends on the ability of the bacteria to regulate the expression of genes coding for enzymes and proteins required for growth and metabolically active in the altered environment.

As described previously, agar well diffusion method was used to assess the antimicrobial activity of proteins secreted by *L. plantarum* ATCC 8014. The antimicrobial study was evaluated against a number of Gram-positive and Gram-negative bacteria including *B. cereus*, *B. subtilis*, *E. faecalis*, *E. coli*, *K. pneumonia* and *S. thyphimurium*. The spectrum of antimicrobial activity was determined based on the size of inhibition zone formed as shown in Figure 3.

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Figure 3: Formation of inhibition zone showed antimicrobial activity.

The microbial proteins without treating with ethyl pentanoate and served as a control were also tested for antimicrobial activity (labeled as PC). The proteins produced by *L. plantarum* ATCC 8014 after treating with ethyl pentanoate (labeled as PS) showed antimicrobial activity against all the six test microorganisms. The synthesized proteins exhibited high antimicrobial activity against *K. pneumonia* and moderate antimicrobial activity toward the other five bacterial strains, including *B. cereus*, *B. subtilis*, *E. faecalis*, *E. coli* and *S. thyphimurium*. Meanwhile, the protein control (PC) as well as control sample (CS) did not show any antimicrobial activity against all the bacteria tested (Table 2).

Table 2: Antimicrobial activity of proteins secreted by *L. plantarum* ATCC 8014

Samples	Test Bacteria					
	B. cereus	*B. subtilis*	*E. faecalis*	*E. coli*	*K. pneumonia*	*S. thyphimurium*
[a]PS	[e]+	+	+	+	[d]++	+
[b]PC	[f]-	-	-	-	-	-
[c]CS	-	-	-	-	-	-

[a]PS Protein sample: protein secreted by *L. plantarum* ATCC 8014 after treated with ethyl pentanoate; [b]PC Protein control: protein produced by *L. plantarum* ATCC 8014 without treating with ethyl pentanoate; [c]CS Control sample: ethyl pentanoate with broth only; [d]++ High antimicrobial activity: inhibition zone > 10 mm in diameter; [e] + Moderate antimicrobial activity: inhibition zone ≤ 10 mm in diameter; [f]- No antimicrobial activity: no inhibition zone

L. plantarum ATCC 8014 proteins exerted an inhibitory effect on all the six strains tested bacteria. It is of importance to the food industry that *L. plantarum* exhibited such a wide range inhibitory effect on non-taxonomically related species, including food-borne pathogens such as *E. coli*, *B. cereus* and *B. subtilis*. In addition, the proteins showed large inhibitory activity towards Gram-negative bacteria, which is a remarkable and an unusual finding since inhibitory effects of Gram-positive strains on Gram-negative bacteria is less prevalent [15]. Notably, the isolated proteins also could inhibit clinical isolates of *E. faecalis* and *K. pneumonia*. This study revealed that the isolated microbial proteins are active against pathogenic and food-spoilage bacteria suggesting for future application in food preservation to control the food pathogens and spoilage causing microorganisms.

Conclusion

Bacteria spend their lives buffeted by stresses and changing environmental conditions. Therefore, the bacteria have developed versatile strategies to deal with the great variety of challenging conditions they are exposed to.

The study on the influence of ethyl pentanoate at sub-MIC on proteins production by *L. plantarum* ATCC 8014 share a knowledge that the transcriptional response of bacteria to changing environmental conditions is an important mechanism for maintaining the bacteria viability. It should be noted that the production of proteins in response to environmental stress revealed that the stress response of *L. plantarum* is a complex process. Some proteins appear to be induced and others repressed. Furthermore, the bioactive proteins displayed antimicrobial activity against Gram-positive and Gram-negative bacteria.

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