Indonesia International Institute for Life Sciences

ENRICHMENT PROGRAM REPORT

The Effects of Spray Drying Inlet Air Temperature on Bile Salt Hydrolase Activity of *Pediococcus acidilactici* for Cholesterol Lowering Property

STUDY PROGRAM
Biotechnology

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INDONESIA INTERNATIONAL INSTITUTE FOR LIFE SCIENCES (i3L)
2022

RESEARCH REPORT

The Effects of Spray Drying Inlet Air Temperature on Bile Salt Hydrolase Activity of *Pediococcus acidilactici* for Cholesterol Lowering Property

by
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Submitted to

i3L – Indonesia International Institute for Life Sciences

School of Life Sciences

in partial fulfillment of the enrichment program for the Bachelor of Science in

Biotechnology

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Jakarta, Indonesia

2022

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Certificate of Approval

Student	:	Regina Natasya
Cohort	:	2019
Title of final thesis project	:	Efek Suhu Inlet Pengeringan Semprot terhadap Aktivitas Enzim Bile Salt Hydrolase (BSH) sebagai Properti Penurunan Kolesterol *The Effects of Spray Drying Inlet Air Temperature on Bile Salt Hydrolase Activity of Pediococcus acidilactici for Cholesterol Lowering Property*

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ABSTRACT

Bile salt hydrolase (BSH) is important to help bile salt deconjugation, converting bile salt into a more hydrophobic form which helps to alleviate cholesterol level in the body. BSH is a N-terminal nucleophile superfamily enzyme, and frequently found in lactic acid bacteria (LAB). Spray drying, as one of preservation methods, is often applied for commercial production, and known to reduce the viability of probiotics. Although there is a rising recognition of lactic acid bacteria, especially *Pediococcus acidilactici*, as potential probiotics, less studies have been conducted to investigate the effect of spray drying temperatures to BSH activity in *P. acidilactici.* Thus, this study aimed to analyze the impact caused by different inlet air temperatures of spray drying towards BSH activity of *P. acidilactici.*

BSH activity derived from spray dried *P. acidilactici* samples produced from three different inlet temperatures (120^{o}C, 150^{o}C, and 170^{o}C) were investigated qualitatively and quantitatively. Qualitative measurement was using a living cell, where all of the samples produced similar bile salt deconjugation activity without any significant differences ($p > 0.05$). On the other hand, quantitative measurement was only able to use the samples supernatant, because the extraction methods were not effective. Quantitative data which showed low specific activity were disputable because the wall material (WM) of the spray dried samples was also detected in the colorimetric assays and resulted in overestimation of concentration and activity. Despite the issue, this study showed promising activity of BSH from *P. acidilactici* after spray drying in all inlet temperatures.

Keywords: probiotics, bile salt hydrolase, bile salt, cholesterol, *Pediococcus acidilactici*, spray drying

ACKNOWLEDGEMENTS

To begin with, the author would like to praise and thank God, the almighty, for His countless blessings, guidance, and protection throughout the whole semester. With His unconditional love, He gives the author and the team strength to overcome all of the difficulties in completing the research. The author would like to deliver greatest appreciation for Dr. Putu Virgina Devanthi Ph.D., and Ihsan Tria Pramanda S.Si., M.Sc. for the opportunity to come abroad to the project, which offers thousands of experience and learnings. Deep gratitude also goes to Mario Donald Bani, S. P., M. Biotech., who became a wonderful advisor which gave the author countless support and guidance throughout the project. The author is also sincerely grateful to have great research assistants also supporting friends, namely Leon Martin, Gabriella Devina Tirta, and Ferren Pratama. To Ms. Esther, Ms. Nancy, and Mr. Ribath, the author would like to thank them for their assistance and help in managing the laboratory equipment and materials.

The author also would like to send gratitude to a wonderful partner, Wenny Novella, who have spent time supporting and accompanying each other through the ups and downs. To dearest teammates in PE Project, the author delivers thanks for the help and memorable time together in the project. Lastly, the author would like to send love to the author's family for mental support and prayers, which enables the author to go through all the hardships.

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
BSH	Bile Salt Hydrolase
CFU	Colony forming units
FC	Free cells *Pediococcus acidilactici*
GA	Gum Arabic
GIT	Gastrointestinal Tract
HC	Hypercholesterolemia
LAB	Lactic acid-producing bacteria
LP+	Lactobacillus plantarum
MRS	de Man-Rogosa-Sharpe
OD	Optical density
pH	Power of hydrogen
RPM	Rotations per minute
SD	Spray Drying
SD120	Spray Dried culture in 120^0C
SD150	Spray Dried culture in 150^0C
SD170	Spray Dried culture in 170^0C
WM	Wall Material
WP	Whey protein

CHAPTER 1: INTRODUCTION

1.1. Background

Hypercholesterolemia (HC), or high blood cholesterol level, poses a major risk factor to many vascular diseases and is associated with 28.5 million adults above 20 years old worldwide (Zahrani et al., 2021). Novel attempt has been done to alleviate HC within patients, and consumption of probiotic is proposed as one of the simple methods available. Sun & Buys (2015) reviewed that 8 out of 10 studies have reported a reduction in cholesterol level after a regular probiotics consumption.

Lactic acid bacteria (LAB) have gained attention on their use as probiotics that are able to decrease cholesterol. One LAB candidate with great probiotic potentials is *P. acidilactici* (Holland et al., 2022). One main probiotic property of *P. acidilactici* is the bacterium's ability to survive harsh human gastrointestinal tract (GIT) environments, such as high bile salt concentration. Interestingly, high bile salt concentration is linked to people with high cholesterol index (Olajugbagbe et al., 2020). The ability to survive high bile salt concentration in *P. acidilactici* is due to bile salt hydrolase (BSH), an enzyme that allows bile salt deconjugation into reduced cholesterol (Reddy et al., 2009; Mandal et al., 2009).

Bile salt is derived from cholesterol which is combined with some other components such as bile acids and phospholipids (Molinero et al., 2019; Cowen & Campbell, 1977). BSH from probiotics help deconjugate bile salt, and convert it into a less soluble form. As a consequence, bile salt is prevented from being reabsorbed and eliminated through feces (Chen & Konhilas, 2012). Thus, measuring the BSH activity of *P. acidilactici* enables an estimation of its cholesterol lowering ability.

