Anti-oxidant and Anti-aging Activities of Fermented Vegetable-Fruit Drink

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Abstract Fermented foods contain beneficial probiotics, which thus results in products with improved nutritional properties and healthy effects. There is however a need for research on clinical trials to measure the effects of fermented foods in clinical applications and in skincare. The composition of fermented vegetable-fruit drink (FVFD) included the fermented vegetable-fruit juice, apple juice, fructose, high methoxyl pectin, citric acid, apple flavor and water. In this study, the effects of FVFD on the free radical scavenging activities, promotion of skin cell proliferation and collagen synthesis were investigated. A randomized, controlled, double-blind study design was used to assess FVFD and basic drink (50 ml of a FVFD/placebo drink daily for 8 weeks for each subject). Thirty subjects (aged 35-55 years old) were recruited for an 8 week-long clinical trial to confirm the efficacy of FVFD in improving the serum biochemical superoxide dismutase (SOD) and catalase levels, and anti-skin age markers, including improving skin moisture, brightness and elasticity, reducing crow’s feet, skin texture, wrinkles, pores and spots, and increasing amount of skin collagen. The results suggest that fermented vegetable-fruit drink has anti-oxidant activities, promotes collagen synthesis and delays the aging of skin with potential applications in food additives.

Keywords: fermented vegetable-fruit drink, skin, anti-oxidant, anti-aging

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1. Introduction

Vegetables and fruits are good sources of vitamins, dietary fibers, minerals, and phytochemicals (such as polyphenols), which impart anti-aging, anti-oxidative, anti-inflammatory, and anti-cancer activities on disease prevention in humans [1]. They are commonly eaten fresh or after cooking as well as may be processed into juices. However, with the accumulation of probiotic-associated studies, researchers have recently forayed into the development of probiotic vegetable/fruit juices through fermentation [2]. It has been unveiled that the lactic acid fermentation with/without yeast can boost the increase of nutritional values (such as amino acids, vitamins, minerals, and antioxidants) of functional vegetable and fruit juices [3-9]. Note that yeast contains abundant nutrients and biofactors, which promote the growth of microorganisms during fermentation [10]. The health benefits of probiotic juices are attributed to probiotic effect or biogenic effect [11]. Biogenic effect refers to the positive influence of the secondary metabolites (such as B vitamins and bioactive peptides) synthesized by bacteria during fermentation on humans [8]. For instance, Rakin et al. demonstrated that Lactobacillus species and brewer’s yeast extract could increase the contents of vitamin C and betanin in beetroot juices as well as reinforce the levels of the minerals and β-carotene in carrot juices [8,9]. In addition, Yang et al. uncovered that lactic acid fermentation might significantly improve the antioxidant capacity of a vegetable-fruit drink (including apples, pears, and carrots) with respect to the enrichment of vitamin C and polyphenols [12].

To date, to the best of our knowledge, most studies of fermented juices are focused on the aspect of development techniques, but few researches point out the advantages of fermented juices in clinical applications. In this clinical evaluation, we attempted to assess the utility of a fermented vegetable-fruit drink for improvement of skin aging.
2. Materials and Methods

2.1. Preparation of Fermented Vegetable-fruit Drink

The vegetable-fruit juice was obtained from the mixture of vegetable juice (i.e., broccoli, celery, asparagus, carrot extracts) and fruit juice (i.e., blue berry, grapes, cranberry, apple, bayberry, mulberry, sugar cane, passion fruit, pineapple, and lemon extracts) in a mass ratio 1:1. The vegetable-fruit juice was then diluted to 50% with water followed by sterilization at 100°C for 30 minutes. Afterwards, the juice was fermented at 35°C for 10 days with 0.5% (w/w) of Saccharomyces cerevisiae and 0.25% (w/v) of Streptococcus thermophilus TCI125. In the end, the fermented vegetable-fruit juice was sterilized at 105°C for 1.5 h.

The composition of a bottle fermented vegetable-fruit drink (FVFD) (50 ml) included the fermented vegetable-fruit juice, apple juice, fructose, high methoxyl pectin, citric acid, apple flavor and water. Placebo drink (50 ml) consisted of apple juice, fructose, high methoxyl pectin, citric acid, apple flavor and water.

