Effect of Different Bacterial Composition of Fermented Cow Milk and Soy Milk on Epidermal Growth Factor and Epidermal Thickness in Female Wistar Rats

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Abstract

Background Skin aging stimulates higher regression of proliferation and architecture of the skin tissue. Isoflavone has been used for countering aging and shown its effect to alter the progression of skin aging, while probiotics have an ability to boost the absorption of nutrient and also have its own ability to maintain the skin tissue. However, there is limited information about the difference of effect between various formulation of fermented cow milk and soy milk towards the skin regeneration via epidermal growth factor (EGF) signalling.

Results Our data showed that there are differences characteristic of stool and its macroscopic appearance after different fermented formula of cow milk and soy milk treatment. Histology results showed that skin epidermal thickness was found thicker in every group tthe same trend as the EGF protein levels.Composition of milk and soy milk fermented by *Bifidobacterium bifidum* potentially stimulate EGF protein levels. This finding inline with

Conclusions Milk fermented by *Bifidobacterium bifidum* showed the greatest effect for the skin tissue regeneration and it might prevent the skin aging process compared to the other formulations. Compared to conventional fermented milk, the composition of soy milk might enhance this positive effect of fermented milk to the skin.

1. Introduction

The epithelial of mammalian skin is a self-regenerating tissue that provides the first line defense mechanism between an organism and its environment [1]. Epidermis should maintain homeostasis between proliferation and differentiation to perform this function greatly [2]. The basal layer borders with an ECM-rich basement membrane below. Proliferative keratinocytes lie in basal layer. These cells express genes encoding integrins, Epidermal Growth Factor Receptor (EGFR; also referred to as ErbB1), and also the structural keratins 5 and 14 (K5 and K14) [3]. Well-ordered transcriptional regulation of those genes can control the epidermal homeostasis [4]. Skin homeostasis rules an important factor in physiological aging process.

Aging is initiated when our body lost its homeostasis control in the cell and regression process more takes place rather than growth. Aging is a slow process that contributes to deterioration of cells and tissues functions, and it could be initiated by both genetics and environment factors contribute to the event of aging [5]. Aging process is happens in every tissue of an organism with no exception include human skin tissue. Skin aging is usually characterized by wrinkles, unwanted spots, and decrease in elasticity. The rise of skin collagen degradation rate makes the collagen structure fragmented. Skin aging happens when the degradation of collagen reduces mechanical tension of the tissue [6].

In human, skin aging become a important issue in modern society because everyone want to keep their beauty and stay young. Thus, there is growing demand of alternative treatment for skin health and aesthetic like : nutrition, exercise, stress management, medication, plastic surgery, etc. Nutrition is one of

the friendly and non invasive approach to counter the skin aging. Recently, fermented milk could addres those expectation, however the link mechanism is remain unclear.

Michelle D. Holmes had reported that dietary behaviour which higher energy, protein, and milk content were associated with higher levels of IGF-I and IGFBP3 levels **(7).** In addition, dietary modification of intestinal microflora by yogurt or Lactobacilli supplementation reduce fecal β-glucuronidase activity, and correlated with estrogen metabolism **(8)**. Thus, by facts that fermented milk could alters and stimulated increase ratio of good gut microbiota and interestingly growth factor like IGF-1, it is interesting to explore its role on Epidermal growth factor (EGF) in the skin.

