Isolation, Identification and Nutritional Activities of Novel *Staphylococcus Succinus* Mf 116251 In Fermented Defatted Soybean Meal (Sbm)

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Abstract
This study aims to isolate, identify, and investigate the nutrient-enhancing properties of the dominant bacteria responsible for defatted soybean meal (SBM) fermentation. For the purpose of isolating bacteria from the fermented SBM, mannitol salt agar with 10-fold dilutions were used and 16S rRNA gene was sequenced for bacterial identification. The crude protein of SBM degradation activity inoculated with *Staphylococcus succinus* MF 116251 was determined using the Bradford assay. The results of the present study indicated that *S. succinus* MF 116251 was the dominant bacteria in fermented, defatted SBM, whereas the measurement of soluble protein demonstrated that the bacteria had improved the nutritional profile of SBM. *S. succinus* MF 116251 was found to increase the nutritional profile of fermented SBM by increasing crude protein (+ 15.24%) and important amino acids such as lysine (+ 7.20), methionine (+ 11.48%), arginine (+ 11.48%), and phenylalanine (+ 11.27%) via solid state fermentation. Furthermore, fermented SBM was also possesses antibacterial and antioxidant properties. The findings of this study suggested that *S. succinus* MF 116251 has great potential to be used as a starter culture in defatted SBM fermentation to overcome antinutritional factors in SBM for fish feed formulation.

Keywords: *Staphylococcus succinus*, soybean meal, fermentation, aquaculture

Introduction
The demand for affordable protein source is increasing as human population is growing rapidly. Fish is considered a cheap protein source; however, the resource is getting depleted due to overexploitation to meet consumers demand. Fish consumption has increased from 9 kg per capita in 1961 to 20.3 kg per capita in 2017 (Food & Agriculture Organization of the United, 2020). Although fish production was projected to increase at the rate 1.2% annually (Food & Agriculture Organization of the United, 2020), this would not be sufficient to meet the incremental demand for fish where fish consumption increases at 1.5% / year (Food & Agriculture

Organization of the United, 2020). Thus, aquaculture is shifting towards intensification to increase fish production. Issues such as overreliance on fishmeal in aquafeed led to high demand for the material and over pricing. As a solution, soybean meal (SBM) was adopted to replace fish meal partially or completely in aqua feed (Miao et al., 2018; Yaghoubi et al., 2016).
Although SBM seems to be a promising alternative, the present of antinutritional factors limit the application of SBM in aquafeed (Hamid et al., 2022; Kari et al., 2022). The antinutritional factors has adversely affect the growth performance and health of aquaculture species (Kari et al., 2021; Sukri et al., 2022). Many studies have shown that Microbial fermentation was able to minimize antinutritional factors in SBM and improve its nutritional profile effectively For example, in the study of (Zhang et al., 2021) revealed that *Bacillus subtilis natto* can reduce antinutritional factors in SBM. Another study by (Morales-Mena et al., 2020) and (Lu et al., 2020) demonstrated fermented SBM can aid in enhancing the growth performance of turkey, poultry and pigs, respectively. Hence, the current study investigates the potential of *S. succinus*in in SBM fermentation.

S. succinus has been reported in many organisms, such as in the resin of Dominican amber (Lambert et al., 1998), the surface of ripened cheese (Place et al., 2002), fermented sausage (Morot-Bizot et al., 2006), small wild animals (Hauschild et al., 2010), human clinical specimens (Novakova et al., 2006) and fermented soybean food (Song et al., 2019). This bacterium is considered as low virulence normal flora in animals (von Eiff et al., 2001), but can posed a threat to the public as it carries antibiotic resistance genes (von Eiff et al., 2001). Nevertheless, in the present study, *S. succinus* was employed to ferment defatted SBM to study its effect on the nutritional profile of SBM. The outcome of this study will be beneficial for aqua feed applications, where the bacterial isolate has great potential to be used as starter culture in improving the nutritional profile of defatted SBM.

