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Research article

Effect of by-product from C-phycocyanin extraction as a prebiotic properties and probiotic microbial population

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Abstract

This study was designed to investigate the efficacy of alternative prebiotic supplements derived from phycocyanin extraction by-product in enhancing the growth of probiotic. These alternative prebiotic supplements included *Spirulina platensis*, the by-product of phycocyanin extraction, and inulin. The efficacy of alternative prebiotics was assessed using beneficial bacteria (*Lactobacillus johnsonii* ck-3 and ck-8), pathogenic bacteria (*Staphylococcus aureus* and *Escherichia coli*), and yeast (*Saccharomyces cerevisiae*). The alternative prebiotics source was used to analyse the chemical composition, prebiotic activity, and bacteria growth. It was demonstrated that the phycocyanin extraction by-product included a high concentration of crude protein (67.74% DM), carbohydrates (31.29% DM). The phycocyanin extraction by-product has a high content of glucose, fructose, and sucrose (63.55, 68.00, and 0.24 µg/ml, respectively). The phycocyanin extraction by-product has presented hydrolysis ability (*in vitro*) at 2.51% while *S. platensis* showed at 1.81 %. The composition of the phycocyanin extraction by-product showed similar activation of probiotic bacteria to inulin. Especially, *Lactobacillus* strain showed the best growth was achieved with the phycocyanin extraction by-product with decrease the final pH and high microbial colony number at 48 hours in 7.5–9.5 $\log_{10}$ colony-forming unit per milliliter (CFU/ml) when compared with the glucose group. The study results revealed that the phycocyanin extraction by-product with the prebiotic potential are effective at increasing numbers of *Lactobacillus* strain. Consequently, the study reveals that phycocyanin extraction by-product are effective as alternative prebiotic supplement, significantly enhancing beneficial probiotic bacteria. These findings suggest the potential use of phycocyanin extraction by-products in creating new prebiotic formulations for gut microbiota modulation through dietary interventions.

Keywords: Agricultural by-product, Blue-green microalgae, Microorganisms, Prebiotic

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INTRODUCTION

The use of feed additives from natural source in the livestock feed industry is very important to substitute the use of antibiotics (Seal et al., 2013). Probiotics being used as feed additives for the development of the food industry (Bai et al., 2017). Probiotics are live microorganisms and an advantage to the health of the host when taken in appropriate amounts (Hill et al., 2014; Sánchez et al., 2017). It inhibits the growth of pathogenic microorganisms (Bodke et al., 2022) and stimulates the immune system of host (Ashraf and Shah, 2014; Yousefi et al., 2019). Supplementation with probiotic *Lactobacillus* strain has been demonstrated to inhibit the growth of pathogenic bacteria, including *Escherichia coli* (Devi et al., 2013; Wang et al., 2018a; Wang et al., 2018b), *Clostridium* (La Ragione et al., 2004; Bolla et al., 2013; Di Gioia et al., 2016), and *Salmonella* (Zhang et al., 2012; Sabo et al., 2020). Pridmore et al. (2008) demonstrated the mediated destructive effects of hydrogen peroxide (H_2O_2) from *Lactobacillus johnsonii* (La1) supernatant was anti-*Salmonella* activity and due to toxic free fatty acids could lead to inhibit the growth of *Salmonella* (Travers et al., 2016). The growth of *L. johnsonii* has been found to inhibit *Brachyspira pilosicoli* (B2904) and *Brachyspira hyodysenteriae* (Mappley et al., 2013). This inhibitory effects is attributed to the release of protein antimicrobial compounds and the production of hydrogen peroxide (Lin et al., 2015). Previous studies showed that supplementation probiotic in rabbit feed promotes growth performance by improved nutrient intake, nutrient digestibility, digestible nutrients, and nitrogen retention (Truong et al., 2024). Probiotics have positive effects on the host animal by promoting intestinal balance, which enhances feed efficiency, nutrient absorption, growth rate, and economics poultry production (Awad et al., 2008; Abd El-Hack et al., 2017; Alagawany et al., 2018; Truong et al., 2024).

Natural prebiotics have become an important object for the animal feed industry of alternative feed supplement. Prebiotics have been shown to play a pivotal role in enhancing host's health, exhibiting resistance to pathogenic organisms (Mohanty et al., 2018), and mitigating metabolic disorders (Giorgino et al., 2023). They effectively promote the growth and activity of beneficial gut microbes, particularly in the large intestine, thereby improving overall host's health. Furthermore, prebiotics are known to stimulate the growth of probiotic strains like *Lactobacillus* and *Bifidobacterium*, contributing significantly to the health and productivity of animals. Sources of prebiotics are oligosaccharides from agricultural residues and by-products such as corncobs (Moniz et al., 2016), rice husks (Bamigbade et al., 2022), and orange rinds (Gomez et al., 2014).