EGF is an important factor for maintaining normal skin growth, regeneration and indirectly helps its physiological function. EGF is a facilitator of the construction of skin tissue through dermal regeneration, proliferation, differentiation, and migration of keratinocyte, endothelial cells, and fibroblast [9]. Interaction between EGF and the EGFR initiates downstream signalling pathways that induce proliferation, differentiation, survival, or motility of the cell [10]. Attachment and binding of EGF ligand to EGFR induces the dimerization of receptor. The process of dimerization then activates phosphorylation of the intracellular domain of tyrosine kinase [11]. Phosphorylated tyrosine kinase then activates various downstream signalling pathways including Ras/MAPK, PLCγ1/PKC, PI(3)kinase/Akt, and STAT pathways [12]. The pathways of the EGF activation have been known to affect the skin physiology. Phospholipase C (PLC) pathway elevates intracellular calcium[13] which improves the formation of tight junction and stratum corneum barrier [14]. PI3K/Akt pathway increases the level of type I procollagen [15] which has an effect of minimalizing intrinsic skin aging [16]. Meanwhile, Src pathway promotes the expression of MMP-1, resulting in the collagen degradation in human fibroblast cells [17] .

Source of fermented milk might determine the nutrient content, and its fermented content results will be correlated with EGF. For instance, Soy, *Glycine max*, contains the great number of phytoestrogen and polyphenols [18]. One of the phytoestrogen is isoflavone. Isoflavone induces high expression of EGFR, thereby improving wrinkle reduction and collagen stimulation [19]. A conduction of cell culture experiment showed that isoflavones had increased collagen, elastin, and tissue inhibitor of metalloproteinases (TIMP) genes expression [20]. Soybean can be processed as soy milk by soaking, draining and grinding and filtering [21]. Various combinations of cow and soya milk fermented by various bacteria have been known to have ability to increase surface area of villi and villus height of the intestine, thus increasing intestinal absorptive function [22]. Probiotics are living microorganisms in gastrointestinal tract which give benefits to the host [23]. Overexpression of pro-inflammatory cytokine IL-6 tends to happen in the aging gut, which affects the inflammatory rate in aging skin [24]. It happens because aging gut shows lower microbial biodiversity, which is associated with an increase of pro-inflammatory bacteria and decrease of beneficial bacteria [25]. Probiotics can improve the condition of -microbial dysbiosis in aging gut and establish a health-promoting strains. Consumption of probiotics consisting Galacto-oligosaccharides (GOS) increases CD44, TIMP-1, and type 1 collagen, which are the markers of dermal cell adhesion and matrix formation, thus improving the rejuvenation of skin. GOS switching unfavourable

amino acid metabolism and phenols production by becoming an additional source of metabolism for gut microbials [26].

Another source is cow milk instead of soy, however there is a different between cow and soy. Formula and bacteria type for fermentating cow milk is different with soy milk. Lactic acid bacteria is used for fermentation because it easily convert lactose into glucose and galactose of cow milk. Others bacteria are also commonly used for the milk probiotics, like *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* [27]. Some products replace *Lactobacillus bulgaricus* with *Bifidobacterium spp.* due to the difference of metabolism isomer product. *Bifidobacterium spp.* produces the isoform of D-(+)-lactic acid which has an active effect in physiology. Meanwhile, *Lactobacillus bulgaricus* produces D-(-)-lactic acid which is physiologically inactive [28]. The digestion of lactose can be enhanced by the presence of *Streptococcus thermophiles* through the activity of its microbial β-galactosidase [29].

By seeing this importance, combination of *Bifidobacterium spp* and *Lactobacillus acidophilus* has shown the potential to increase increase the activity of lipase and protease [30]. The yoghurt formulation of *Lactobacillus acidophilus* and *Bifidobacterium spp.* also increase the non-pathogenic population and decrease the pathogenic bacteria in colon [31] In the other hand, adding beneficial microorganisms to the gut could prevent or delay some age-associated diseases by improving the immune response or by producing bioactive metabolites. Unfortunately, there is limited information about effect of formulated fermented milk and green bean on skin physiological homeostasis .

Thus, in this present study, we want to explore different formulation of bacteria starter in cow milk and soy milk for initiating fermentation process and stimulates optimal formula to skin maintenance.

2. Materials And Methods

2.1. Fermented soy milk and cow milk

Four different formulations of fermented soy milk and cow milk were obtained from Faculty of Animal Husbandry, Universitas Padjadjaran. All of the formulation was given with the dosage of 1.25% of body weight. Each millimeter of the formulation contained 10^7 probiotics. Four formulation used in this study were shown on the Table.