One problem of probiotic products is to make sure that the cells can survive long enough until they reach the human GIT. Adaptation of spray drying has been commercially used to overcome this problem (Bhagwat et al., 2020). However, spray drying induces stress through heat and dehydration exposure, leading to reduced viability. Spray drying settings such as different inlet temperatures and encapsulation materials, are known to affect the properties of the bacteria culture (Arepally & Goswami, 2018). However, the effect of spray drying on *P. acidilactici* BSH activity has not

yet been investigated comprehensively. Therefore, this study focused on measuring the effect of spray drying, especially inlet air temperatures (120°C, 150oC, 170oC) on BSH activity of *P. acidilactici*.

1.2. Research Questions

In regards to the objectives of the study, the following research questions are to be investigated:

1. What are the effects produced from different spray drying inlet air temperatures to the concentration of BSH in *P. acidilactici*?
2. What are the effects produced from different spray drying inlet air temperatures to the activity of BSH in *P. acidilactici*?

1.3. Research Objectives

This study aimed to achieve following schemes:

1. Investigate and analyze the effects of using different spray drying inlet air temperature to the concentration of BSH in *P. acidilactici*.
2. Investigate and analyze the effects of using different spray drying inlet air temperature to the activity of BSH in *P. acidilactici.*

1.4. Hypothesis

These were following hypotheses in relation to the research questions:

1. H_0: The difference in BSH concentration between spray dried *P. acidilactici* produced from different inlet air temperature will not be significant.

   $H_{1:}$ The difference in BSH concentration between spray dried *P. acidilactici* produced from different inlet air temperatures were significant from each other.
2. H_0: The difference in BSH activity between spray dried *P. acidilactici* produced from different inlet air temperature will not be significant.

   $H_{1:}$ The difference in BSH activity between spray dried *P. acidilactici* produced from different inlet air temperatures were significant from each other.

1.5. Research Scope

The research scopes of this study consisted of:

1. Preparation of *P. acidilactici* free cells.
2. Production of spray dried *P. acidilactici* culture with three different inlet air temperatures (120°C, 150°C, 170°C).
3. Qualitative comparative study between BSH in *P. acidilactici* free cells and spray dried *P. acidilactici* from different inlet air temperatures in bile salt deconjugation.
4. Quantitative comparative analysis between BSH concentration and activity of *P. acidilactici* free cells and spray dried *P. acidilactici* from different inlet air temperatures.

CHAPTER 2: LITERATURE REVIEW

2.1. Introduction to Probiotics

Probiotic was firstly introduced in 1965 by Lilley and Stillwell as a secreted substance from one microorganism which can induce growth of another (Fuller, 1992). Later, probiotics are defined as live microorganisms that provide lucrative effects on hosts by the World Health Organization or WHO (Fijan, 2014). In recent years, the definition of probiotic is specified for particular bacteria strains that confer health benefits (Reid et al., 2019).

Desirable probiotic isolates are often obtained from mouth, gut, and/or human fecal because probiotics should be elements derived from healthy normal microbiota and regarded as safe for consumption (Kumar & Salminen, 2016). WHO highlights that the primary characterization of probiotics properties are often strain-specific (Ganguly et al., 2011). For a strain to be accepted as probiotic, it should confer gastric acid and bile acid resistance, adherence to epithelial cells, antimicrobial activity against pathogens, hydrophobicity, bile salt hydrolase activity, also aggregation activity (Ganguly et al., 2011). Strains from *Lactobacillus* and *Bifidobacterium* genera occupy the majority of probiotics products. Both genera are notable for probiotics due to their well-known safety and strong probiotic attributes (Kumar & Salminen, 2016).

Probiotics are often discovered in many food products such as fermented food, dairy, beverages, and also supplements (O'Toole & Cooney, 2008). Minimum probiotics incorporated into food products has been suggested to be at least 10^7 CFU/g or CFU/mL upon consumption to be able to effectively exert health benefits (Desmond et al., 2001). Noteworthy health benefits that have been claimed upon probiotic consumption may include prevention of infectious disease, modulation of host immune system, alleviation of lactose intolerance, and reduction of cholesterol (Dicks & Botes, 2010; Azad et al., 2018).

2.2 *Pediococcus acidilactici*

P. acidilactici belong to *Pediococcus* genera, that is known as homofermentative, gram-positive, non-motile, and catalase negative (Sarma, 1998; Gupta & Sharma, 2017). *P. acidilactici*, along with *P. pentosaceus,* are mostly associated with food fermentations, and might naturally exist as indigenous microflora (Papagianni & Anastasiadou, 2009). Recently, *P. acidilactici* able to be acquired from numerous food products, including *gajami-sikhae* (Jang et al., 2021); *wara* (Olajugbagbe et al., 2020); and also gouda cheese (García-Cano et al., 2020). It grows optimally under temperature of 40°C, and is able to grow under temperature up to 50°C (Papagianni & Anastasiadou, 2009). Most of the time, it is grown in De Man, Rogosa and Sharpe (MRS) media with pH range of 4.2 to 8.0 (De Vos et al., 2009).

Currently, *P. acidilactici* is extensively studied for its potential uses as probiotic supplements due to its properties. *P. acidilactici* is an eminent pediocin producer, including Pediocin AcH and Pediocin SA-1 (Wang et al., 2015; Anastasiadou et al., 2008; Motlagh et al., 1992). Pediocin is a class IIa bacteriocin, a small cationic protein that serves as antimicrobial properties (Papagianni & Anastasiadou, 2009). Its ability to survive acidic conditions (at pH range of 2 - 4) makes *P. acidilactici* may also give additional benefit as probiotic (Olajugbagbe et al., 2020).

Some studies have reported *P. acidilactici* potential for cholesterol-lowering property for hypercholesterolemia (Ribeiro et al., 2014; Kim et al., 2021). *P. acidilactici* is also notable for its resistance to bile salts, and reported to be tolerant up to 1% bile salt concentration (Ribeiro et al., 2014). Bile salt tolerance is a significant property for probiotics to survive within hosts with high cholesterol index, as bile salt synthesized from cholesterol conjugated with some other components such as bile acids and phospholipids (Molinero et al., 2019; Cowen & Campbell, 1977). In addition, *P. acidilactici* SC25 obtained from curd was shown to confer bile salt deconjugation activity on plate assay (Gil-Rodriguez et al., 2021). However, this property was not observed in *P. acidilactici* CITKHZ7, which signify that bile salt deconjugation activity by BSH is strain specific (Basumatary et al., 2022).