Spirulina platensis are blue-green microalgae that has been used in food commercially cultivated for a long time. It has been used for many years as human food with high protein content and nutritional value. *S. platensis* contains about 20% carbohydrates, 50-70% protein, 5% lipid, 7% minerals and 3 to 6% moisture (Richmond, 2013; Ak et al., 2016; Abdel-Latif et al., 2022; Liu et al., 2022). Among the proteins present in *S. platensis* are phycobiliproteins which are intensely pigmented, highly fluorescent (Stanic-Vucinic et al., 2018), and water-soluble fluorescent proteins (de Morais et al., 2018; Pagels et al., 2019). The *S. platensis* produces only two phycobiliproteins, i.e. allophycocyanin, and C-phycocyanin (Stanic-Vucinic et al., 2018). The main elements of the phycobiliprotein family are C-Phycocyanin. C-Phycocyanin has a high aggregated value and production is a potentially attractive characteristic. It is not only used as an ingredient of nutrient and natural dyes for food and cosmetics, but it is also used as potent therapeutic agent and fluorescent markers (de Morais et al., 2018). Phycocyanin has garnered attention in biomedical research due to its antiviral (McCarty and DiNicolantonio, 2020; Raj et al., 2020), anti-tumoral (Cotas et al., 2020; Dev et al., 2020; Pagels et al., 2021), and anti-inflammatory properties (Guo et al., 2022; Liu et al., 2022). However, it is important to note that the phycocyanin extraction process from *S. platensis* results in a significant amount of by-products. These by-products are presumed to maintain nutritional value and have potential as effective alternative

prebiotic in animal diets. Based on these benefits, this study was conducted to determine the potential of phycocyanin extraction by-product from *S. platensis* as alternative feed additives in livestock feed.

MATERIALS AND METHODS

Preparation of phycocyanin extraction by-product from *Spirulina platensis*

This research used a by-product of phycocyanin extraction from *S. platensis* by freezing and thawing method (Silveira et al., 2007) which remain the by-product after extraction around 56-57% yield (primary data through experiments). *S. platensis* powder was prepared using commercial manufacturing techniques. The samples, including phycocyanin extraction by-product, *S. platensis*, and Inulin, were finely ground in a laboratory mill equipped with 1-mm mesh screen. The treatments are divided into 8 group with 9 replicates: W (buffered peptone water), SP0.4 (*S. platensis* at 0.4% concentrations), SP0.8 (*S. platensis* at 0.8% concentrations), PB0.4 (phycocyanin extraction by-product at 0.4%), PB0.8 (phycocyanin extraction by-product at 0.8%), IN0.4 (Inulin at 0.4%), IN0.8 (Inulin at 0.8%), and G (buffered peptone water with 2 mM glucose). The buffered peptone water used for bacterial growth did not contain glucose.

Microorganisms, Culture Conditions and Microbial Enumeration

The beneficial bacteria (*Lactobacillus johnsonii* ck-3 and ck-8), pathogenic bacteria (*Staphylococcus aureus* ST398 and *Escherichia coli* O157:H7), and yeast (*Saccharomyces cerevisiae*) were used to evaluate their optical density and the growth of microbial population. *L. johnsonii* was cultivated in de Man, Rogosa & Sharpe (MRS) broth (Merck, Germany), *S. aureus* and *S. cerevisiae* in Tryptic Soy Broth (TSB) (Merck, Germany), and *E. coli* in Eosin Methylene Blue (EMB) broth (Merck, Germany). After incubation at 0, 24, and 48 hours in optimum temperature (37°C), the relative cell growth was measured spectrophotometric at 600 nm then compared to the cell growth in basal medium supplemented with and without glucose (Taylor et al., 2021). Subsequently, 0.1 ml was pipetted out from each of the broth and serial dilutions were performed for plating using spread plate method in duplicates. All plates were incubated at 37°C for 24 hours under aerobic or anaerobic conditions. The population of *L. johnsonii* was determined by counting viable cells on MRS agar plates under anaerobic condition. Similarly, viable cell counts of *S. aureus* and *S. cerevisiae* were determined on TSB agar plates, and *E. coli* count was determined on EMB agar plates. Each sample was analysed in duplicates, and the experiments were conducted three times. The data were expressed in terms of colony-forming unit per milliliter (CFU/ml), calculated using the following formula:

CFU/ml = (Average Number of colonies*dilution factor) / volume of culture plate

Chemical component analysis of phycocyanin extraction by-product from *Spirulina platensis*

The chemical composition of each product was analyzed. Dry matter (DM) content was determined by drying at 100 °C for 6 h in a hot air oven. Crude protein (CP) content in each sample was analyzed following the Kjeldahl method. Physiochemical parameters such as moisture, ash, total fat, and fiber contents were determined according to the standard AOAC 1990 methods (AOAC methods

Nos. 925.10, 923.03, 2003.05 and 993.19 respectively). Neutral-detergent fiber (NDF), acid-detergent fiber (ADF), and acid-detergent lignin (ADL) concentrations were determined according to the method of Goering and Van Soest (1970). The determination of reducing sugar content in our study was conducted using the Modified dinitrosalicylic acid method (Miller, 1959), and total sugar content was quantified employing the modified phenol sulfuric acid method (Wongputtisin et al., 2014).

Hydrolysis of phycocyanin extraction by-product from *Spirulina platensis*

The *in vitro* digestion simulation of the samples was carried out using a method modified from Saura-Calixto et al. (2000). Initially, proteins were digested using pepsin (Sigma Chemical Co., St. Louis, MO) at 40 °C for 15 minutes in an acidic environment (pH 1.5). This step was followed by incubation with amylase (Sigma Chemical Co., St. Louis, MO) at 37 °C for 30 minutes to hydrolyze digestible starch. Subsequently, the insoluble residue was treated with 2 M KOH. The digestion process continued with an incubation with amyloglucosidase (Sigma Chemical Co., St. Louis, MO) at 41 °C for 45 minutes at pH 4.75. Finally, glucose levels were quantified using a glucose oxidase peroxidase assay.