Table 1
Different composition of fermented cow milk and soymilk.

No	Group	Composition	Probiotics
1	P0	Control	-
2	P1	Cow Milk	*Lactobacillus bulgaricus* *Streptococcus thermophiles* *Lactobacillus acidophilus*
3	P2	Cow Milk 75% + Soymilk 25%	*Lactobacillus bulgaricus* *Streptococcus thermophiles* *Lactobacillus acidophilus*
4	P3	Cow Milk 50% + Soymilk 50%	*Lactobacillus bulgaricus* *Streptococcus thermophiles* *Lactobacillus acidophilus*
5	P4	Cow Milk	*Lactobacillus acidophilus* *Bifidobacterium bifidum*

2.2. Animal treatment

The treatment given to animal including handling, maintenance, and euthanasia were performed after approved by the Ethics Committee, Faculty of Medicine, Universitas Padjadjaran with registration number 1088/UN6.KEP/EC/2019. This experiment used 25 female Wistar rats aged 12 weeks. Before conducted in the experiment, the rats were bred in the Animal Facility of PT Bio Farma, Indonesia. The rats were kept in condition of 24 °C experiment under the 12 hour dark and light cycle. Food and water were given ad libitum in Animal Laboratory, Physiology Division, Faculty of Medicine, Universitas Padjadjaran.

The rats were randomly divided into control and treatment group. Four different formulations of fermented soy milk and cow milk were given to the treatment group for 12 weeks, and water was given to the control group by gavaging. The dose administered to the treatment group was 1.25% of body weight. The treatment group was divided into four groups (P_1, P_2, P_3, and P_4) based on the formulation. Treatment and water were given daily every morning at the same time for 12 weeks. The control group was given normal diet. The rat was sacrificed using carbon dioxide anaesthesia, then dorsal skin was obtained, weighed, and rapidly frozen in liquid nitrogen and stored at − 80 °C until use for analysis.

2.3. Protein extraction and western blot

About 25 mg for each of the stored samples was taken and stored in an Eppendorf tube. About 300 µL of ice cold lysis buffer was then added rapidly to the tube. After being homogenized, the samples were centrifuged in 12,000 rpm at 4 °C. Supernatant were then aspirated and stored before analysed by

Western blotting. 10 uL of protein were separated on SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare) by electrophoresis for two and a half hour. 2% blocking reagent (GE Healthcare) were added to the membrane and incubated at 4 ^{O}C overnight. Membrane immunoblotting was conducted using primary antibodies: EGF (Ab77851) purchased from Abcam, and β-actin (MA5-15739) were purchased from Thermo Scientific. The visualization was enhanced by chemiluminescence reagent (GE Healthcare) and imaged by LI-COR C-DiGit Chemiluminescence Western Blot Scanner. Protein band thickness were analysed using ImageJ software (NIH). β-actin antibody was used as internal control for protein levels monitoring.

2.4. Histological analysis

The skin samples were cut into about 0.3 × 0.4 × 0.5 cm in size and put into the formalin for 24 hours. After being dehydrated, the samples were set into paraffin block and cut by microtome, before being rehydrated and stained by hematoxylin and eosin.

The observed skin tissue specimen was examined by AxioImager Z2 microscope using Brigfield lighting technique. The image of the tissue was taken by Axiocam MR3 microscope camera. The image then reviewed in ZEN 2 PRO software.

2.5. Statistical analysis

Data were analysed by One-Way Analysis of Variance (ANOVA) test using SPSS version 20.0 software for Windows. Data were presented as means ± standard error of mean (means ± SEM). Significant is considered by p-values < 0.05.