2.3 Cholesterol Lowering Properties of Probiotics

Cholesterol lowering ability of probiotic strain in milk was first claimed by Mann and Spoerry in 1974. This claim was based on diminishing cholesterol serum in African subjects with hypercholesterolemia (Mann & Spoerry, 1974). Hypercholesterolemia, or high blood cholesterol level, known to be one of the risk factors which increase the likelihood for vascular diseases to emerge (Zahrani et al., 2021). This condition may be derived from familial or acquired factors, where age may also exacerbate the possibility to gain hypercholesterolemia in both sex (Zahrani et al., 2021). Eventually, investigation upon this criteria was extensively conducted through feeding studies and animal models (Miremadi et al., 2014). Complete and definite mechanisms of action of cholesterol lowering remain undeciphered, however some potential mechanisms have been suggested (Lye, Rusul, & Lion, 2010). Some bacterial strains have been reported to actively collect cholesterol from their growth media, which introduces a potential cholesterol lowering mechanism (Aloğlu & Öner, 2006). In a study conducted by Duchesneau et al. (2014), *Lactobacillus* strains were analyzed for their ability to absorb cholesterol, thus underlying cholesterol assimilation mechanism. From five different species tested, *Lactobacillus plantarum* was observed to exhibit the highest assimilation activity (Duchesneau et al., 2014).

Another notable mechanism is through production of inhibitory compounds that promote cholesterol lowering action, such as ferulic acid. *Lactobacillus* bacteria are known to be able to produce ferulic acid, which restrain hepatic HMG-CoA reductase and also induce removal of acidic sterol (Duchesneau et al., 2012). Last well-known mechanism is bile salt hydrolase (BSH) activity. BSH induces bile salt deconjugation, and converts it into a less soluble form. As a consequence, bile salt is prevented from being reabsorbed and eliminated through feces which induce cholesterol removal from the host (Chen & Konhilas, 2012).

2.3.1 Bile Salt Hydrolase (BSH)

BSH is an enzyme that requires N-terminal processing for buried cysteine to resurface and induce catalytic activity, thus considered as a member of N-terminal nucleophile superfamily enzyme (Foley et al., 2019). BSH is often discovered in homotetrameric form in bacterial cytoplasm (Foley et al., 2019). However, some studies have observed the existence of oligomeric extracellular and secreted BSH (Bustos et al., 2018). BSH has been identified to have optimum temperature at 41°C and pH of 5.5 (Wang et al., 2012). According to Dong & Lee (2018), BSH molecular weights are ranging in between 34-42 kDa. This is also observed from purified BSH derived from LAB, such as purified BSH of *L. plantarum* CK 102 and *Lactobacillus gasseri* FR4 appeared to have molecular weight of 37 kDa (Ha et al., 2006; Rani et al., 2017).

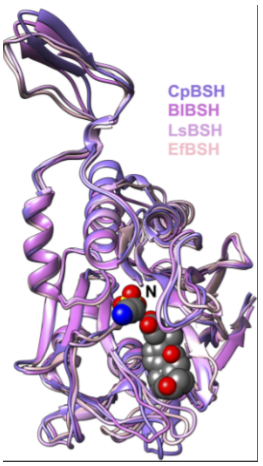

Figure 1. the structure of overlaid BSH. The structure was derived and overlaid from *Bifidobacterium longum, Enterococcus faecalis, Lactobacillus salivarus,* and *Clostridium perfringens* by (Foley et al., 2019).

Wide range of GIT microbes often harbored BSH genes, although most studies particularly assessed BSH activity from *Lactobacillus* species (Foley et al., 2021; Foley et al., 2019). Gil-Rodriguez and Beresford (2021) measured the BSH producing ability of several lactic acid strains through plate precipitation assay and compared the length of precipitation halos produced in each strain. In the experiment, *L. plantarum* isolated from cheese produced a high average of precipitation halos (4-10mm) during three times tested (Gil-Rodriguez & Beresford, 2021).

2.4 Spray Drying

Spray drying is a greatly versatile method incorporated in manufacturing industries to convert emulsion into solid powder. Spray drying is sometimes favored over freeze-drying due to lower specific energy cost, while achieving higher productivity (Huang et al., 2017). It involves encapsulation of the solution which is followed by atomization, air drying, and evaporation of droplets moisture in high temperatures (Selvamuthukumaran, 2019). Spray drying employs rapid water evaporation and also enables particle retention at a low temperature.

The spray drying process brought probiotics into heat stress, and frequently developed damage into the cell wall, DNA, and RNA of the cell, plus disturbance to its metabolic activity (Perdana et al., 2013; Tripathi & Giri, 2014; Ortega, 2017). The encapsulation material, or coating material, would act as protective materials that reduce the effects from biotic and abiotic stresses, including the heat stress (Feng et al., 2017). Incorporation of probiotic encapsulation offers improvement of stability, prolonged shelf life, sustained and controlled release (Kailasapathy, 2014; Zhao et al., 2020).

In promoting high quality and quantity of spray dried probiotics for commercialized products, selection for optimum spray drying settings should be performed. Several parameters that should take into consideration: flow rate of feed and air, temperature of inlet and outlet, atomizer, concentrations, and carrier agents (Pui & Saleena, 2022).

2.5 Protein and Amino Acid Detection Assay

Quantifying protein concentration within samples is necessary in many fields, often when analyses of quantitative protein expression is required (Lu et al., 2010). The preference of protein concentration detection assay reflects on the protein structure which is intended to be measured (Lu et al., 2010).

2.5.1 Lowry Assay

The Lowry method was developed by Oliver H. Lowry in 1951 through improvement of biuret reaction (Lowry et al., 1951). Lowry method extends the original Biuret reaction into two steps: a) Biuret reaction, which involves copper reduction by protein within alkaline solutions, and b) enhancement of the color by reduction of the Folin-Ciocalteu reagent (Noble & Bailey, 2009; Lu et al., 2010). The reaction with Folin-Ciocalteu reagent resulted in blue characterized color, with absorbance at 750 nm. This additional step boosts the protein detection sensitivity, with a range in between 5-500 µg/ml, although the exact mechanism is still obscure (Shen, 2019; Noble & Bailey, 2009).

