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FULL PAPER

Formulation of sprouted cowpea yoghurt rich in antioxidant, as functional drink for diabetics

[1,*]Winarsi, H., [2]Erminawati, E. and [1]Ramadhan, G.R.

[1]*Department of Nutrition, Faculty of Health Sciences, Universitas Jenderal Soedirman, Purwokerto, 53123, Central Java, Indonesia*
[2]*Department of Food Technology, Faculty of Agriculture, Universitas Jenderal Soedirman, Purwokerto, 53123, Central Java, Indonesia*

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Abstract

This study aimed to obtain a formula of Cowpea sprouted yoghurt (Cops-Yo) rich in phenolic antioxidants, fibre, vitamin C, and protein based on Lactic Acids Bacteria (LAB) levels and the proportion of sprouted cowpea milk (Cops-milk) and skim milk. This factorial study used a completely randomized design (CRD), with a factor of LAB levels and the proportion of sprouted cowpea milk and skim milk. Cops-Yo product was obtained and tested for phenolic content (Folin Ciocialteau method), fibre (oven method), vitamin C (Iodometry), and soluble protein (Lowry method). Data was tested using ANOVA, followed by DMRT if there was significance at a 5% level. The best Cops-Yo formula was obtained from a proportion of 70 parts of sprouted cowpea milk and 30 parts of skim milk with 0.3% LAB. The product has characteristics that contain 529.75 mg GAE/L of phenolic, 3.09% dietary fibre, 100.55 mg/100 g vitamin C, and 36.22% soluble protein. The result reflects that Cops-Yo could help improve sugar levels in diabetic patients.

1. Introduction

The incidence of type-2 diabetes mellitus (T2DM) increases from year to year and is mostly experienced at the age of more than 30 years (Gómez-Huelgas *et al*., 2018). Barengolts (2016) that oxidative stress (OS) contributes to the pathogenesis of diabetic complications, which interferes with glucose absorption (Salman *et al*., 2013), insulin secretion, and even causes damage to blood vessel walls (Wong *et al*., 2013). Oxidative stress is induced by hyperglycemia conditions. Antioxidant-rich functional foods are reported to be able to overcome OS and improve blood sugar levels (Winarsi *et al*., 2013; Burkard *et al*., 2017). One type of antioxidant-rich nut is Cowpea *(Vigna unguiculata subsp. Unguiculata)*.

Cowpea reported Thangadurai (2005) rich in protein (29.2%), fat 29.2%, fibre 4.9%, ash 3.6%, and energy 1737 KJ/100 g, and minerals of K 11.18 mg, Ca 22.63 mg, Na 96.4 mg, Zn 9.5 mg, Fe 4.47 mg, Cu 9.31 mg, Pb 2.24 mg, and P 421.58 mg/ 100 g (Gerrano *et al*., 2017). In addition to their potential as antioxidants, Cowpea has various pharmacological effects such as antimicrobial (Ashraduzzaman *et al*., 2016), hypocholesterolemic (Weththasinghe *et al*., 2014), and antidiabetic (Ashraduzzaman *et al*., 2011). The high Zn content proves that Cowpea has antioxidant potential; however, the acceptance of the beans is limited by the presence of beany flavour and anti-nutritional content.

Germination, in addition to minimizing the beany flavour, also increases the antioxidant and protein content (Winarsi *et al*., 2020). During germination, the constituent components of the bean are degraded, therefore the content of glucose, fatty acids, and amino acids increases. These compounds are very good for the growth of lactic acid bacteria (LAB) in yoghurt fermentation.