Materials and methods

Isolation and identification of *Staphylococcus succinus*

Isolation and identification of *Staphylococcus succinus* was performed as described by (Seong Wei et al., 2021) and (Thomson et al., 2022). 1 g of fermented SBM was diluted in 9 ml physiological saline followed by homogenization using a Stomacher (Seward, UK). The sample was then subjected to a 10-fold serial dilution. 100 µl of each of dilution was pipetted and spread plated onto mannitol salt agar (Himedia, India) followed by 24 h – 48 h incubation at room temperature in triplicates. After the incubation period, the total colony forming unit was calculated for the agar plates with bacterial colonies ranging from 25 – 250 single and pure colonies. The bacterial isolates were kept in Tryptic Soy agar (Merck, Germany) deep tube until further usage.

Biochemical tests

The bacterial isolates were identified using biochemical tests, which include Gram staining, coagulase test, catalase test, D – mannose, adenosine, inosine, D – sorbitol and D – alanine utilisation tests.

Blood Hemolysis assay

In the blood hemolysis assay was conducted as described by (Jeong et al., 2014). Fresh cultured bacterial was streaked on TSA supplemented with 5% human blood followed by 24 h incubation at room temperature. The result was observed and recorded after incubation period.

Bacterial DNA preparation

Bacterial isolates were inoculated on fresh Tryptic Soy agar (Merck, Germany) plate and were incubated for 24 h at room temperature. The fresh bacterial colonies were subjected to DNA extraction by using QIAmp DNA minikit (Qiagen). The procedure DNA extraction was conducted as described in the manufacturer guidelines. The final volume of DNA extract was 100 µl and was kept in the freezer at – 20 °C until further usage.

Amplification 16S rRNA gene

Amplification of 16S rRNA gene of the bacterial isolates was carried out as described

by (Jenkins et al., 2012). Polymerase Chain Reaction (PCR) mixture consists of 1.0 U Taq polymerase, 2 mM $MgCl_2$, 10 mM KCl PCR buffer, 200 µM of each dNTP and 0.5 µM primers (Woo et al., 2001). The primers used were 5' – AGTTTGATCCTGGCTCAG – 3' and 5' – AGGCCCGGGA ACGT ATTCAC – 3'. 10 µl of bacterial DNA was added into PCR mixture and was subjected to the following amplification procedures: 5 mins at 94 ºC for DNA denaturation, 1 min of DNA denaturation at 94 ºC for 40 cycles, 1 min at 55 ºC of primer annealing and 2 min at 72 ºC strand extension. All amplification procedures were carried out by using thermal cycler (Eppendorf, Germany). The PCR products were electrophoresed on a 1.5% agarose gel with ethidium bromide as staining agent. The negative control was tap water, whereas the positive control was *Escherichia coli* DNA extract.

PCR products sequence analysis

Sequencing analysis of purified PCR products were carried out as described by (Xu et al., 2004). Two ways (forward and reverse) sequencing were applied using primers 5' – CAGACTCCTACGGGAGGCAGCAGT – 3' and 5' – ACTTAACCCAACATC TCACG ACAC – 3'. 40 µg DNA template was added into PCR reaction mixture and subjected to the following amplification procedures: 1 min at 96 ºC for heat, 10 s at 96 ºC for 25 cycles, 5 s at 50 ºC and 4 s at 60 ºC. The products were then purified using ethanol followed by pelleted and air dried. The sequencing products were rehydrated in 15 µl formamide and was analysed using 3130 Genetic Analyser Capillary Array for detection (Applied Biosystems). The sequenced of 16S rRNA gene obtained were compared to a database library in GenBank, and 100% similarity was considered to match the bacterial isolates.

Minimum Inhibitory Concetration (MIC) determination

MIC assay was conducted by using broth microdilution method as described by Clinical and Laboratory Standards Institute (Wikler et al., 2009). A total of 6 antibiotics was used to characterize antibiogram of the isolated bacterial. The antibiotics used in the present study were ampicillin, chloramphenicol, erythromycin, kanamycin, penicillin G and tetracycline. These antibiotics were commonly used in antibiotic test for *Staphylococcus* spp. as reported in the study of (Jeong & Lee, 2017) and (Wang et al., 2019). In the MIC assay, two-fold serial dilution was conducted to each antibiotic by using deionized water with the final concentrations in each well ranging from 0.03 to 512 mg/l. Bacterial inoculum was cultured in TSB for overnight and its turbidity was adjusted by comparing to 1.0 McFarland turbidity standard (bioMerieux, France). The bacterial inoculum was diluted by using TSB broth to a final concentration in each well at 5 X 10^9 colony forming unit (CFU) / ml. The inoculated microplates were incubated at room temperature for 24 h. The MIC of each antibiotic was defined as the lowest concentration at which no bacteria growth / no turbidity was observed in the wells. Finally, the results were then compared to breakpoint values for staphylococci by referred to (Wikler et al., 2009). All the tests were conducted in triplicate.