2.2. SOD Measurement

The analysis of SOD activity is based on the inhibition of pyrogallol autoxidation that is catalyzed by the superoxide radical [13]. 0.2 ml of FVFD sample solution was mixed with 2.35 ml of pyrogalol solution (56.7 mg pyrogalol in 10 mM HCl solution) and 0.15 ml of 0.15 ml of Tris-ethylenediaminetetraacetic acid buffer (1.2114 g Tris and 37.2 mg ethylenediaminetetraacetic acid disodium salt dehydrate in 0.1 M HCl solution), and the absorbance at 325 nm (BioTek, Synergy™2, U.S.A.) was obtained.

2.3. Free Radical Scavenging Activities Assays

The ability of FVFD to scavenge 1,1-Diphenyl-2-picrylhydrazyl (DPPH·) and 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS+·) free radicals was assessed. In brief, 4 ml of FVFD was mixed with 1 ml of 10 mM DPPH· (dissolved in methanol to a final concentration of 2 mM). The mixture was left in the dark at room temperature for 30 min. The remaining DPPH· radical was determined on a spectrophotometer at 517 nm (BioTek, Synergy™2, U.S.A.). Distilled water (4 ml) was used to substitute for FVFD sample as background (absorption control).

The inhibition percentage of ABTS+· radical as determined by the extent of decolorization was taken to be proportional to the concentration of antioxidants. ABTS+· radical solution was prepared by oxidation of reagents with peroxidase (44 U/ml), 75 μM H2O2, 750 μM ABTS-·, and H2O with a volume ratio of 1:1:1:6. The mixture was placed in the dark at room temperature for 1 h. For antioxidant activity determination, 180 μl of the ABTS+· radical solution was added with 20 μl of samples of different-concentrations and incubated for 10 min. Absorbance was recorded on a spectrophotometer at 620 nm (BioTek, Synergy™2, U.S.A.).

L-Ascorbic acid (AA) was utilized as standard. DPPH· and ABTS+· radical scavenging activity was calculated as follows: ABTS+· radical scavenging activity (%) = [(absorption of blank sample - absorption of tested sample)/(absorption of blank sample)] × 100.

2.4. Cell Viability Assay

Human fibroblast Hs68 cells (2 × 10⁵ cells/ml) were each seeded in 100 μl of 96-well plates for at least 24 h prior to use. The cells were treated with FVFD for 72 h. The cell viability was measured by the MTT-[3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay according to the manufacturer’s instructions (Promega, Madison, WI). After the cells had been incubated for the indicated times, they were incubated with MTT solution (0.5 mg/ml) for 4 h. The formazan precipitate was dissolved in 20 μl DMSO, and the absorbance at 570 nm was measuring using an automated microplate reader (BioTek, Synergy™2, U.S.A.). Values are expressed as the mean cell viability as a percentage of that of the vehicle ddH2O (0.1% final volume)-treated cultures.

2.5. Collagen Synthesis Assay

Human fibroblast Hs68 cells (2 × 10⁵ cells/ml) were seeded in 24-well plates and FVFD (1, 5 and 10%) were added to wells of the plates and incubated in serum-free medium for 72 h. Then, the medium in each well was collected to measure collagen levels using Sircol soluble collagen assay (Biocolor Assays, Ireland). Briefly, to the samples of the medium Sircol dye reagent was added and mixed for 30 min on an orbital shaker. The samples were then centrifuged and the dye bound to the collagen pellet was solubilized with an alkali reagent. The absorbance of the samples was measured at 555 nm using a microplate reader (Infinite M200, TECAN). A calibration standard of type I collagen was used to obtain the standard curve.

2.6. Double-blind, Randomized, Placebo-controlled Trial Study

This clinical research was approved by the ethics committee of the Antai Medical Care Corporation Antai Tian-Sheng Memorial Hospital (TSMH-IRB17-095-A). All subjects recruited in this trial returned the written consent forms. Thirty healthy subjects were enrolled in this clinical study, and they were randomly assigned to FVFD or placebo group as a result of 15 subjects in FVFD group and 15 subjects in placebo group. Each subject was informed to intake one drink daily for 8 weeks.