Many factors affect the production of yoghurt, including levels of LAB and milk. Yoghurt production is affected by several factors, including LAB levels and milk (dairy milk) addition. The study by Winarsi *et al.* (2022) used the proportions of sprouted mung bean milk and skim milk of 100:0, 90:10, 80:20, and 70:30, and LAB levels of 0.25 and 0.5%; found the best sprouted-mung bean yoghurt formula in the proportion of 70:30 and 0.5% LAB. Winarsi *et al*. (2019) reported that the best sprouted-red bean yoghurt formula with the proportion of 90% sprouted-red bean milk and 10% skim milk, with 2% LAB content and 24 hrs fermentation,

*Corresponding author.
Email: *winarsi12@gmail.com*

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containing 993.08 ppm phenolic antioxidants and 6.29% fibre. Based on the regulations of the Codex Alimentarius Committee 2003, the levels of LAB used in probiotic drinks are 2-6%. Winarsi *et al*. (2019) added that the use of varying levels of LAB affects the antioxidant content in yoghurt. The problem is the LAB level and the proportion of sprouted cowpea milk and skim milk to obtain yoghurt rich in phenolic antioxidants, vitamin C, fibre, and soluble protein. This study aimed to determine the effect of LAB levels and the proportion of sprouted cowpea milk and skim on the levels of phenolic antioxidants, vitamin C, fibre, and soluble protein of Cowpea sprout yoghurt (Cops-Yo). This research also aimed to get the best Cops-Yo formula.

2. Materials and methods

The research design used a factorial randomized block design. There are two factors; the proportion of Sprouted cowpea milk and skim milk (S) with 4 levels; S1 = 100:0; S2 = 90:10; S3 = 80:20; S4 = 70:30; and levels of LAB (B) with 2 levels; B1 = 0.3%; B2 = 0.25%), with 3 replications, so a total of 24 experimental units.

2.1 Production of sprouted cowpea milk yoghurt

This method was conducted according to Winarsi *et al*. (2022) with slight modifications. The cowpea was washed, soaked for 10 hrs, drained, and germinated for 12 hrs at room temperature without light. With a ratio of 1: 8, the sprouts and water were blended until smooth, then filtered is called Cowpea sprout milk (Cops-milk). To each proportion of skimmed milk, 10% sucrose was added, then pasteurized at 80-90 \°C for 10 mins, and then cooled to 45°C. The addition of starter LAB was 0.25% and 0.3% of total volume, then incubated at room temperature for 24 hrs, and then Cops-Yo was stored in the refrigerator.

2.2 Determination of phenolic content of Cops-yo

A total of 1 mL of Cops-Yo was put into a test tube and 4 mL of distilled water was added. An aliquot of 0.2 mL of Folin-Ciocalteu reagent was added to it and allowed to stand for 5 mins. A total of 1 mL of 5% Na_2CO_3 was added to the reaction mixture and allowed to stand for 60 mins at room temperature and in the dark. The absorbance was read at a wavelength of 747 nm (Orak, 2006).

2.3 Determination of vitamin C content of Cops-yo by Iodometry method

As much as 10 mL of Cops-Yo was placed in a 50 mL volumetric flask and distilled water was added. Approximately, three drops of 1% starch solution were then added and titration was carried out using a standard Iodine solution until the final solution turns blue (Techinamuti and Pratiwi, 2018).

2.4 Determination of fibre content

The Cops-Yo fibre content was measured using the SNI 01-2891-1992 method. A total of 5 g of Cops-Yo was put into a 50 mL Erlenmeyer, 0.313 N H_2SO_4 was added, then covered with aluminium foil and boiled for 30 mins. About 50 mL of 0.255 N NaOH was then added and covered with aluminium foil, then boiled again for 30 mins. It was filtered using a previously weighed filter paper. The filter paper was doused with 1.25% hot H_2SO_4, hot water, and 96% ethanol. The filter paper was placed in a petri dish and heated in the oven. After that, the filter paper was weighed and the residual weight was calculated, using the final weight of the filter paper being reduced by the initial weight therefore the amount of fibre in the Cops-Yo was obtained.

Fibre content (%) = residual weight (g) / sample weight (g) × 100%

2.5 Determination of dissolved protein content by Lowry method

The sample was diluted 10 times first, then 1 mL was taken and added to 10 mL of distilled water. Six mL of Biuret solution was added to 4 mL of the sample solution and allowed to stand at 37°C for 10 mins. The absorbance was read at a wavelength of 600 nm using a UV-Visible spectrophotometer (Goretti and Purwanto, 2014).

2.6 Data analysis

The data obtained from the results of this study were continued by testing using ANOVA. If there is a significant difference with an error of 5%, then proceed with Duncan's Multiple Range Test (DMRT).