Prebiotic activity score

The prebiotic activity assays were performed according to the procedure established by Huebner et al. (2007). This involved the addition of 1% (vol/vol) of an overnight culture of *Lactobacillus* strain to separate tubes containing MRS broth with 1% (wt/vol) glucose or 1% (wt/vol) of the test prebiotic. The cultures were incubated under anaerobic conditions at 37°C. Viable cell counts of samples were performed on MRS agar after 0 and 24 hours of incubation. In a parallel setup, overnight cultures of *E. coli* strains were introduced at 1% (vol/vol) into separate tubes containing EMB broth with either 1% (wt/vol) glucose or 1% (wt/vol) prebiotic. These cultures were incubated at 37°C in an ambient atmosphere. Viable cell counts for *E. coli* were then conducted on EMB agar following incubation periods of 0 and 24 hours. The prebiotic activity score was determined using the following equation:

$$\text{Prebiotic activity score} = \frac{(L0-L24 \text{ on the prebiotic})}{(L0-L24 \text{ on glucose})} - \frac{(E0-E24 \text{ on the prebiotic})}{(E0-E24\ on\ glucose)}$$

when L0 mean *Lactobacillus* colony ($\log_{10}$ CFU/ml) at 0 hours.
L24 mean *Lactobacillus* colony ($\log_{10}$ CFU/ml) at 24 hours.
E0 mean *E. coli* colony ($\log_{10}$ CFU/ml) at 0 hours.
E24 mean *E. coli* colony ($\log_{10}$ CFU/ml) at 24 hours.

By definition, probiotics are microorganisms that benefit the host by improving its intestinal microbial balance. In this context of this study, probiotic bacteria, such as *Lactobacillus* strains are expected to thrive on substrates with high prebiotic activity, resulting in cell densities ($\log_{10}$ CFU/ml) that are comparable to those grown on glucose substrate. However, the cell densities of enteric bacteria strains grown on prebiotics should be significantly lower than those grown on glucose.

Optical density and pH-value measurements

An overnight culture (5 ml) of each probiotic bacterial strain was added to a bottle with the corresponding medium and incubated at 37°C. Anaerobic conditions were used to incubate the *L. johnsonii* and ambient atmosphere were used to incubate *S. aureus*, *E. coli*, and *S. cerevisiae*. For the optical density readings, pH-

value measuring, and the missing media were replaced. Optical density readings were done every 4 hours over 48 hours, and the pH-value of all samples was measured by using a pH meter.

Statistical analysis

The study was analyzed as a completely randomized design with eight treatments and nine replicates per treatment. Data were using analysis of variance (ANOVA) in SAS version 9.2 and means compared by Duncan's new multiple range test. The enumerations of bacterial colony-forming units (CFU/ml) of beneficial bacteria (*L. johnsonii* ck-3 and ck-8), pathogen bacteria (*S. aureus* and *E. coli* (O157:H7), and yeast (*S. cerevisiae*) were exposed to logarithmic transformation (log_{10} CFU/ml) for normality when variances were not homogeneous, and the transformed data were presented.

RESULTS

Nutritional composition of phycocyanin extraction by-product from *S. platensis*

The nutritional compositions of phycocyanin extraction by-product (PB) are shown in Table 1. The PB contained dry matter 89.74 %, ash 1.62 %DM, CP 67.74 % DM, EE 6.16% DM, CF 0.40% DM, NDF 1.88% DM, ADF 0.31% DM, and ADL 0.77% DM. The major dietary fiber components, which included cellulose, hemicellulose, and lignin, were 0.46% DM, 1.46% DM, and 0.06% DM, respectively. The carbohydrate compositions of the phycocyanin extraction by-product were measured in a broader spectrum of attributes in comparison to the inulin. The PB contained reducing sugar 130.51 mg/g (glucose 63.55 µg/ml and fructose 68.00 µg/ml) and non-reducing sugar 91.09 mg/g (sucrose 0.24 µg/ml) all compounds were similar with *S. platensis* (SP) raw material (Table 1).

Table 1. Nutritional composition of phycocyanin extraction by-product from *Spirulina platensis*, *Spirulina platensis* and Inulin (Means ± SD)

Items	PB	SP	IN
Dry Matter	89.74 ± 0.02	89.44 ± 0.15	98.02 ± 0.76
Ash (% DM)	1.62 ± 0.18	1.68 ± 0.06	nd
Crude Protein (% DM)	67.74 ± 0.27	77.71 ± 1.31	0.06 ± 0.00
Ether Extract (% DM)	6.16 ± 0.03	2.90 ± 0.08	nd
Crude Fibe r (% DM)	0.40 ± 0.02	0.56 ± 0.04	nd
%NDF	1.88 ± 0.02	2.58± 0.37	nd
%ADF	0.31 ± 0.34	1.37 ± 0.58	nd
%ADL	0.77 ± 0.63	2.07 ±1.04	nd
NFE (%)	31.29 ± 0.42	25.39 ± 1.11	nd
Fiber content			
%Hemicelluloses	1.46 ± 0.34	1.13 ± 0.19	nd
%Cellulose	0.46 ± 0.29	0.69 ± 0.45	nd
%Lignin	0.06 ± 0.04	0.74 ± 0.21	nd
Total Phenolic compound (ug GAE/g DW)	607.36 ± 73.19	629.94 ± 54.18	17 ± 1.91
Sugar content (mg/g extracted)			
Total Sugar	221.59 ± 54.10	222.83 ± 15.57	nd
Reducing Sugar	130.51 ± 14.83	132.05 ± 5.57	nd
Non- Reducing Sugar	91.09 ± 39.40	90.78 ± 10.64	nd

*PB: Phycocyanin extraction by-product from *Spirulina platensis*, SP: *Spirulina platensis*, IN: Inulin.
nd: Not detected; DM: Dry matter; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; ADL: Acid detergent lignin; NFE: Nitrogen free extract; GAE: Gallic acid extraction

Prebiotic activity and Percentage of hydrolysis between prebiotic source

The potential of prebiotic activity was showed in Table 2. *S. platensis* and phycocyanin extraction by-product from *S. platensis* showed prebiotic activity at 10.32 ± 0.77 and 11.47 ± 0.81, respectively. The prebiotic activity was similar with inulin powder (12.98 ± 0.18). In case of percentage of hydrolysis showed less value in inulin powder (0.10%) than those of *S. platensis* and phycocyanin extraction by-product (1.81% and 2.51%, respectively).