Defatted soybean meal (SBM) protein degradation assay

Bacterial suspension preparation

Staphylococcus succinus was cultured in Tryptic Soy Broth (TSB) (Merck, Germany) for 24 h at room temperature. The bacterial cells were harvested by centrifugation using a minispin (Eppendorf, Germany) and the concentration of bacterial cells was adjusted into 1 X 10^6 CFU / mL using physiological saline.

Defatted soybean meal (SBM) protein mixture preparation

Defatted soybean meal (SBM) protein mixture was prepared by mixing 1 g of defatted SBM in 8 ml of distilled water. 1 ml of bacterial suspension of *Staphylococcus succinus* was added into the mixture. The mixture was homogenised and incubated at room temperature together with mixture without bacterial inoculation as control. All the experiments were carried out in triplicates. 1 ml of the mixture was sampled and

subjected to soluble protein measurement with an hour as interval time to monitor total soluble protein content for 8 h.

Soluble protein measurement

Soluble protein measurement was carried out as described by (Shen et al., 2020). 1 ml of the mixture was harvested and centrifuged at high speed. The supernatant was used to measure protein content using Bradford method (Kruger, 2009).

Solid state fermentation of defatted SBM and nutritional analysis

Bacterial cells of *S. succinus* were prepared as described in the section bacterial suspension preparation. Solid state fermentation of defatted SBM was carried out as described by (Zhang et al., 2021). 100 g of defatted SBM was purchased from local commercial factory (Soon Soon, Malaysia) and mixed with the prepared bacterial cells. The mixture was incubated at room temperature for a week followed by nutritional analysis. All the experiments were conducted with triplicates.

After a week of fermentation process, non-fermented and fermented defatted SBM were sent for nutritional analysis of dry matter, crude protein, crude lipid and fiber. The nutritional analysis was conducted according to AOAC procedure as described by (Latimer & International, 2012) and (Thiex et al., 2002). The total amino acid of both non-fermented and fermented defatted SBM was performed as described by (Teshima et al., 1986) and (Kader et al., 2010). All the experiments were performed in triplicates.

Antibacterial assay of FSBM

FSBM crude peptide preparation

FSBM crude peptide was prepared as described in the study of Li et al. (2019) (Li et al., 2019). FSBM solution was obtained by added FSBM into sterile water with final concentration 40 ppm. The solution was shaked at 180 rpm for 2 h followed by centrifuged at 4000 rpm at 4 °C for 30 mins. The supernatant was then adjusted pH to 2.0 by using 3 mol/L of HCl followed by overnight incubation at 4 °C. After incubation, the supertant was subjected centrifuged to get sediment. The sediment was then mixed homogenously with 90% ethanol. The mixture pH was adjusted pH to 7.0 by using 3 mol/L of NaOH followed by incubation for 5 h at room temperature. After incubation, the mixture was centrifuged to get supernatant. The supernatant was evaporated to obtain crude peptide.

Antibacterial assay of FSBM peptide

Antibacterial property of FSBM peptide was revealed by using MIC assay as described in the above. *Escherichia coli* and *Staphylococcus aureus* were used as microorganisms whereas antibiotic erythromycin was used as control. The concentration of FSBM peptide used in the assay was ranged from 0.03 to 512 mg/l.

Determination of FSBM antioxidant activity

Antioxidant assay in the present study was carried out as described in the study of Lee et al. (2011) (Wei et al., 2011). α, α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging method was conducted by using a 96 wells ELISA plate with triplicates. 5 µl of FSBM (0.5 mg/ml) was added into each ELISA well followed 200 µl DPPH. The absorbance reading was viewed at 450 nm spectrophotometry method by using ELISA reader (Bio-Rad, USA). Antioxidant activity in soybean meal (SBM) was also determined for comparation. The percentage of DPPH inhibition was determined based on the absorbance reading calculation.