3. Results and discussion

3.1 Effect of LAB levels and proportion of sprouted cowpea milk – skim milk on phenolic content of Cops-Yo

The higher the LAB level, the higher the phenolic content of Cops-Yo, from 402.81±153.44 to 581.67±164.41 mgGAE/L (p = 0.041). During fermentation, the LAB releases microbial enzymes that produce free-form chemical compounds, such as phenolics. Similarly, when Cowpea is germinated, spontaneous fermentation occurs, and phenolic compounds are also formed, especially when continued with LAB. Therefore, the phenolic content of Cops-Yo is higher than that of Cops-milk. In this case, LAB also contributes to the synthesis of phenolic compounds. The most abundant component found in phenolic compounds is oleuropein, which is then hydrolyzed into aglycone form by glucosidase produced by LAB. Furthermore, these aglycone compounds are metabolized into hydroxytyrosol and tyrosol acids by LAB. Ozcan and Ekinci (2016) reported that these two compounds were the most commonly detected phenolic compounds.

The LAB can metabolize phenolic compounds, which in the process can increase LAB biomass, by supplying energy, thereby optimizing LAB nutrient consumption, and providing a stimulating effect on the growth of *Lactobacillus acidophilus* (Ozcan and Ekinci, 2016). Lactic acid bacteria can form organic acids, such as lactic acid, which are then converted into phenolics through cinnamic acid and ferulic acid (Primurdia and Kusnadi, 2014). Phenolic acid is a derivative of cinnamic acid or benzoic acid (Awika and Doudu, 2016).

Lactic acid bacteria such as *L. plantarum* and *L. brevis* contain the enzyme phenolic acid decarboxylase (PAD) which can hydrolyze phenolic acid, hydroxycinnamic acid, and hydroxybenzoic acid into phenolics. *Lactobacillus plantarum* bacteria can metabolize hydroxybenzoic acid in food by the PAD enzyme. Meanwhile, *L. brevis* degraded hydroxycinnamic acid into catechol and pyrogallol. Ozcan and Ekinci (2016) confirmed that pyrogallol is the most powerful antioxidant found in simple phenolic compounds.

Nuts, including cowpea, contain phenolic acids, flavonoids, and tannins. These compounds are widely distributed in the seed coat and cotyledons. Therefore, the skin used in the manufacture of Cops-Yo can increase the phenolic content. This finding was reinforced by Winarsi *et al*. (2019) that the phenolic content of skinned red bean sprouted milk was higher than that of skinless red bean sprouted milk.

In producing Cops-Yo, 10% of sugar of the total volume was added to it. The presence of added sugar can stimulate the growth of LAB. Furthermore, LAB breaks down sugar into primary metabolites (lactic acid) and secondary metabolites (polyphenols) (Primurdia and Kusnadi, 2014), therefore phenolic levels increase.

The germination process can increase phenolic compounds. This can be attributed to the availability of water and the activity of enzymes in it. Absorption of water at the time of germination can hydrate the beans, and then activate the enzymes amylase, protease, and lipase. The starch, protein, and lipids of Cowpeas are degraded into simpler compounds. These compounds become nutrients for the synthesis of new compounds, namely phenolics (Xu *et al*., 2018) therefore their levels increase. During the germination process, phenolic acids and flavonoids act as precursors for the synthesis of plant tissues such as lignin, which is a part of phenolics. Therefore, the content of phenolic compounds in Cops-Yo increases. In addition, the content of gentisic acid and trihydroxyflavones increased during the germination process, both of which are phenolic compounds that contribute to the increase in antioxidant levels.

Meanwhile, the proportion of Cops-milk did not affect the phenolic content of Cops-Yo ($p = 0.428$), as well as the combination of LAB levels and the proportion of Cops-milk and skim also did not affect the levels ($p = 0.863$). During the fermentation process, LAB requires nutrients for growth, cell formation, and the synthesis of metabolite products (Zubaidah *et al*., 2010). For its growth, organic substances such as carbohydrates, proteins, and fats are needed as a source of energy. The availability of macronutrients derived from Cops-milk and skim milk, may not meet their nutritional needs, therefore growth is not optimal, as well as phenolic synthesis.