Table 2. Prebiotic activity and Percentage of hydrolysis in gastrointestinal digestion (Means ± SD)

Items	PB	SP	IN
Prebiotic activity	11.47 ± 0.81	10.32 ± 0.77	12.98 ± 0.18
% Hydrolysis	2.51 ± 0.61	1.81 ± 0.63	0.10 ± 0.05

*PB: Phycocyanin extraction by-product from *Spirulina platensis*, SP: *Spirulina platensis*, IN: Inulin.

Growth of probiotic bacteria on different prebiotic sources

The optical density in this studied probiotic bacterial strains showed better growth within 48 hours, with an expected response curve showed the ability of phycocyanin extraction by-product to become an alternative prebiotic (Figure 1). At 0 hour before incubation, the optical density of *S. platensis* 0.8 % group showed high optical density value than other group with all bacterial strains except in *E. coli* ($p < 0.05$; Table 3). At 24 and 48 hours after incubation *L. johnsonii* (ck-3(showed the highest optical density value when used with inulin 0.8 % group than the other groups. ($p < 0.05$; Table 3 and Figure 1A). While *L. johnsonii* (ck-8(and *S. cerevisiae* showed the highest optical density value when used with *S. platensis* 0.8% group at 0, 24, and 48 hours ($p < 0.05$; Table 3 and Figure 1B and E). At 0 hours before incubation, the optical density of phycocyanin extraction by-product 0.8% group showed high optical density value than other group with *E. coli* strains (Table 3 and Figure 1D). However, the optical density value of *E. coli* strains at 24 and 48 hours of phycocyanin extraction by-product 0.8 % group showed low value than *S. platensis* 0.8 % group ($p < 0.05$; Table 3).

The strains of *S. aureus* at 48 hours after incubation, the optical density of inulin 0.4 % group showed high optical density value that other group ($p < 0.05$; Table 3 and Figure 1C). In the phycocyanin extraction by-product and *S. platensis* groups showed almost same growth rate with *E. coli* substance (Figure 1D). Furthermore, when comparing with the incubation times at 0, 24, and 48 hours after incubation, there was a significant difference in the optical density at 600 nm of the probiotic strain in all treatment ($p < 0.05$).

The pH value at 0 hour before incubation in different prebiotic sources was the same with each bacteria strain (Figure 2A). At 24 and 48 hours after incubation the high pH values showed the media without additional substrate group and media with Glucose 2 Mm group with *L. johnsonii* (ck-3) and *L. johnsonii* (ck-8) strains ($p < 0.05$; Figure 2B and C). While, at 24 and 48 hours after incubation the high pH values of *S. aureus* strains showed in media without additional substrate group than the other groups ($p < 0.05$; Figure 2B and C). The *E. coli* at 24 hours after incubation in *S. platensis* 0.4 % group showed the high pH value than the other groups ($p < 0.05$; Figure 2B). However, at 48 hours after incubation in without additional substrate group showed the high pH value than the other groups ($p < 0.05$; Figure 2C). The phycocyanin extraction by-product, *S. platensis*, and inulin group showed nearly pH value at 24 and 48 hours with *L. johnsonii* (ck-3) and *L. johnsonii* (ck-8) substance (Figure 2B and C).