2.5.2 Ninhydrin Assay

Long discovered ninhydrin method is one of alternative to detect α-amino acids through a reaction with ninhydrin (Perret & Nayuni, 2014; Friedman, 2004). Ninhydrin ($C_9H_6O_4$) is a light yellow crystals that is soluble in water with optimum pH of 5.5 that is frequently utilized in bioanalytical techniques in many fields, such as microbiology, agricultures, histochemistry, even forensics (Friedman, 2004, Lennard, 2013). The Ruhemman's Purple produced through decarboxylation reaction occurs between α-amino acids with ninhydrin, which also resulting in CO_2 and an aldehyde (Friedman, 2004). Despite its popularity, this method may also detect free amines, and be limited into small samples, with inability to detect protein with high molecular weight (Kerr, 2011).

CHAPTER 3: MATERIALS AND METHODS

3.1 Design, Location, and Time of the Study

This experiment was conducted at Microbiology Laboratory and Pilot Plant in Indonesia International Institute for Life Sciences (i3L) since September 2022 up to early December 2022. The experimental design of this research is shown in Figure 2, which is initiated by preparation of bacterial culture, spray drying, then proceeding to the BSH analysis.

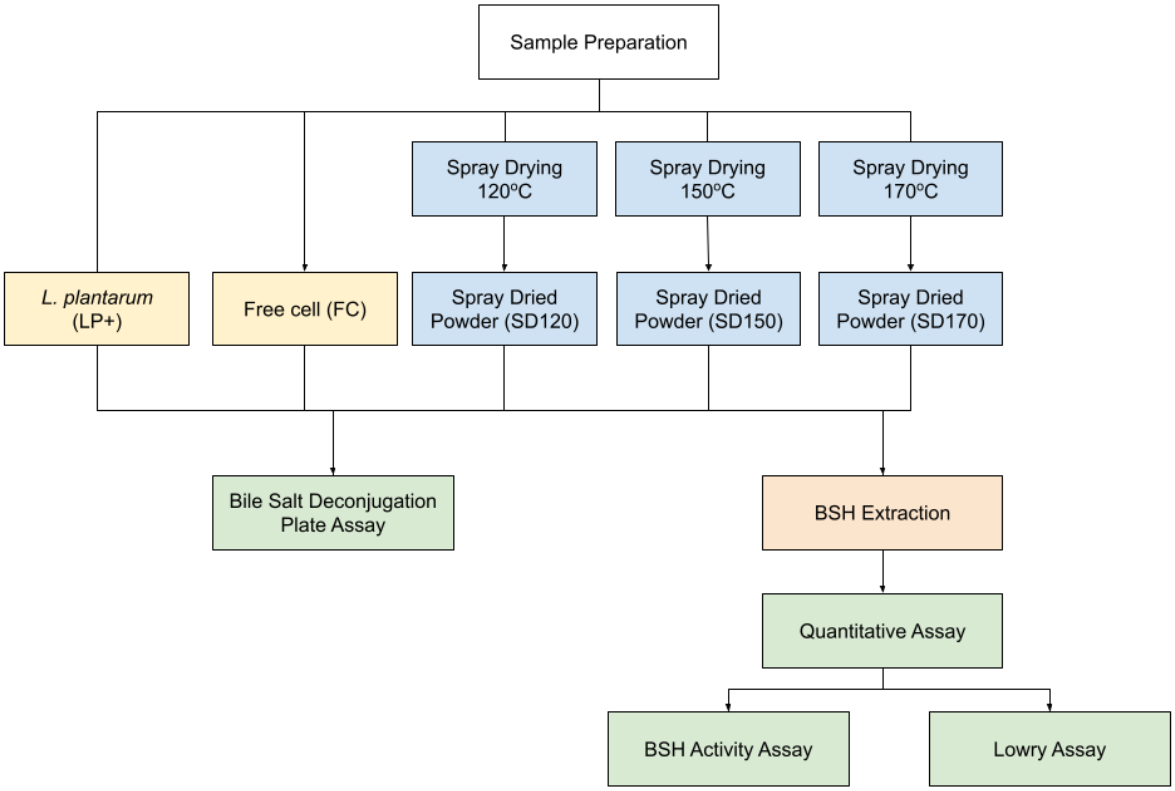

Figure 2. Research Experimental Design

3.2. Sample Preparation

3.2.1. Culture Activation and Maintenance

P. acidilactici culture was bought from Universitas Gadjah Mada Culture Collection, then was activated (10% v/v) in de Man-Rogosa-Sharpe (MRS) broth (Merck KGaA, Darmstadt, Germany,) at 30°C then incubated for 18 hours, without aeration. The positive control, *L. plantarum,* was also cultured (10% v/v) in MRS broth at 30°C and incubated for 18 h, without aeration. Both cultures were gram stained to confirm their identity, and the culture purity was maintained prior to the experiment. Partials of cultures were transferred into 50% glycerol stock to create stock culture and stored at -80°C until usage. Agar stock culture also was made in the form of quadrant streak plates and stored at 4°C after 2 days incubation. Resuscitation of dormant bacteria from stock culture was prepared through transferring culture (10% v/v) into MRS broth and incubated at 30°C for 18 h. Each was prepared in biological replicates.

3.2.2. Feed Preparation for Spray Drying

Spray drying feed will start with harvesting overnight culture bacteria through centrifugation and washing twice. The culture was then resuspended in 10 mL of sterile 0.9% NaCl solution to achieve final concentration of log 10 CFU/mL. Encapsulation material (20% w/v) was prepared by mixing gum arabic and whey protein in a ratio of 1:1, where it was added with concentrated bacteria culture. Philips Hand Mixer 1552 was utilized to homogenize the bacteria with encapsulation material for 5 minutes.

3.3. Spray Drying

A pilot-scale spray dryer (LPG-5, Changzhou Huaihai Drying Equipment Co., Ltd., Changzhou, China) was used for spray drying. Homogenized spray dry feed was flowed at constant flow rate (15 rpm) through a peristaltic pump (BT100S Lead Fluid basic variable-speed peristaltic pump). The inlet air temperature was set into three different temperatures: 120°C ± 2°C, 150°C ± 4°C, and 170°C ± 5°C, while the outlet air temperature will remain the default. Other parameters were in default (Table

1). Resulting spray dried samples were stored in plastic zipper bags, and were kept inside aluminum foil ziplocks to avert sun exposure. All of the spray dried samples were stored at 4°C in the refrigerator.