3.2 Effect of lactic acid bacteria levels and proportion of sprouted cowpea milk – skim milk on the dietary fibre content of Cops-Yo

LAB levels increased the fibre content of Cops-Yo, from 1.57±0.24 to 2.15±0.56% ($p = 0.018$). LAB growth can produce cellulose can produce high fibre supported by Winarsi *et al*. (2019). In addition, the epidermis carried during the yoghurt-making process can also increase the fibre content.

In the fermentation process, LAB undergoes growth and increases metabolic processes. In its metabolism, LAB converts sugar into cellulose, therefore, the longer the fermentation process, the greater its ability to produce cellulose (fibre) (Jasmine *et al*., 2020). The germination process activates enzymes that can break down complex compounds into simpler ones; amylase enzymes convert carbohydrates into glucose (Saputro *et al*., 2015). Increased glucose levels can increase the cellulose of legumes during the germination process (Benítez *et al*., 2013). The increase in fibre content is related to the occurrence of structural carbohydrate synthesis, such as cellulose and hemicellulose which are the largest fibre components (Shah *et al*., 2011).

Unlike the case with the proportion of Cops-milk and skim, which did not affect the fibre content of yoghurt ($p = 0.194$), similarly what happened to the combination of LAB levels and the proportion of Cops-milk and skim also did not affect its content ($p = 0.594$). Skim milk, which is the substrate for LAB, does not contain fibre, therefore, the higher proportion of skim milk does not change the fibre content of Cops-Yo. The main content of skim milk is protein, so the availability of carbohydrates to be converted by LAB into cellulose is inadequate.

3.3 Effect of lactic acid bacteria levels and proportion of sprouted cowpea milk – skim milk on soluble protein levels of Cops-Yo

In this study, LAB levels increased the soluble protein content of Cops-Yo, from 27.99 to 33.46% ($p = 0.024$). The more LAB is used, the more the microbial population increases (Khoiriyah and Fatchiyah, 2013), therefore, the metabolic products produced are also higher. The metabolic activity of LAB increases the growth of LAB (Undugoda *et al*., 2019). The LAB consists of *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *Lactobacillus acidophilus*. Amani *et al*. (2017) explained that the combination of *L. bulgaricus* and *S. thermophilus* in making yoghurt has high proteolytic activity. During the fermentation process, LAB produces protease enzymes and degrades proteins into simple amino acids (Anggraini *et al*., 2018). Ahtesh *et al*. (2017) added that the level of the proteolytic activity of LAB depends on the length of fermentation. The Cops-Yo products were fermented for 24 hrs and the possibility of LAB growth was optimal (Isleten and Karagul, 2008), therefore, its proteolytic activity is also optimal and increases the levels of dissolved protein in yoghurt. Soluble protein is part of the total protein contained in water-soluble foodstuffs (Andarwulan *et al*., 2012). Soluble protein has less than 10 amino acid chains, it is easier for humans to digest.

The germination process of Cowpea also increases the soluble protein content (Fouad *et al*., 2015). Germination is a spontaneous fermentation process, without the addition of microorganisms in the process. Fouad and Rehab (2015) explained that the germination process increases the activity of breaking down complex compounds in beans that are not water-soluble to become water-soluble. During the germination process the protease enzyme was activated and hydrolyzed protein into amino acids which had a smaller molecular weight and were water-soluble (Anggraini *et al*., 2018). Therefore, the germination process in Cowpea also plays a role in increasing the soluble protein content of yoghurt.

The proportion of Cops-milk and skim milk increased soluble protein content compared to control ($p = 0.03$), but the proportions 90:10, 80:20, 70:30 there was no difference ($p = 0.644$) (Figure 1). The soluble protein content of Cops-Yo increased along with the increase in the proportion of skimmed milk because the lactose in the skim became the substrate for LAB. During growth, LAB breaks down protein into peptides and simple amino acids (Kusnadi *et al*., 2015), the more skim milk is used, the more Cops-Yo soluble protein increases.