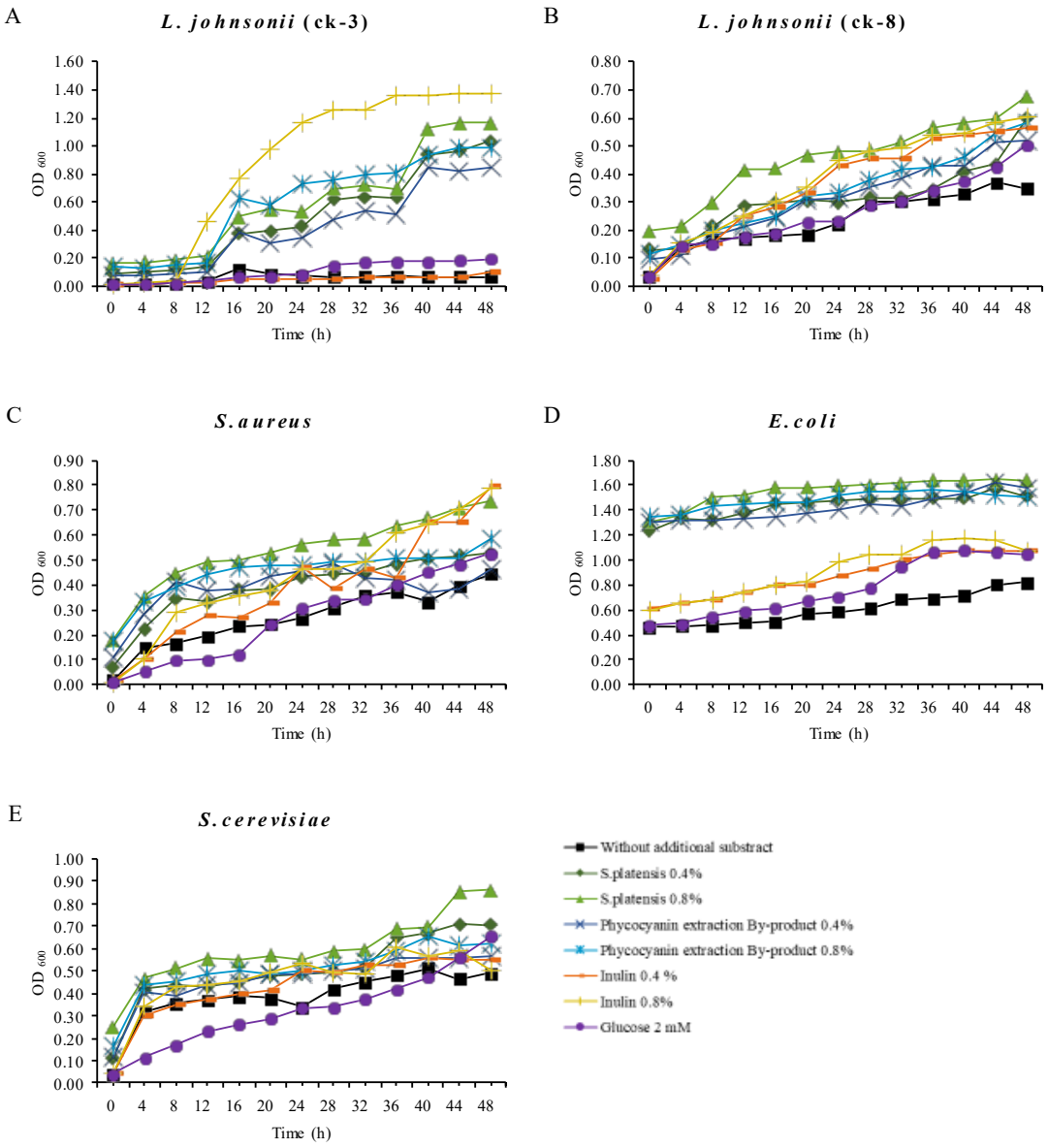

Figure 1. Probiotic activity of different prebiotic sources was expressed as optical density at 600 nm with A, *Lactobacillus johnsonii* (ck-3); B, *Lactobacillus johnsonii* (ck-8); C, *Staphylococcus aureus*; D, *Escherichia coli*; E, *Saccharomyces cerevisiae*. The value are the means. W: Buffered peptone water, SP0.4: *Spirulina platensis* 0.4%, SP0.8: *Spirulina platensis* 0.8%, PB0.4: Phycocyanin extraction by-product from *Spirulina platensis* 0.4%, PB0.8: Phycocyanin extraction by-product from *Spirulina platensis* 0.8%, IN0.4: Inulin 0.4 %, IN0.8: Inulin 0.8 %, G: Buffered peptone water with glucose 2 mM

Table 3. Effect of different prebiotic sources on optical density of microbial activity (600 nm)

Items	W	SP0.4	SP0.8	PB0.4	PB0.8	IN0.4	IN0.8	G	SEM	P-value
Lactobacillus johnsonii (ck-3)										
0 h	0.0152^{f}	0.0932^{c}	0.1658^{a}	0.0773^{d}	0.1411^{b}	0.0163^{e}	0.0157^{ef}	0.0166^{e}	0.0103	≤0.001
24 h	0.0753^{g}	0.4256^{d}	0.5292^{c}	0.3512^{e}	0.7353^{b}	0.0579^{h}	1.1689^{a}	0.0897^{f}	0.0646	≤0.001
48 h	0.0663^{h}	1.0367^{c}	1.1683^{b}	0.8502^{e}	0.9910^{d}	0.1013^{g}	1.3789^{a}	0.1971^{f}	0.0877	≤0.001
SEM	0.0079	0.1178	0.1249	0.0965	0.1073	0.0105	0.1807	0.0223		
P-value	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001		
Lactobacillus johnsonii (ck-8(										
0 h	0.0360^{e}	0.1309^{b}	0.1971^{a}	0.0980^{d}	0.1159^{c}	0.0246^{f}	0.0379^{e}	0.0352^{e}	0.0103	≤0.001
24 h	0.2238^{h}	0.3010^{f}	0.4800^{a}	0.3136^{e}	0.3363^{d}	0.4328^{c}	0.4481^{b}	0.2339^{g}	0.0164	≤0.001
48 h	0.3485^{g}	0.6031^{b}	0.6782^{a}	0.5230^{e}	0.5827^{c}	0.5625^{d}	0.6038^{b}	0.5064^{f}	0.0164	≤0.001
SEM	0.0387	0.0588	0.0595	0.0523	0.0574	0.0691	0.0719	0.0582		
P-value	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001		
Staphylococcus aureus										
0 h	0.0177^{e}	0.0725^{d}	0.1790^{a}	0.1069^{c}	0.1715^{b}	0.0172^{e}	0.0086^{g}	0.0122^{f}	0.0121	≤0.001
24 h	0.2662^{h}	0.4332^{f}	0.5626^{a}	0.4561^{e}	0.4750^{b}	0.4704^{c}	0.4627^{d}	0.3080^{g}	0.0162	≤0.001
48 h	0.4458^{h}	0.5263^{f}	0.7358^{c}	0.4622^{g}	0.5846^{d}	0.7947^{a}	0.7885^{b}	0.5281^{e}	0.0242	≤0.001
SEM	0.0529	0.0590	0.0701	0.0501	0.0526	0.0961	0.0964	0.0637		
P-value	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001		
Escherichia coli										
0 h	0.4622^{h}	1.2319^{d}	1.3065^{b}	1.3019^{c}	1.3516^{a}	0.6085^{e}	0.5991^{f}	0.4774^{g}	0.0691	≤0.001
24 h	0.5832^{h}	1.4827^{c}	1.5964^{a}	1.4074^{d}	1.5241^{b}	0.8727^{f}	0.9856^{e}	0.7073^{g}	0.0677	≤0.001
48 h	0.8248^{h}	1.5080^{c}	1.6424^{a}	1.5827^{b}	1.5043^{d}	1.0670^{f}	1.0735^{e}	1.0477^{g}	0.0521	≤0.001
SEM	0.0455	0.0376	0.0448	0.0349	0.0232	0.0567	0.0621	0.0706		
P-value	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001		
Saccharomyces cerevisiae										
0 h	0.0427^{e}	0.1157^{c}	0.2501^{a}	0.1172^{c}	0.1625^{b}	0.0451^{d}	0.0431^{e}	0.0434^{e}	0.0126	≤0.001
24 h	0.3399^{g}	0.4859^{f}	0.5507^{a}	0.4882^{e}	0.5036^{c}	0.4994^{d}	0.5361^{b}	0.3340^{h}	0.0140	≤0.001
48 h	0.4929^{h}	0.7051^{b}	0.8596^{a}	0.5651^{e}	0.6183^{d}	0.5479^{f}	0.5013^{g}	0.6563^{c}	0.0200	≤0.001
SEM	0.0564	0.0733	0.0750	0.0589	0.0583	0.0683	0.0677	0.0755		
P-value	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001		