Table 1. Spray Drying Parameters settings

Parameter	Value
Fan Speed	45 Hz
Air Hammer	1 second/ 20 seconds
Pump Rate	15 rpm
Atomization	250 Hz

3.4 Bile Salt Deconjugation Plate Assay

Bile salt deconjugation plate assay was adapted from Abdel-Haleem et al. (2018) with minor changes. The media was prepared by mixing MRS agar with 0.3% (w/v) taurodeoxycholate with $CaCl_2$ (0.037 g/L), and poured into petri dishes within Biological Safety Cabinet 1300 Series A2 - 1386. After the agar solidifies, triplicates of 6 mm diameter holes were made within each petri dish (figure 3). The assay was performed by inoculating *P. acidilactici* free cell, *L. plantarum* isolate and spray dried cultures (120°C, 150°C, and 170°C) to PBS and standardized. Then, 10 µL of standardized free cells, *L. plantarum* and spray dried cultures were aliquoted and transferred into the previously made holes inside corresponding MRS+bile salt agar. Following the transfer, dishes were incubated at 37°C for 24-48 hours, and observed for detectable precipitated white halos formation surrounding the punctured location. Bacterial cultures also were grown on MRS agar without bile salts as a negative control. The approximate area deconjugated was measured through the formula below, the d is the diameter of the precipitate halo:

$$Area\ of\ deconjugated\ bile\ salt\ = \frac{1}{4}\pi d^2 - \ area\ of\ hole$$

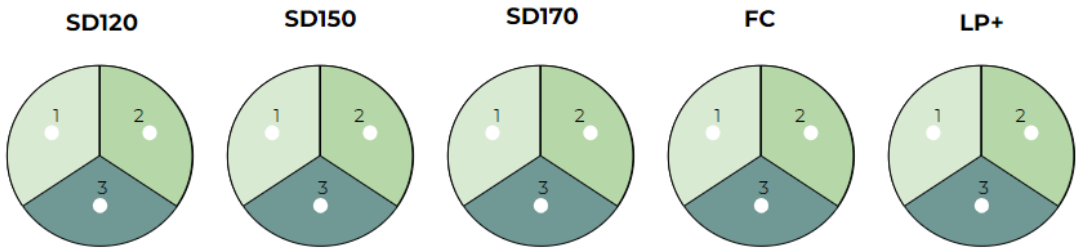

Figure 3. The layout of plate assay

3.5 Quantitative Measurement of BSH Activity

3.5.1 BSH Extract Preparation

Extraction method followed a combination of methodology from previous research (Tanaka et al., 2003; Shehata et al., 2016; Liong & Shah, 2005) with some adjustment. FC, LP+, and SD cultures (SD120, SD150, and SD170) were washed twice with centrifugation (10000 *x g*, 4°C) and resuspended in 0.1 M PBS. All of the cultures then were standardized into 10^7 cfu/mL prior to extraction. Addition of freeze thaw cycles using liquid nitrogen was done to improve the extraction method. The extraction was performed using sonication with Ultrasonic Bath Sonorex RK52 for 10 minutes and constant cooling on ice. The sonication was followed by centrifugation for 15 minutes (9700 *x g*), then supernatant was collected and underwent confirmation using Lowry assay, which was deciphered below. Additional extraction using lysozyme was also performed for comparison.

3.5.2 BSH Activity Measurement

The BSH activity assay was using ninhydrin reagent and followed previous methods (Tanaka et al., 2003; Shehata et al., 2016; Liong & Shah, 2005) combined.

3.5.2.1 Sample and Reagent Preparation

Initial mixture was prepared by combining 100 µL supernatant from each culture (FC, LP+, and SD) sonication with 10 mM DTT, 100 µL bile salt, and 1.8 mL 0.1 M PBS (pH 6). Bile salt used was 6 mM taurodeoxycholate. Prior to the assay, ninhydrin reagent was also prepared by mixing 5 mg ninhydrin powder in 0.7 mL 0.5 M citrate buffer pH 5.5 and 1.2 mL of 30% glycerol.

3.5.2.2 BSH Activity Assay

Previously prepared initial mixture was incubated at 37°C for 30 minutes to carry out enzymatic reaction. After incubation, reaction was terminated by introducing 0.5 mL of trichloroacetic acid (15% w/v) to the sample (thus creating solution 1), and centrifuged. Following centrifugation, 200 µL of solution 1 was allotted and dispensed into 200 µL distilled water, which was added with 1 mL ninhydrin reagent. This final mixture was vortexed and boiled for 15 minutes, followed by cooling in tap water. Absorbance of samples was measured in 570 nm using a microplate reader (Infinite® 200 PRO NanoQuant, TECAN) with taurine as standard. Total activity unit was (U/mL) stipulated as the amount of enzyme which discharge 1 mmol of amino acid from substrate every minute.

3.5.3 Lowry Assay

The Lowry solution was prepared by mixing 2% Na_2CO_3 in 0.2N NaOH with 10 mg/mL Potassium Sodium Tartrate and 5 mg/ mL $CuSO_4$ in water (ratio 50:1). Bovine Serum albumin (BSA) was used as standard in triplicate. All of the samples and standards were then vortexed and will have 0.2 mL transferred into separate microcentrifuge tubes. To each sample and standard, 0.4 mL Lowry solution was added, which was continued with vortexing and incubating for 20 minutes in the dark (RTP). Within the last minutes of incubation period, Folin Reagent was made by transferring 5 mL 2N

Folin and Ciocalteu's Phenol Reagent into 5 mL distilled water. Each tube then was added by 0.1 mL Folin Reagent, vortexed, and incubated for 30 minutes or longer. Proceeding the incubation, OD750 measurement of sample was performed.