3. Result

3.1. Stool length and weight between groups of rats

Stools of the rats were collected, weighed, and measured from both groups. Stool weight was decreased from P1 to P4 group. We also observed that stool length was decreased from control group to P3 group; apparently there was an increasement of length in P4 (Fig. 1). We analyzed the stool length and weight to explore whether the treatment differences affected the stool morphology through the changes in gut microbial diversity.The stools of the rats showed the observable differences in appearance, consistency, size, color, and smell as shown in Fig. 1. The control group showed the larger, paler, and harder stool, while the groups of treatment shows the smaller, darker, and softer stool with the presence of unique smell.

3.2 Cow milk fermented by *Bifidobacterium bacterium* increased epidermal thickness the most

The skin samples were taken from dorsal area of the rats. Skin was collected, weighed, and processed as histology specimen. Plenty of epithelium cells can be found in the epidermis in a relatively regular way.

The epidermis look denser and thicker than the other layers. The dermis can be divided into two layers, the papillary and reticular dermis. The papillary dermis is located upper to the reticular dermis and lower to the epidermis. Generally papillary dermis contains more cells than the reticular layers, while the reticular layers contain denser structure of connective tissue. The collagen fibres and elastic fibres of the reticular layer shows stronger staining and more intense density. There were differences in epidermal thickness among the different formulations as shown in Fig. 2.

The average of epidermal thickness of the treatment group was higher than the control group. This may project the direct effect of EGF into the rate of epidermal proliferation. The treatment group exhibited thicker epidermal thickness compared to control group. It was shown by how the rise of soy milk percentage was directly proportional to the epidermal thickness. However, the formula of *Bifidobacterium bifidum* showed the highest epidermal thickness regardless the presence of isoflavone. We analyzed the epidermal thickness to explore the significant phenotype differences that are affected by the the effect of EGF.

3.3 EGF was increased the most by the formulation of *Bifidobacterium bifidum*

Analyzing protein level of EGF may correlate with epidermal thickness increase from control group to P4 group. There were a parallel increase of EGF protein level and epidermal thickness as shown in Fig. 3. There was a rise in epidermal thickness from control to P4 in sequencing manner as shown in Fig. 2 Probiotics surely exhibited potential to increase level of EGF as well as to thicken the epidermis. The soy milk enhance the properties as the 50% composition of soy milk thickened the epidermis and increased level of EGF more than the 25% soy milk. As the comparison of the bacterial composition, *Bifidobacterium bifidum* showed the greatest effect compared to *Lactobacillus bulgaricus, Streptococcus thermophiles*, and *Lactobacillus acidophilus* even though this treatment group was not enhanced by the substance of soy milk.

4. Discussion

Epidermal homeostasis is a renewal process of the epidermis and its appendages as a replacement mechanism of lost cells and matrix during the turnover or subsequent to injury. [31]. The modulation of EGFR ligand system must be evoked to activate the downstream pathway by various ligands and initiate the skin maintenance process, ligand are namely:, transforming growth factor-α (TGF-α), amphiregulin (AREG), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), epiregulin (EREG), and epigen (EPGN) [32]. EGFR binding to its ligands promotes signalling of intrinsic kinase domain, followed by specific tyrosine kinase residues phosphorylation in the cytoplasmic tail that leads to activation of multiple pathways [33]. Epidermal homeostasis are affected by proliferation, keratinization, and detachment of epidermal cells. External factors such as UV and medication also affect the epidermal homeostasis by thinning the epidermis in remarkable fashion [34]. Study shows that aged group take longer time to undergo the turnover process [35]. In this study, the consumption of probiotics might

improve the epidermal thickness (Fig. 2). It showed their potential of maintaining the homeostasis and overcoming the effect from external factors.