[a-h] Means in a row with different superscripts differ significantly (P<0.05).
*W: Buffered peptone water, SP0.4: *Spirulina platensis* 0.4%, SP0.8: *Spirulina platensis* 0.8%, PB0.4: Phycocyanin extraction by-product from *Spirulina platensis* 0.4%, PB0.8: Phycocyanin extraction by-product from *Spirulina platensis* 0.8%, IN0.4: Inulin 0.4 %, IN0.8: Inulin 0.8 %, G: Buffered peptone water with glucose 2 mM

The growth of microbial was measured and described in colony number was showed in Table 4. The number of each microbial colony strain were not significant difference at initial value ($p > 0.05$). At 24 and 48 hours after incubation the high microbial colony number showed in the phycocyanin extraction by-product, *S. platensis*, and Inulin group with *L. johnsonii* (ck-3) and *L. johnsonii* (ck-8) strains ($p < 0.05$). Except the *S. cerevisiae* strain was showed significant difference microbial colony number at 48 hours. While, *S. aureus* and *E. coli* strain were not effect with different prebiotic sources ($p > 0.05$). However, when comparing with the incubation times at 0, 24, and 48 hours after incubation, there was a significantly difference in the microbial colony number of the probiotic strain in all treatment ($p < 0.05$).

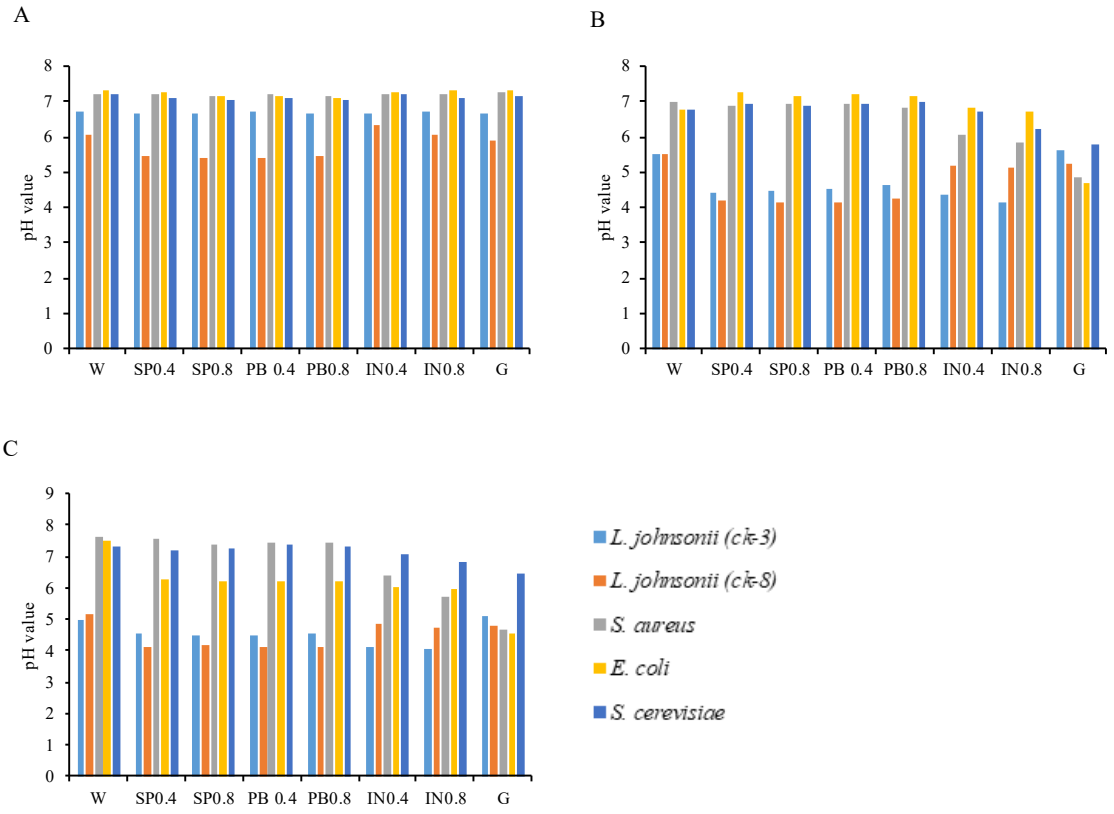

Figure 2. Determination of pH-value after incubation of bacterial strains with different prebiotic sources with A, 0 hour of incubation; B, after 24 hours of incubation; C, after 48 hours of incubation. The value are the means. W, Buffered peptone water; SP0.4, *Spirulina platensis* 0.4%; SP0.8, *Spirulina platensis* 0.8%; PB0.4, Phycocyanin extraction by-product from *Spirulina platensis* 0.4%; PB0.8, Phycocyanin extraction by-product from *Spirulina platensis* 0.8%; IN0.4, Inulin 0.4 %; IN0.8, Inulin 0.8 % and G, Buffered peptone water with glucose 2 mM.

DISCUSSION

The *S. platensis* and phycocyanin extraction by-product include a high concentration of crude protein 77.71 ± 1.31 %DM and 67.74 ± 0.27 %DM respectively (Table 1), which is in agreement with another research where a crude protein content was obtained under similar conditions. The researchers reported that using the quantitative Kjeldahl method, they found a protein content of 76% in *S. platensis* samples (Seghiri et al., 2019). The most carbohydrates contained in *S. platensis* were 25.39 ± 1.11 %DM and phycocyanin extraction by-product was 31.29 ± 0.42 %DM. This value was considerably higher than the carbohydrate concentration in previous reported which found the carbohydrate concentration at 6.46 ± 0.32% (Seghiri et al., 2019). The majority of the total carbohydrates in higher plants are composed of insoluble carbohydrates, whereas in seaweeds, soluble carbohydrates are compose the majority of the total carbohydrates (Kumar and Sahoo, 2017; Verma et al., 2017). The researchers (Bohn, 2014) reported that the non-soluble starch content, structure, and composition of plant samples from individual species (Lynch et al., 2016), could be a factor affecting the enzymatic degradation of agricultural residues as well as the different levels of hydrolysis was