Statistical analysis

The total soluble protein data was analysed by using SPSS (Version 13). T – test was used to analyse the variance between control and treatment, and $p<0.05$ was considered as significant

Results

Bacterial isolation and identification

The present bacterial isolate of *Staphylococcus succinus* was able to utilise D – mannose, adenosine, inosine and D – sorbitol but failed in the D – alanine utilisation test. The bacterial isolate was shown to be Gram positive and tested negative in coagulase test and, but tested positive in catalase. The bacterial isolate was

then sequenced for 16S rRNA and was identified as *Staphylococcus succinus* MF 116251. Figure 1 shows the DNA amplification of *Staphylococcus succinus* that was later used for sequencing. The present bacterial isolate showed weak or α-hemolytic against human blood.

Soluble protein measurement

Fermented defatted SBM with *Staphylococcus succinus* MF 116251 had shown significantly higher soluble protein after 5 h of incubation (Table 1) (Figure 2). The total soluble protein increased rapidly after 7 h of incubation. In the meantime, showed little variation in total soluble protein.

Nutritional profile of non-fermented and fermented SBM

The results showed that fermentation of SBM using *S. succinus* (MF 116251) as a starter culture had improved the nutrient content of the SBM by up to +15.24% for crude protein and 0.99% for total dry matter. In the meantime, solid state fermentation of SBM was able to successfully reduce antinutritional factors such as total lipid and total fibre with changing rate of 17.58% and 11.54%, respectively. The total essential amino acids such as methionine, arginine and phenylalanine in the fermented SBM were improved more than 10%, whereas the increment total lysine was recorded as +7.20% (Table 2).

Minimum Inhibitory Concentration (MIC)

Table 1 showed minimum inhibitory concentration (MIC) of antibiotic results. The values of MIC against the present bacterial isolate were ranged from 0.25 to 16 mg/l where ampicillin performed the lowest and chloramphenicol showed the highest concentration. The present bacterial isolate was sensitive to all tested antibiotics.

Antibacterial and antioxidant properties of FSBM

MIC values of FSBM peptide against *E. coli* and *S. aureus* was 8 and 16 mg/l, respectively. Antioxidant capacity in FSBM (1.68 ± 0.12 mmol/g) is significantly higher than SBM (0.48 ± 0.06 mmol/g).

Table 1. Minimum Inhibitory Concentration of the present bacterial isolate

Antibiotics	MIC (mg/l) – case	Breakpoint (mg/l) CLSI (2014)
Ampicillin	0.25 – sensitive	R ≥ 0.5
Chloramphenicol	16 – sensitive	R ≥ 32
Erythromycin	2 – sensitive	R ≥ 8
Kanamycin	8 – sensitive	R ≥ 64
Penicillin G	2 – sensitive	R ≥ 0.25
Tetracycline	0.5 – sensitive	R ≥ 16

Table 2. Total soluble protein with or without *Staphyloccoccus succinus* MF 116251 inoculation

Incubation period (hour)	µg / g Soluble Protein (with *Staphyloccoccus succinus* MF 116251)	µg / g Soluble Protein (control)
0	40.17±1.002	40.17±1.002
1	42.83±1.270	41.37±1.312
2	46.53±1.375	42.90±1.601
3	58.40±1.429*	48.23±0.436
4	82.83±1.997*	51.23±1.002
5	94.20±5.773*	53.60±0.902
6	111.37±7.267*	56.77±1.721
7	216.97±30.649*	59.43±1.266
8	310.50±27.826*	61.50±1.442