One of the causative aspect of skin aging is the presence of Reactive Oxygen Species (ROS). ROS triggers expression of matrix metalloproteinases (MMP-1, MMP-3, and MMP-9). Collagen that construct the skin tissue will be degraded by MMPs. The degradation process will decrease the skin tension and increase the formation of more ROS. This process takes place in the looping manner and activated via the MAPK pathway [6]. MAPK pathway is also a downstream pathway of EGFR activation [12]. EGF promotes the extracellular-signal-regulated MAPK while the ROS induces the stress-activated MAPK. The activity of both type of MAPK will be elevated if there is an enhancement in the activation [6]. Thus, the elevation of EGF can inhibit the process of collagen degradation that leads to skin aging.

Soy isoflavones bind to the estrogenic receptor [36]. The estrogenic receptor itself is classified into two classes, the nuclear receptor and the membrane receptor [37]. Previous studies showed that there was decline in estrogenic receptor-α as the female mice grew old [38]. This study showed that the formulation with higher proportion of soy milk shows greater thickness of epidermis (Fig. 2). This might represent that considerable amount of isoflavone binds to the estrogenic receptor. As the epidermis become thinner with aging, the process is halted by the presence of isoflavones. This finding might be due to the increase in rate of collagen synthesis [39]. This antiaging effect of isoflavone show the potential of soybean to be an alternative for preserving skin health and preventing skin aging.

It is not limited to soybean takes role in aging, we observed that bacteria formulation might determine the metabolite active compound resulted by fermentation process. For instance, *Bifidobacterium bifidum* increases the largest surface area, elevate the most brush border enzyme, and give the best improvement to the transport system compared to *Lactobacillus bulgaricus, Streptococcus thermophiles*, and *Lactobacillus acidophilus* [29] and produce more active metabolic products than *Lactobacillus spp* which initiates other signalling mechanism in tissue [27]

As the aging process affect integrity, humidity, pH and elsatisity of skin tissue, and ultimately the epidermis gradually becomes thinner [39], [40]. The thinning of epidermis is caused by the rise of Epidermal Fas expression in aging skin that induce Fas-mediated apotosis [41]. The thicker the epidermal thickness means the less Fas-mediated apoptosis take place. Mechanism that has been known to have the ability to inhibit the Fas-mediated apoptosis is the Akt pathway of EGF [42], [43]. This study showed that the composition consisting of 100% cow milk fermented by *Bifidobacterium bifidum* and *Lactobacillus acidophilus* exhibited the thickest epidermis. This result was also parallel with the trends in EGF protein level observed in this study as shown in Fig. 3. The property of *Bifidobacterium bifidum* in enhancing gut enzymatic activity and improving gut microbial dysbiosis may be better if the isoflavone is added by adding the soy milk to the formula.

The interaction between EGF and EGFR plays an important role in maintaining skin homeostasis, proliferation, and maintenance through various interactions of the receptor and the ligands that leads to the activation of various downstream pathways. The changes that happen in the skin can be caused by

various factors, including the internal and external factor. In this research, we are proposing that probiotics with enhancement of soy isoflavones may maintain the skin homeostasis. This is achieved by elevating the level of EGF.

5. Conclusion

The consumption of various formulation of fermented milk and fermented soy milk may maintain skin structure and delay aging in skin by elevating the level of EGF. It was shown in an experiment in rats supported by the difference in epidermal thickness. Formulation of *Bifidobacterium bifidum* exhibited the greatest potential as skin homeostasis modulator.

Declarations

Availability of Data and Material

The data of this research was provided by corresponding author upon permission and request.

Competing Interest

Authors declare that there are non financial and financial interest.