found in this study. This result is consistent with other reports showing that whole dried microalgae can be used as food due to their higher carbohydrate and protein digestibility.

Table 4. Effect of different prebiotic sources on microbial colony number (log cfu $^{mL-1}$)

Items	W	SP0.4	SP0.8	PB0.4	PB0.8	IN0.4	IN0.8	G	SEM	P-value
Lactobacillus johnsonii (ck-3(										
0 h	2.803	2.725	2.7145	2.761	2.690	2.681	2.789	2.831	0.044	0.983
24 h	3.717^{b}	6.169^{a}	6.357^{a}	6.179^{a}	6.288^{a}	4.006^{b}	6.657^{a}	3.796^{b}	0.239	≤0.001
48 h	4.671^{c}	7.596^{ab}	7.720^{ab}	7.509^{ab}	7.618^{ab}	5.513^{bc}	8.365^{a}	4.857^{c}	0.346	0.016
SEM	0.5547	0.5074	0.5139	0.4828	0.5116	0.5167	0.5711	0.5787		
P-value	0.413	≤0.001	≤0.001	≤0.001	≤0.001	0.073	≤0.001	0.381		
Lactobacillus johnsonii (ck-8(										
0 h	2.406	2.304	2.296	2.315	2.325	2.310	2.348	2.347	0.049	0.997
24 h	5.892^{c}	6.178^{bc}	6.407^{ab}	6.154^{bc}	6.468^{ab}	6.787^{a}	6.867^{a}	6.009^{bc}	0.071	≤0.001
48 h	7.858^{c}	9.380^{ab}	9.601^{a}	9.284^{ab}	9.576^{a}	9.696^{a}	9.795^{a}	8.991^{b}	0.102	≤0.001
SEM	0.5589	0.7062	0.7294	0.6986	0.7255	0.7407	0.7476	0.6626		
P-value	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001		
Items	**W**	**SP0.4**	**SP0.8**	**PB0.4**	**PB0.8**	**IN0.4**	**IN0.8**	**G**	**SEM**	**P-value**
Staphylococcus aureus										
0 h	3.127	2.983	3.007	3.008	3.066	2.984	3.005	2.982	0.063	0.993
24 h	4.644	5.650	5.809	5.591	5.811	5.938	6.029	4.805	0.179	0.395
48 h	5.538	6.522	6.582	6.494	6.527	6.866	7.024	6.680	0.459	0.998
SEM	0.6682	0.5561	0.5635	0.5501	0.5525	0.5946	0.6049	0.6379		
P-value	0.351	0.015	0.014	0.016	0.015	0.010	0.010	0.050		
Escherichia coli										
0 h	2.754	2.746	2.758	2.718	2.742	2.768	2.717	2.767	0.036	0.997
24 h	5.265	5.486	5.618	4.444	4.717	6.048	6.093	6.212	0.178	0.095
48 h	5.596	5.490	5.639	5.230	5.333	6.483	6.677	6.714	0.298	0.826
SEM	0.4732	0.4725	0.4849	0.5044	0.5237	0.4091	0.4256	0.4288		
P-value	0.016	0.013	0.010	0.110	0.103	≤0.001	≤0.001	≤0.001		
Saccharomyces cerevisiae										
0 h	3.104	3.038	3.020	3.112	3.115	3.131	3.136	3.070	0.025	0.927
24 h	4.526	6.541	6.626	6.357	6.495	8.567	8.771	5.995	0.417	0.216
48 h	5.996^{b}	9.567^{a}	9.608^{a}	9.364^{a}	9.513^{a}	10.514^{a}	10.629^{a}	7.795^{ab}	0.354	0.012
SEM	0.8009	0.7687	0.7768	0.7386	0.7578	0.7598	0.7756	0.7781		
P-value	0.358	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	0.032		