*Significantly different at $p < 0.05$

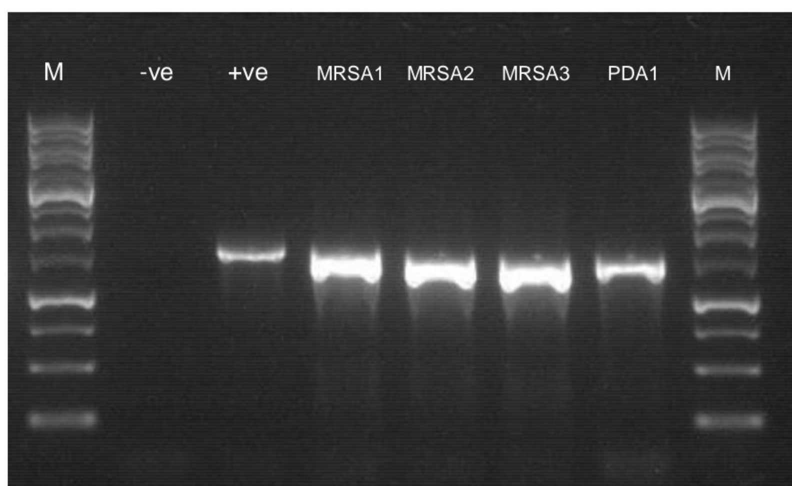

Figure 1. DNA amplification of *Staphylococcus succinus* for sequencing. - ve: PCR no template control (water to replace DNA template); +ve: Positive control (DNA extracted from *Escherichia coli* as template); M: marker 1000 Kbp; MRSA 1, 2, 3 & PDA 1: DNA from bacterial samples

Table 3. Nutritional profile of non-fermented and fermented SBM

Nutrient	Non-fermented SBM %	Fermented SBM %	Changing percentage %
Crude protein	40.21±0.11	46.34±0.31	+ 15.24
Total lipid	1.98±0.09	1.63±0.29	- 17.68
Total fibre	5.63±0.13	4.98±0.14	- 11.54
Total dry matter	89.32±0.09	90.21±0.45	+ 0.99
Lysine	2.64±0.04	2.83±0.12	+ 7.20
Methionine	0.61±0.08	0.68±0.09	+ 11.48
Arginine	3.31±0.12	3.69±0.13	+ 11.48
Phenylalanine	2.13±0.09	2.37±0.15	+ 11.27

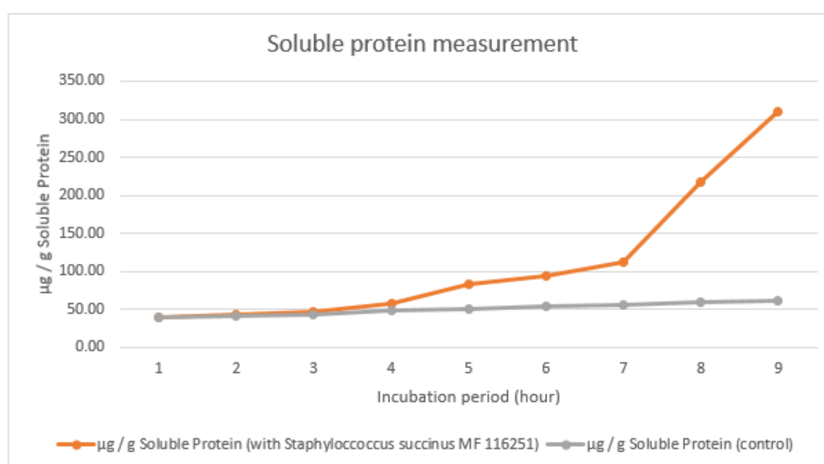

Figure 2. Soluble protein measurement with and without bacterial incubation at room temperature

Discussion

Staphylococcus succinus MF 116251, the pres ent bacterial isolate, performed similarly in bi ochemical tests as the bacterial isolate in the s tudy by (Place et al., 2002). Both bacteria were able to produce acid by utilising D – mannose, adenosine, inosine and D – sorbitol. Generally, coagulase positive staphylococci (CPS) are considered as pathogenic and can lead to pneumonia and food poisoning (Archer, 1998). However, the present bacterial isolate of *S. succinus* belonged the group of non-virulence coagulase negative staphylococci (CNS). This was also supported by (Heo et al., 2020), who concluded that CNS group of bacteria can be used as starter in fermentation of dairy and meat-based products such as sausage (Leroy et al., 2006),

seafood (Guan et al., 2011), soybean food (Jeong et al., 2014) and cheese (Heo et al., 2020). *S. succinus* also has been used commercially as starter culture for soybean food fermentations (Jeong & Lee, 2017). Furthermore, the application of CNS group of bacteria has been reported to enhance the taste, aroma and color of fermented food. Besides, heat – inactivated virulence factor free *S. succinus* can administered orally as treatment for asthmatic patients (Song et al., 2019). Thus, this indicates that the current bacterial isolate can be used as starter culture for defatted SBM fermentation for aquaculture feed formulation.