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Authors Contribution

RL, LA, ZH, are responsible for designing experiment, treatment, analyzing and writing manuscript, western blot and histology staining; HG, YSP, NS are animal handling analizing, sampling and collecting data, LA and US are responsible for writing, histology study and stool analysis

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Figures

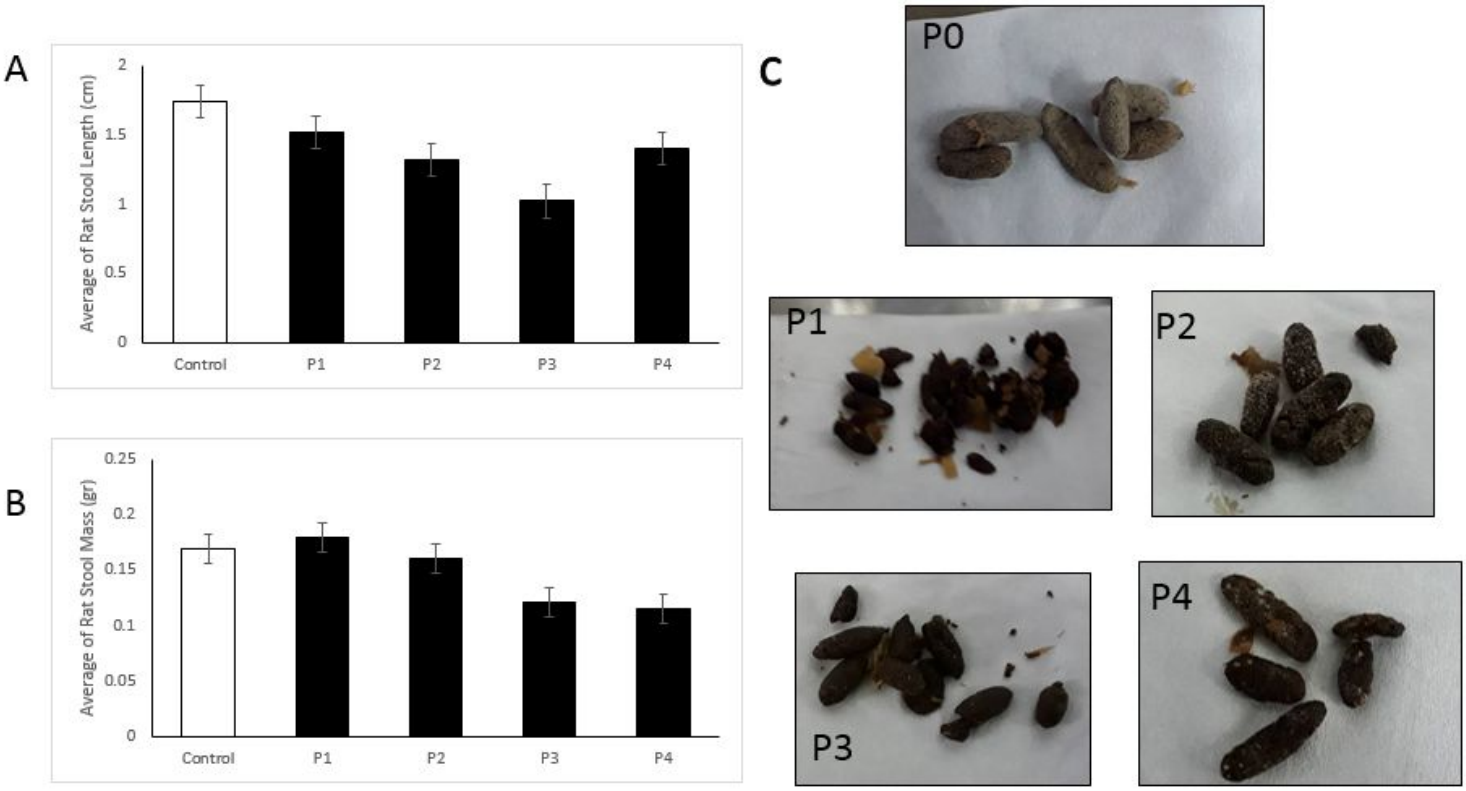

Figure 1

Different formula of fermented cow and soy milk affects rat faeces length, weight and colour after 12 weeks of treatment. Data were represented as average mean ± SEM; (A) average length of stool; (B) average weight of faeces; (C) Changes color of the faeces.