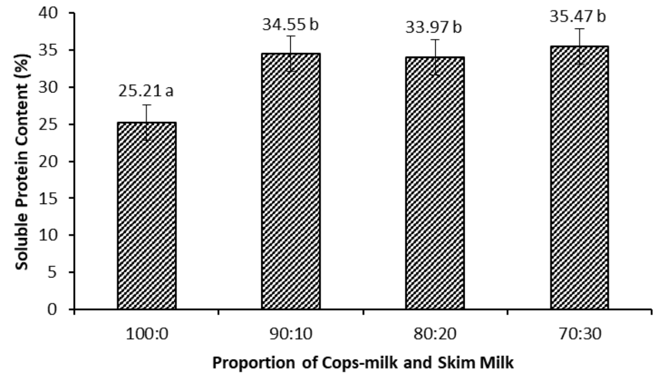

Figure 1. The effect of the proportion of Cops-milk and skim milk on the soluble protein of Cops-Yo. Values with different alphabets are significantly different ($\alpha = 0.05$). Cops-milk: Cowpea sprout milk, Cops-Yo: Cowpea sprout milk yoghurt.

The combination of LAB concentration and the proportion of skim milk had no significant effect on the soluble protein content of Cops-Yo ($p = 0.498$). However, there was a tendency for an increase in soluble protein content along with an increase in LAB concentration and the proportion of skim milk. This happens, because LAB has 4 growth phases, including the lag and log phases. In the lag phase, the bacteria adapt to the new growth medium, there has not been an increase in the number of bacteria, but metabolic activity begins to occur although it is not optimal. Significant bacterial growth occurred in the log phase. In this phase, there is also proteolytic activity by LAB (Purkan *et al*., 2017). LAB breaks down proteins into water-soluble peptides and simple amino acids.

Metabolic activity in LAB depends on the growth medium (Undugoda *et al*., 2019). Lactose in skim milk is a good medium for LAB growth. It is possible, that the utilization of lactose by LAB is not optimal, so the activity of protein hydrolysis by LAB is also not optimal and ultimately does not affect the levels of dissolved protein in Cops-Yo.

3.4 Effect of lactic acid bacteria Levels and proportion of Cops-milk and skim milk on vitamin C levels of Cops-Yo

LAB levels are affected by the increasing vitamin C levels of Cops-Yo ($p = 0.034$) (Figure 2). The results of this study are in line with Kaprasob *et al*. (2017) who found that fermentation using LAB increases vitamin C levels. Khasanah *et al*. (2017) in their research reported that *L. acidophilus* bacteria could increase the vitamin C content in yoghurt with the addition of Tawangmangu orange powder.

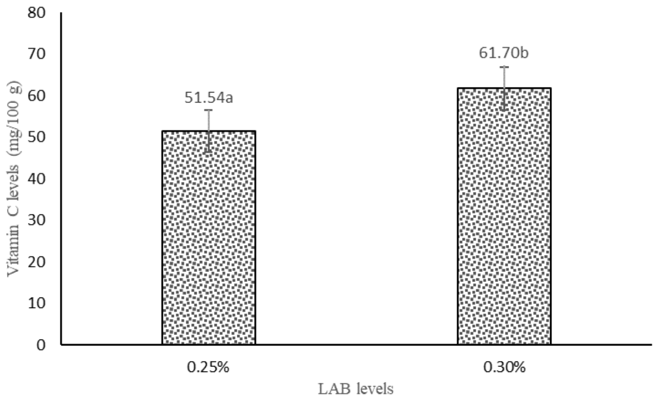

Figure 2. Effect of LAB Levels on Vitamin C Levels of Cops-Yo. Values with different alphabets are significantly different (α = 0.05). Cops-Yo: Cowpea sprout milk yoghurt.