This study is the first report on antibiogram of *S. succinus* isolated from FBSM. The bacterial isolate was found sensitive to all tested antibiotics. We may conclude that the antibiotic resistance genes were absence in the bacterial. However, further study needs to be carried to investigate the present of antibiotic resistance genes in the bacterial isolate before we can conclude. This was also agreed in the study of (Kong et al., 2022) where the incidence of antibiotic sensitive case of *Staphylococcus* spp. was associated with the absence of antibiotic resistance genes in the bacterial. In the present study, the bacterial isolate exhibited weak blood hemolytic activity indicating do not posed high risk or non-pathogenic bacterial strain. Weak blood hemolytic activity is usually related to strong lipolytic activity that caused erythrocyte membrane phospholipids degradation (Kong et al., 2022). Based on hemolytic activity, the present bacterial isolate can categorise as non-pathogenic bacterial. Thus, this bacterium can be used as FSBM starter culture.

FSBM was found possesses antibacterial property. The property is due to the presence of alkaline amino acids such as arginine, histidine and lysine which contribute to antibacterial property of FSBM. This finding was supported by the study of (Sanjukta & Rai, 2016) and (Cheng et al., 2017). High antioxidant capacity of FSBM was observed in the present study. Similar finding was also reported in the study of (Li et al., 2019). Based on literature survey, generally fermented soybean foods possess higher antioxidant activity as claimed in the study of (Zhu et al., 2008), (Kim et al., 2008; Moktan et al., 2008). Fermentation process release amino acids in SBM and these free amino acids contribute to antioxidant activity of FSBM.

Protein is an expensive ingredient in aquaculture feed formulation. Traditionally, fish meal was the primary source of protein for feed formulation. However, the increased demand for fish meal had caused a price increase for this ingredient. Furthermore, fish meal resource is depleting as fisheries production declines. Alternative protein sources, such as soybean meal (SBM), were utilised as aquafeed due to higher protein content However, most of aquaculture species cannot digest plant-based protein including SBM, thus. Due to the fact, protein degradation, such as fermentation, needs to be applied prior to formulation of aquafeed. In the present study, *S. succinus* MF 116251 was shown to improve nutritional profile of defatted SBM effectively. Similar findings were observed by (Sarkar et al., 1997) and (Zhang et al., 2021), fermentation was shown to improve the nutritional values of SBM. It is possible that the microbial communities involved in the process may contain protease enzymes that can hydrolysed protein to amino acids (Guo et al., 2007) and biogenic amines (Gallego et al., 2018; Liu et al., 2011). *S. succinus* MF 116251 was found to increase important essential amino acids (EAA), particularly methionine in defatted SBM. Similar findings were observed in the study by (Zhang et al., 2021), which revealed that *Bacillus subtilis natto* was able to improve EAA in fermented SBM, particularly methionine, an important EAA that is typically absent in many raw materials for aquafeed. However, (Han et al., 2012) found that where *B. subtilis* was unable to increase methionine in fermented SBM.

Conclusion

This study is the first to propose theuse *S. succinus* MF 116251 in SBM fermentation process for aquafeed formulation. Our findings showed that the bacterial isolate has great potential to improve nutrition profile of SBM, thus supports SBM to replace conventional fish meal as protein source in aquafeed formulation. Complete genome sequence of the current bacteria *S. succinus* MF 116251 needs to be carried out in the very near future to ensure this bacterium is free from any virulence factor before it can be used as a culture starter in soybean meal fermentation for aquacultures.

Declaration of competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contribution

Zulhisyam Abdul Kari: Conceptualisation, Writing – review and editing; **Muhammad Khairulanam Zakaria:** Writing – review and editing; **Wendy Wee:** Writing – review and editing; **Guillermo Téllez-Isaías:** Writing – review and editing, data analysis; **Nik Nur Azwanida Zakaria**-Writing-review and editing; **Khang Wen Goh:** Writing-review and editing; **Romalee Cheadoloh:** Writing-review and editing; **Lee Seong Wei**: Project administration, Writing - Original draft, Writing – review and editing

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