$^{a-h}$ Means in a row with different superscripts differ significantly ($p<0.05$).
*W: Buffered peptone water, SP0.4: *Spirulina platensis* 0.4%, SP0.8: *Spirulina platensis* 0.8%, PB0.4: Phycocyanin extraction by-product from *Spirulina platensis* 0.4%, PB0.8: Phycocyanin extraction by-product from *Spirulina platensis* 0.8%, IN0.4: Inulin 0.4 %, IN0.8: Inulin 0.8 %, G: Buffered peptone water with glucose 2 mM

Generally, *S. platensis* was considered to be a dietary supplement that has various benefits for human health, and a feed supplement for animals. The authors reported that Lactulose, fructo-oligosaccharides, galacto-oligosaccharides, inulin and its hydrolysates, malto-oligosaccharides, and resistant starch are commonly prebiotics was utilized in human food (Gupta et al., 2017). Prebiotic activity

indicates the ability of a substrate to support the growth of organisms in parallel to other organisms and regard to growth on non-prebiotic substrates (Huebner et al., 2007; Rubel et al., 2014; Zhang et al., 2018). Consistent with this result, *S. platensis* and phycocyanin extraction by-product have prebiotic activity at 10.32 ± 0.77 and 11.47 ± 0.81 respectively (Table 2). It was similar to the prebiotic activity of inulin at 12.98 ± 0.18. Supplementing *S. platensis* containing diets may increase the lactobacillus population (Park et al., 2018; Khan et al., 2020; Alwaleed et al., 2021). Prebiotics are foods for probiotic bacteria. They are defined as low digestible ingredients that benefit the host organism by stimulating the growth performance or improving the activity of probiotic microorganism within the colon (Manning and Gibson, 2004; Gupta et al., 2017; Ashwini et al., 2019). As consequence, phycocyanin extraction by-product from *S. platensis* with their high potential is suitable for prebiotic use.

The combination of probiotics and prebiotics, known as synbiotics resulted in better bacterial survival than probiotics or prebiotics alone (Al-Khalaifah, 2018; Markowiak and Śliżewska, 2018). Zou et al. (2020) demonstrated that *L. johnsonii* has the capacity to decrease pathogen load and preserve metabolic balance. *L. johnsonii* is a probiotic strain that is cohesive onto intestinal epithelial cells and could produce bactericidal responses against destructive bacteria such as *S. aureus*, *S. enteritidis*, and *E. coli* (Bintsis, 2018; Wang et al., 2018a; Wang et al., 2018b). The prebiotic effect of the substrate could be measured as a selective effect on the encouragement of the main group of common bacteria in the gut. In particular, the selection of increased numbers of lactobacillus group was not compared to that of pathogen microorganisms (Castellano et al., 2017; Ding et al., 2019). Therefore, it is necessary to find the right combination of probiotics with prebiotics to maximize the health benefits. As a result, *S. platensis* and phycocyanin extraction by-product demonstrated this ability due to the growth-stimulating activity of the probiotic genus *Lactobacillus* as measured by the optical density of microbial activity (600 nm) (Figure 1 and Table 3) and microbial colony number (log CFU/ml) (Figure 3 and Table 4) compared to glucose and inulin. Samanta et al. (2015) and Piyadeatsoontorn et al. (2018) have described the numerous beneficial effects of prebiotic on human and livestock health, primarily through stimulating the growth of gut microbiota. Specifically, supplementation with probiotic genus *Lactobacillus* has been shown to enhance gas production *in vitro* at 12 and 24 hours. Furthermore, it increases rumen fermentation products and substrate degradability (Fitriyah et al., 2024). In contrast to *Lactobacillus* strain, the growth of the other probiotic bacteria strain *S. aureus* and *E. coli* were not improved when compared between prebiotic sources (Figure 1 and Figure 3). Truong et al. (2024) mentioned that the group of rabbits fed without probiotics exhibited a significantly higher fecal density of *E. coli* compared to those in the probiotic-supplemented group, highlighting the role of probiotics in modulating gut microbial populations. Pattananandecha et al. (2015) report that inulin extraction from artichoke could have an increased modulating effect on the probiotic activity, specifically for *L. plantarum*, whereas inhibitory effect against *S. enterica* and *E. coli*. In this study, the researchers assessed only bacterial strains and yeast species to study the alternative prebiotics potential. These observations are base on the similar pattern of *S. platensis*, phycocyanin extraction by-product, and inulin have the potentiality to stimulate the growth of probiotic microorganisms.

CONCLUSIONS

The phycocyanin extraction by-product from *S. platensis* could be effective as a prebiotic and promote the growth of probiotic strains, since their effect showed a better-defined result in optical density of microbial activity (600 nm), and the pH value was decreased. Especially, the phycocyanin extraction by-product can increase the microbial colony growth rate of *L. johnsonii* ck8 strain to 9.57 $\log_{10}$ CFU/ml at 48hours after incubation. Therefore, future studies should address the

question of whether synbiotic of phycocyanin extraction by-product would attenuate the impact on the growth performance, meat quality, and intestinal morphology of livestock.

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AUTHOR CONTRIBUTIONS

KH: Research implementation, statistical analysis, and drafted and revised the manuscript. **BM** and **WJ**: Designed and investigated the study. **JP**: Supervision of the study and critical revision of the manuscript for important intellectual content. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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