3.6 Data Analysis

Throughout the experiment, data was collected and analyzed for significance by GraphPad Prism 8 for Windows 10. All of the data were performed as triplicates. One-way analysis of variance (ANOVA) method was employed to analyze the BSH activity and concentration, and continued with Tukey's Honestly Significant Different Test to compare between means. Significance is achieved when ($p < 0.05$), and enables rejection of null hypothesis

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Qualitative Measurement of Bile Salt Deconjugation

Three batches of experiment were conducted, with each in biological triplicates. Bile salt deconjugation activity was measured through the area of precipitation halos surrounding the sample. The produced BSH was used to deconjugate taurodeoxycholate, liberating taurine and deoxycholic acid. Formation of precipitation occurred as a result of reaction between Cl_2 with acid, leaving insoluble calcium salts (Gil-Rodriguez & Beresford, 2021). All of the samples appeared to possess bile salt deconjugation activity, as precipitation was observed in all plates. SD120 achieved the highest average area of deconjugation by 1.452 cm^2, followed by SD170 and SD150 with average area of deconjugation 1.351 cm^2 and 1.085 cm^2 respectively. Despite the differences, it was not significant between inlet temperatures ($p > 0.05$). LP+ with the highest deconjugation activity (avg: 1.872 cm^2), was significantly higher compared to SD150 ($p < 0.05$).

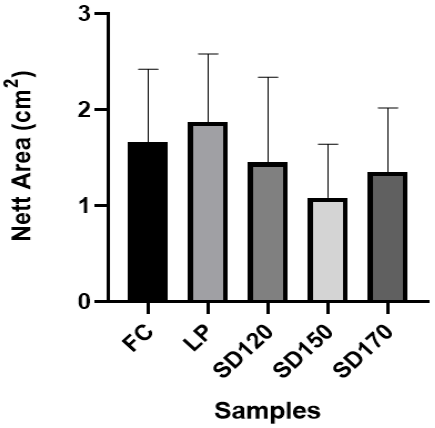

Figure 4. Bile salt deconjugation area in plate assay. LP+ produced the highest average of area, whilst SD150 gave the lowest average of area.

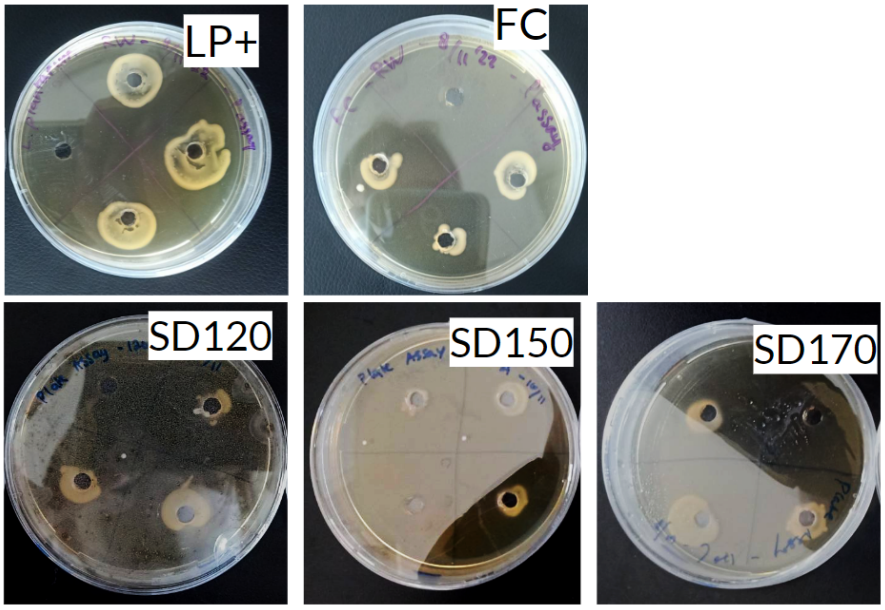

Figure 5. Bile salt deconjugation in taurodeoxycholate embedded agar. The pictures were only the representative from triplicates of three batches of experiment.

Ability of all spray dried samples to exhibit comparable bile salt deconjugation activity may be related to the presence of encapsulation during spray drying. Retained bile salt tolerance in probiotics due to encapsulation has been reported in *L. casei* ATCC393 (Phupaboon et al., 2022); *L. lactis* (Bi et al., 2016); and also *P. acidilactici* ATCC 8042 (Halim et al., 2017). Similar result was also observed in the encapsulated *L. paracasei*, which were able to survive 12 hours after spray drying in 2% bile salts with only 2.17 log CFU/mL decrease in viability (Ilha et al., 2014). This ability to survive high bile salt is highly connected to the BSH expression, where a BSH reaction with bile salt induces formation of micelles containing bile acid to cope with bile salt stress (Bi et al., 2016).

4.2 Quantitative Measurement of BSH Specific Activity

Through protein extraction (including enzymatic digestion with lysozyme, freeze-thaw cycles, and sonication), all of them were ineffective. Lysozyme was shown to interfere with the protein activity estimation (see Appendix 2). Lysozyme which was able to be detected with Lowry assay may cause overestimation of protein concentration (Wulandari et al., 2015). On the other hand, negative protein activity (after reduced with blank) for bile salt deconjugation was also observed (see

Appendix 2). This can be caused by inhibition activity of bile to lysozyme, hence reducing the bile amount to react with BSH (Vantrappen et al., 1976; Hall et al., 2013). These results therefore created a necessity for downstream purification or improvement on BSH sonication.

In addition, the amount of total protein and activity in resting cells detected were indifferent with the samples which underwent the extraction process using sonication (see Appendix 3). Cells that were suspended in PBS were no longer growing due to the absence of nutrition in PBS, and were referred to as resting cells (Julsing et al., 2012). This finding may indicate incompatible extraction equipment and methods, which prevented proper cell lysis and extracted protein for later detection. The sonicator (Ultrasonic Bath Sonorex RK52) is initially dedicated for cleaning purposes and unable to give direct ultrasonic exposure to the cells, which hinder cell lysis (Ferdous et al., 2021). Therefore, this study narrowed down the assessment only to BSH activity of supernatants derived from resting cells.

Numerous studies have assessed genetic characterization of BSH, and within particular strains, it is located intracellularly within cytoplasm (Foley, 2019; Begley et al., 2006). This resonates with the absence of enzyme activity in the supernatant of the overnight culture of *Bifidobacterium longum* SBT2928, where high activity was noticed in the cell extract (Tanaka et al., 2000; Shehata et al., 2016). However, Kumar et al. (2012) were able to assess activity derived from resting cells without protein extraction, although activity observed was ten times lower compared to activity from cell-free extracts. Ability to secrete BSH from some strains also supported by Hernandez-Gomez et al. (2021) through BSH purification secreted by resting cells of six different probiotics using High-Performance Thin-Layer Chromatography (HPTLC). The method using resting cells can assume the activity were produced similarly in natural conditions (Kumar et al., 2012). Thus, these findings correspond to the plate assay which is able to form deconjugation activity without need of extraction.