Another study by Palupi *et al*. (2020) showed that *L. bulgaricus* also played a role in increasing vitamin C content. There was an increase in vitamin C levels in yoghurt because, during fermentation, LAB synthesizes lactic acid (Degrain *et al*., 2020), which affects the taste of the product (Zhou *et al*., 2020). The sour taste produced by LAB activity indicates the presence of vitamin C (Palupi *et al*., 2020). The increased production of lactic acid during fermentation makes the conditions acidic and maintains the viability of LAB and inhibits the autoxidation reaction (Filannino *et al*., 2012; Palupi *et al*., 2020). Therefore, increasing the concentration of BAL increases the levels of vitamin C Cops-Yo, which is an antioxidant.

The opposite happened from the effect of LAB levels, that the higher the proportion of skim milk or the lower the proportion of Cops-milk, the lower the vitamin C content of Cops-Yo (p = 0.002) (Figure 3). The germination process in Cowpea causes the enzyme L-Galactono-1,4-lactone dehydrogenase (GLDH) to be activated and oxidizes L-galactono-1,4-lactone to

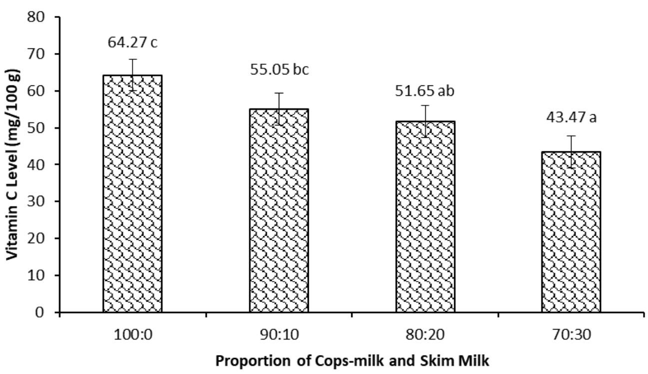

Figure 3. Effect of Proportion of Cops-milk and skim milk on vitamin C levels of Cops-Yo. Values with different alphabets are significantly different (α = 0.05). Cops-milk: Cowpea sprout milk, Cops-Yo: Cowpea sprout milk yoghurt.

ascorbic acid (Vitamin C) (Masood *et al*., 2014). Therefore, the use of less and less proportion of Cops-milk reduces the vitamin C content of Cops-Yo.

Furthermore, the addition of sucrose to Cops-milk also contributed to the increase in vitamin C levels of Cops-Yo. LAB uses sucrose as a growth medium (Zhou *et al*., 2021), and synthesizes lactic acid (Kaprasob *et al*., 2017). The production of lactic acid results in an acidic condition of yoghurt, which indicates the presence of vitamin C (Palupi *et al*., 2020). Therefore, the proportion of Cops-milk results in more yoghurt products with higher levels of vitamin C, and conversely the lower proportion also reduces vitamin C levels.

The combination of LAB levels and the proportion of Cops-milk and skim milk reduced the vitamin C content of Cops-Yo (p = 0.001) (Figure 4). The use of more and more Cops-milk increased the vitamin C content. The proportion of 70:30 with 0.3% LAB level had the highest vitamin C content of 76.05 mg/100 g. Palupi *et al*. (2020) stated that the LAB population increased along with the increase in *Indigoferaa zollingeriana*. In this study, more use of Cops-milk increased the levels of vitamin C. The high levels of vitamin C became a good medium for the growth of LAB (Tayo and Akpeji, 2016). During LAB fermentation produces organic acid (lactic acid), therefore the more LAB contained in yoghurt, the higher the lactic acid (Purbowati *et al*., 2021). Increased production of lactic acid creates an acidic environment. This can be advantageous because the oxidation rate of vitamin C decreases under acidic conditions. In addition, the sour taste resulting from the activity of lactic acid synthesis by LAB also indicates the presence of vitamin