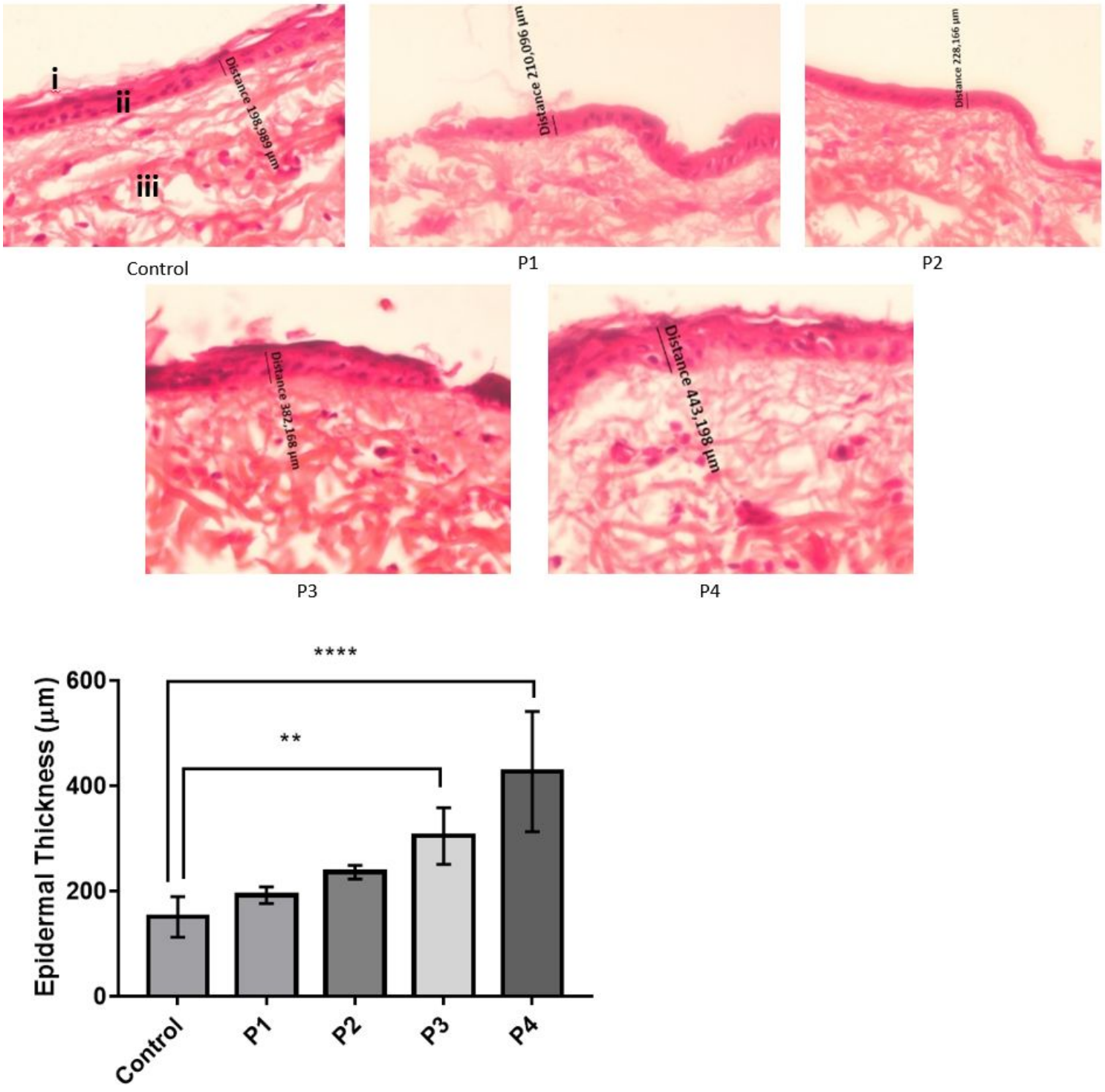

Figure 2

Effect of different formulation of fermented milk and soymilk on rat epidermal thickness (A). The differences in average of epidermal thickness (B) (i) Stratum corneum. (ii) epidermis. (iii) dermis. (B). Data were represented in average mean ± SEM with **P < 0.01; ***P < 0,0001.