Table 2. Specific activity from resting cells of FC, LP+, SD120, SD150, SD170.

Samples	Protein		
	Total Protein (mg/mL)	Total Activity (U/mL)	Specific Activity (U/mg)
FC	0.13 ± 0.01[b]	0.03 ± 0.02[b]	0.25 ± 0.18[b]
LP+	0.16 ± 0.01[b]	0.04 ± 0.02[a]	0.24 ± 0.08[a]
120	1.49 ± 0.74[a]	0.08 ± 0.03[a]	0.02 ± 0.12[b]
150	1.38 ± 0.60[a]	0.09 ± 0.07[a]	0.01 ± 0.02[b]
170	1.46 ± 0.48[a]	0.09 ± 0.04[a]	0.03 ± 0.05[b]
WM	1.40 ± 0.46[a]	0.15 ± 0.01[a]	0.08 ± 0.02[b]

Results are depicted in means (n=9) of triplicates from three batches with ± Standard Deviation of means (*SD*).
WM stands for wall material.
[ab] different uppercase letters beside the means within the same column denote significant differences ($p < 0.05$).

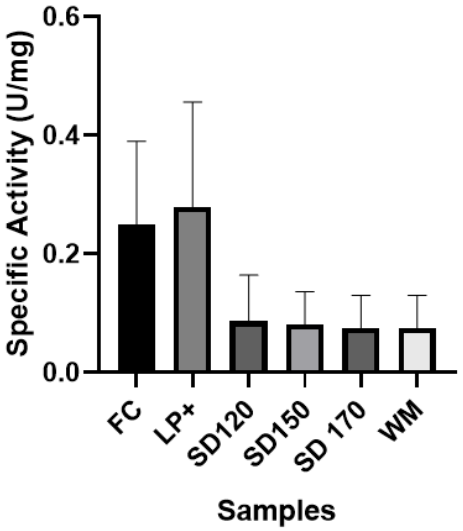

Figure 6. Specific activity of BSH from samples.

Both FC and LP+ were standardized into log 7 CFU/mL to create similar concentration with spray dried samples. The activity of both controls were significantly higher ($p < 0.05$) compared to spray dried samples, whereas the difference between inlet temperature was insignificant ($p > 0.05$). However, these findings are disputable because of the presence of the wall material (whey protein and gum arabic) in the spray dried samples, which also contributed to the total protein concentration

and total activity detected in the assays (Friedman, 2004; Kaushik & Kumar, 2011). Comparatively high concentration of wall material might have reduced the final specific activity of the spray dried samples.

Aforementioned issue thus generated the requirement in the separation of the wall material from the bacterial cells. In addition, both assays were not specific to only BSH detection. Lowry assay is based on detection of tyrosine, tryptophan, also cysteine, cystine, and histidine residues, but in lesser amounts (Noble & Bailey, 2009). Ninhydrin assay also allows overestimation of BSH activity due to its ability to detect free amine residues (Kerr, 2011).

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

The present study compared BSH activity of spray dried *P. acidilactici* derived from different inlet temperatures: 120°C, 150°C, and 170°C. Through qualitative examination on deconjugation ability in taurodeoxycholate embedded agar, all of the spray dried samples manifested similar BSH activity (deconjugation area ranges from 1.085 cm^2 to 1.452 cm^2) without significant differences with FC. It can be inferred that BSH activity is well retained in all spray dried samples. Although the quantitative measurement was unable to give specific results, this finding revealed that spray drying inlet temperature did not affect the BSH activity in *P. acidilactici*.

To support the data, future studies should proceed with specific quantification on BSH concentration and activity in specific strains with a wider range of bile salts (e.g. glycodeoxycholate, taurocholate, etc.). Application of HPLC, SDS-PAGE, and other purification methods BSH activity retention by wall material presence in spray drying should be investigated to ensure the statement. For long term product development, *in-vivo* studies are fundamental in order to screen the BSH activity in the actual environment. Additionally, identification of specific strain is necessary to create probiotic products with complete and optimum properties.

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APPENDICES

Appendix 1. Raw data for plate assay.

	Nett Deconjugation Area (cm^2)				
	FC	**LP+**	**SD120**	**SD150**	**SD170**
FIRST BATCH - A	1.532	1.58	1.414	0.668	2.553
	1.532	1.653	1.391	0.22	0.738
	1.323	1.257	1.213	0.22	1.369
FIRST BATCH - B	1.484	1.856	0.472	0.65	0.984
	1.346	1.128	0.426	0.886	1.323
	1.604	0.964	0.382	0.616	0.426
FIRST BATCH - C	2.068	1.369	1.804	0.925	1.461
	1.004	1.149	1.629	0.867	1.778
	0.271	1.391	1.213	0.829	1.461
SECOND BATCH - A	0.685	2.376	1.391	1.934	0.616
	0.55	2.095	0.65	0.285	0.984
	1.024	1.323	1.323	0.792	0.426
SECOND BATCH - B	1.83	1.703	0.411	1.532	1.856
	1.778	1.369	0.285	1.703	1.856
	1.629	1.604	0.668	1.508	0.382
SECOND BATCH - C	1.778	2.704	1.369	0.583	0.583
	1.604	2.095	1.213	0.867	0.456
	1.369	1.17	0.633	1.323	1.301
THIRD BATCH - A	1.604	1.484	1.414	0.925	1.556
	2.15	2.523	2.041	0.397	1.934
	0.456	2.922	1.703	1.257	2.068
THIRD BATCH - B	2.89	3.729	3.181	1.908	2.319
	2.319	0.964	2.765	1.804	0.984
	2.954	2.89	2.434	1.438	1.128

THIRD BATCH - C	2.206	2.206	1.391	1.934	2.319
	3.588	2.643	2.765	1.83	1.191
	2.178	2.405	3.623	1.391	2.434

Appendix 2. Total protein concentration and activity of FC from lysozyme extraction

	Total Concentration (mg/mL)	**Total Activity (U/mL)**	**Specific Activity (U/mL)**
FC - A	2.836 ± 0.02	0.021 ± 0.01	0.07 ± 0.02
FC - B	2.934 ± 0.09	0.022 ± 0.01	0.07 ± 0.01
Lysozyme only (Blank)	2.796 ± 0.04	0.024 ± 0.04	0.08 ± 0.01

Appendix 3. Comparison of total concentration and activity of FC and LP+ resting cells with sonicated cells ($p > 0.05$).