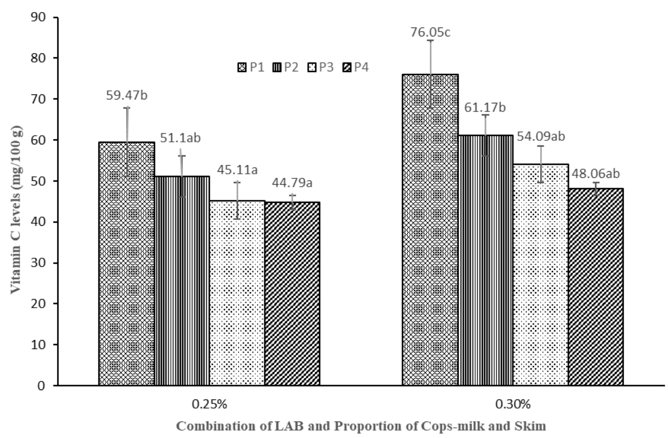

Figure 4. Effect of combination of LAB levels and proportion of Cops-milk and skim milk on Vitamin C levels of Cops-Yo. Values with different alphabets are significantly different (α = 0.05). Cops-milk: Cowpea sprout milk, Cops-Yo: Cowpea sprout milk yoghurt, P1: Proportion of Cops-milk and skim milk: 100:0, P2: Proportion of Cops-milk and skim milk: 90:10, P3: Proportion of Cops-milk and skim milk: 80:20, P4: Proportion of Cops-milk and skim milk: 70:30.

C in it.

Glucose in Cops-milk also plays a role in increasing Cops-Yo vitamin C levels. The LAB converts D-Glucose to D-glucuronolactone, then to L-gluconolactone, and finally, L-ascorbic acid is formed (Nkhata *et al*., 2014). Therefore, the higher the proportion of Cops-milk, the higher the vitamin C content; on the other hand, the higher the proportion of skim, the lower the vitamin C content of the Cops-Yo.

3.5 The best formula of Cops-Yo and its potential

The best formula of Cops-Yo was determined using the effectiveness index test (de Garmo *et al*., 1994). Selection of the best formula based on the content of phenolic, fibre, vitamin C, and soluble protein. Based on the effectiveness index test, the best formula was obtained, Cops-Yo made with 0.3% LAB level, and the proportion of Cops-milk and skim milk was 70:30. The best Cops-Yo product contained 529.75 mg GAE/L phenolic, 3.09% dietary fibre, 100.55 mg/100 g vitamin C, and 36.22% soluble protein.

Phenolics are compounds produced by plants that act as natural antioxidants. When the oxidation reaction takes place, phenolics can stabilize phenoxy radicals by donating hydrogen atoms to free radicals (Dhurhania and Novianto, 2018). Phenoxy radicals are compounds that, when resonated in the phenolic ring, are stable and do not form new free radicals, thereby stopping the chain oxidation reaction (Purbowati *et al*., 2018). Diabetes mellitus is a metabolic disease that is triggered by high levels of free radicals.

The Cops-Yo dietary fibre is 3.09%. Azrimaidaliza (2011) states that the consumption of fibre-rich food products in adequate amounts provides metabolic benefits in the form of controlling blood glucose. Mir and Griffing (2016) added that the consumption of high-protein foods can also help control blood glucose by improving insulin response in the body. It was also mentioned that vitamin C supplementation controls hyperglycemia and lowers blood pressure in hypertension, namely by increasing the formation of prostaglandin E1 (PGE1), PGI2 (prostacyclin), endothelial nitric oxide (eNO), and normalizing essential fatty acid metabolism (Das, 2019). Afkhami-Ardekani and Shojaoddiny-Ardekani (2007) also reported that T2-DM patients who were given a 1000 mg vitamin C supplement for 6 weeks had significantly decreased fasting blood sugar, triglycerides, LDL, HbA1c, and serum insulin levels. Cops-Yo may be rich in phenolic antioxidants, fibre, vitamin C, and protein, which is beneficial for people with diabetes.

4. Conclusion

The LAB levels increase the content of phenolic, fibre, vitamin C, and soluble protein of Cops-Yo. The proportion of Cops-milk and skimmed and its combination with LAB levels lower Cops-Yo vitamin C levels. The best Cops-Yo formula is made from 70 parts of Cops-milk and 30 parts of skim and has a LAB content of 0.3%. It contains 529.75 mg GAE/L phenolic, 3.09% dietary fibre, 100.55 mg/100 g vitamin C, and 36.22% soluble protein. Based on its content, Cops-Yo is useful for improving sugar levels and the antioxidant status of diabetics.

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