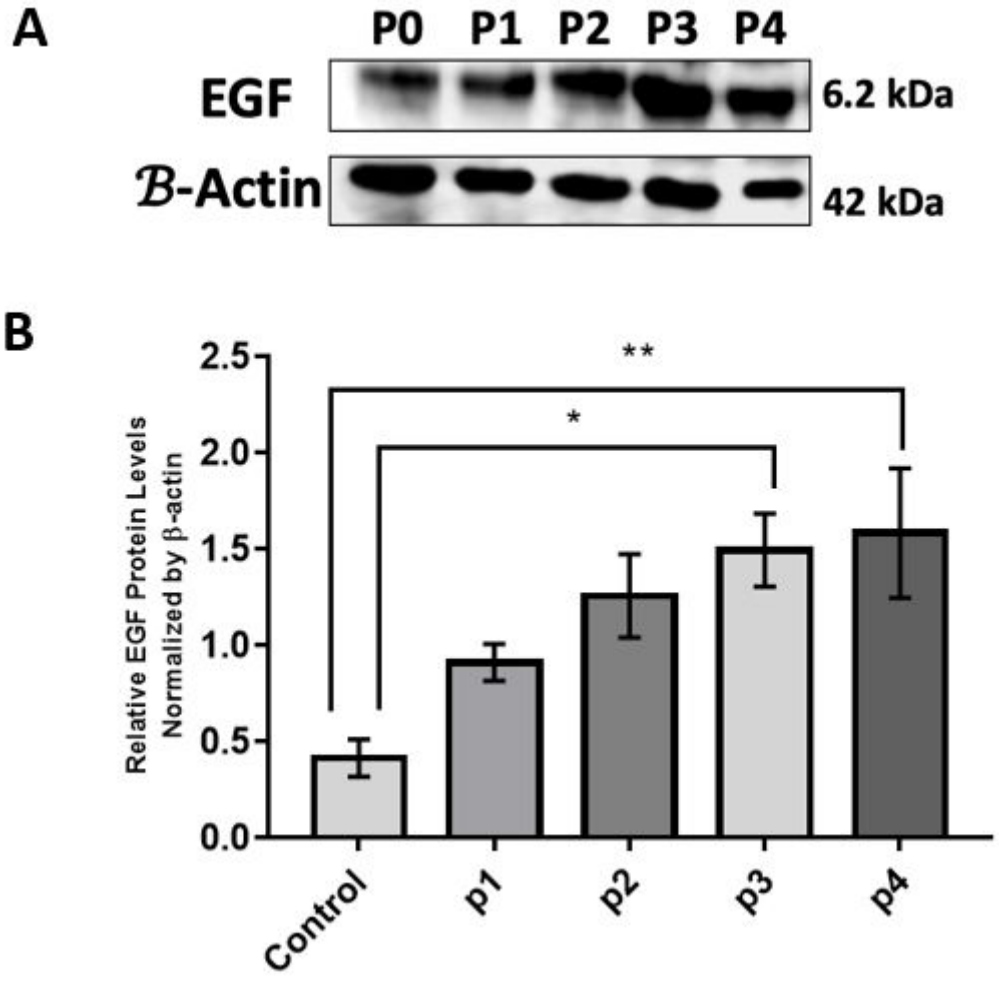

Figure 3

Increase of epidermal thickness and EGF level took effect in parallel manner. This induction stimulated epidermal proliferation in skin tissue. Representative immunoblotting was shown (A) and densitometric quantification was shown in the graph normalized by β-actin (B). Data were represented as average ratio ± SEM with *($P < 0.05$) **($P < 0.01$).