	Total Concentration (mg/mL)	**Total Activity (U/mL)**	**Specific Activity (U/mL)**
FC Resting Cells	0.205 ± 0.01	0.07 ± 0.01	0.352 ± 0.01
FC Sonicated Cells	0.264 ± 0.01	0.06 ± 0.06	0.233 ± 0.06
LP+ Resting Cells	0.245 ± 0.10	0.07 ± 0.01	0.318 ± 0.12
LP+ Sonicated Cells	0.248 ± 0.01	0.08 ± 0.11	0.331 ± 0.09

Appendix 4. ANOVA analysis of Lowry Assay

ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	18.09	5	3.618	F (5, 48) = 23.54	P<0.0001
Residual (within columns)	7.379	48	0.1537		
Total	25.47	53			

Tukey's multiple comparisons test	**Mean Difference**	**95,00% Confidence Intervals**	**Significance**	**Summary**	**Adjusted P Value**
FC vs. LP+	-0.03091	-0.5795 to 0.5176	No	ns	>0.9999
FC vs. SD120	-1.281	-1.829 to -0.7322	Yes	****	<0.0001

FC vs. SD150	-1.201	-1.749 to -0.6523	Yes	****	<0.0001
FC vs. SD 170	-1.277	-1.826 to -0.7288	Yes	****	<0.0001
FC vs. WM	-1.207	-1.755 to -0.6581	Yes	****	<0.0001
LP+ vs. SD120	-1.250	-1.798 to -0.7013	Yes	****	<0.0001
LP+ vs. SD150	-1.170	-1.718 to -0.6214	Yes	****	<0.0001
LP+ vs. SD 170	-1.246	-1.795 to -0.6979	Yes	****	<0.0001
LP+ vs. WM	-1.176	-1.724 to -0.6272	Yes	****	<0.0001
SD120 vs. SD150	0.07988	-0.4687 to 0.6284	No	ns	0.9980
SD120 vs. SD 170	0.003350	-0.5452 to 0.5519	No	ns	>0.9999
SD120 vs. WM	0.07409	-0.4745 to 0.6226	No	ns	0.9986
SD150 vs. SD 170	-0.07653	-0.6251 to 0.4720	No	ns	0.9983
SD150 vs. WM	-0.005794	-0.5543 to 0.5428	No	ns	>0.9999
SD 170 vs. WM	0.07074	-0.4778 to 0.6193	No	ns	0.9989

Appendix 5. ANOVA analysis of Ninhydrin Test

ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	0.03518	5	0.007037	F (5, 48) = 3.917	P=0.0047
Residual (within columns)	0.08622	48	0.001796		
Total	0.1214	53			

Tukey's multiple comparisons test	**Mean Difference**	**95,00% Confidence Intervals**	**Significance**	**Summary**	**Adjusted P Value**
FC vs. LP+	-0.01272	-0.07202 to 0.04658	No	ns	0.9876
FC vs. SD120	-0.05849	-0.1178 to 0.0008044	No	ns	0.0552
FC vs. SD150	-0.06197	-0.1213 to -0.002671	Yes	*	0.0358
FC vs. SD 170	-0.06381	-0.1231 to -0.004510	Yes	*	0.0282
FC vs. WM	-0.05391	-0.1132 to 0.005385	No	ns	0.0942
LP+ vs. SD120	-0.04577	-0.1051 to 0.01352	No	ns	0.2178

LP+ vs. SD150	-0.04925	-0.1085 to 0.01005	No	ns	0.1552
LP+ vs. SD 170	-0.05109	-0.1104 to 0.008211	No	ns	0.1283
LP+ vs. WM	-0.04119	-0.1005 to 0.01811	No	ns	0.3243
SD120 vs. SD150	-0.003475	-0.06277 to 0.05582	No	ns	>0.9999
SD120 vs. SD 170	-0.005314	-0.06461 to 0.05398	No	ns	0.9998
SD120 vs. WM	0.004580	-0.05472 to 0.06388	No	ns	>0.9999
SD150 vs. SD 170	-0.001839	-0.06114 to 0.05746	No	ns	>0.9999
SD150 vs. WM	0.008055	-0.05124 to 0.06735	No	ns	0.9985
SD 170 vs. WM	0.009894	-0.04940 to 0.06919	No	ns	0.9961

Appendix 6. ANOVA analysis of specific activity

ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	0.3807	5	0.07614	F (5, 48) = 5.752	P=0.0003
Residual (within columns)	0.6354	48	0.01324		
Total	1.016	53			

Tukey's multiple comparisons test	**Mean Difference**	**95,00% Confidence Intervals**	**Significance**	**Summary**	**Adjusted P Value**
FC vs. LP+	-0.05183	-0.2128 to 0.1091	No	ns	0.9295
FC vs. SD120	0.1467	-0.01432 to 0.3076	No	ns	0.0931
FC vs. SD150	0.1460	-0.01496 to 0.3070	No	ns	0.0956
FC vs. SD 170	0.1522	-0.008758 to 0.3132	No	ns	0.0736
FC vs. WM	0.1522	-0.008758 to 0.3132	No	ns	0.0736
LP+ vs. SD120	0.1985	0.03752 to 0.3595	Yes	**	0.0078
LP+ vs. SD150	0.1978	0.03687 to 0.3588	Yes	**	0.0081
LP+ vs. SD 170	0.2040	0.04307 to 0.3650	Yes	**	0.0058
LP+ vs. WM	0.2040	0.04307 to 0.3650	Yes	**	0.0058
SD120 vs. SD150	-0.0006420	-0.1616 to 0.1603	No	ns	>0.9999

SD120 vs. SD 170	0.005557	-0.1554 to 0.1665	No	ns	>0.9999
SD120 vs. WM	0.005557	-0.1554 to 0.1665	No	ns	>0.9999
SD150 vs. SD 170	0.006199	-0.1548 to 0.1672	No	ns	>0.9999
SD150 vs. WM	0.006199	-0.1548 to 0.1672	No	ns	>0.9999
SD 170 vs. WM	0.000	-0.1610 to 0.1610	No	ns	>0.9999