Among the 30 subjects, there are 13 males and 17 females. The inclusion criteria were as follows: i) healthy volunteer in the age range of 35-55 years old; ii) yellow-brown skin tone: lightness (L*) value < 65; iii) visible black spots; iv) dry skin (moisture content < 75); and, v) visible wrinkles. The exclusion criteria were as follows: i) pale skin: L* value > 65; ii) retinoids used either externally and/or orally; iii) dihydroxyacetone applied externally; iv) the presence of other dermatoses; v) hypersensitivity to any component present in the assigned product; vi) pregnancy and lactation; vii) other
2.7. Serum Biochemical Parameters of SOD and Catalase and Routine Test Items

The fasting blood of each participant was collected at weeks 0 and 8 for the following analysis of physiological parameters. The blood samples were centrifuged at 2000 ×g for 15 min at 4°C. The levels of the serum biochemical parameters including SOD and catalase were monitored. Other parameters in serum, including the levels of cholesterol, triglyceride, serum glutamate oxaloacetate transaminase (SGOT), blood urine nitrogen (BUN), uric acid and creatinine were monitored. SOD (Superoxide Dismutase Assay Kit, Cayman #706020, U.S.A.) and catalase (Catalase Assay Kit, Cayman #707002, U.S.A.) were analyzed using an automatic analyzer (Hitachi 7180, Japan).

2.8. Measurement of Skin Moisture, Brightness and Elasticity

Corneometer CM825 (Courage + Khazaka Electroni, Germany) was used to measure the moisture of upper cheek. The degree of improvement in skin moisture is positively correlated with the increase of measurement value. Chroma Meter MM500 (Minolta, Japan) was employed to measure the brightness of upper cheek. The degree of improvement in skin brightness/tone is positively correlated with the increase of L* value (L* range: 0-100). Soft Plus (Callegari 1930, Italy) was employed to measure the skin elasticity of upper cheek. Measurement principle: stress/deformation. Field: 0-50 u.c.

2.9. Measurement of Crow’s Feet Depth, Skin Texture, Wrinkles, Pores and Spots

Soft Plus (Callegari 1930, Italy) was used to measure crown’s feet on the subjects’ faces. The depth of the crow’s feet was obtained by determining the change in area of skin shadow. VISIA® Complexion Analysis (Canfield Scientific, U.S.A.) was used to measure skin texture, wrinkles, pores and spots on the whole face. Skin smoothness is indicated by color gradations with peaks shown in yellow, and valleys shown in blue. Wrinkles are characterized by the long and narrow shape areas; the green lines in the software reflect the presence and depth of wrinkles. The purple dots in the software indicate the pores. The degree of improvement in skin pores is inversely correlated with the numbers of purple dots. The blue dots in the software indicate the spots. The degree of improvement in skin spots is inversely correlated with the numbers of blue dots. The degree of improvement in skin textures, wrinkles, pores and spots are positively correlated with the measured values.

2.10. Measurement of Skin Collagen Content

DermaLab® Series SkinLab Combo (Cortex, Denmark) was employed to skin collagen content of upper cheek. The instrument uses ultrasound to analyze the collagen density of upper cheek.

2.11. Statistical Analysis

The experimental results are presented as mean ± standard deviation (SD). Statistical differences were estimated by one-way analysis of variance (ANOVA) followed by Student’s t test and Tukey’s multiple comparison test. Statistical significance is indicated as *, p < 0.05; **, p < 0.01; ***, p < 0.001 vs. week 0 for the group, and #, p < 0.05; ##, p < 0.01; ###, p < 0.001 vs. placebo at week 0. Data were analyzed and relevant figures plotted using SigmaPlot Version 8.0 (San Rafael, CA, USA).

3. Results

3.1. Antioxidant Activity of FVFD

To verify the advantage of antioxidant activity in FVFD, we analyzed the contents of SOD of vegetable-fruit juice before and after fermentation (TCI vegetable and fruit enzyme). The level of SOD of FVFD was approximately 0.7 times higher than that of conventional vegetable-fruit juice (Table S1). It suggests that lactic fermentation may substantially fortify the antioxidant capacity of vegetable-fruit juice. We further prepared the FVFD with the fermented vegetable-fruit juice (as a concentrated liquid) for the following experiments.

Additionally, we also investigated the antioxidant activities of FVFD and placebo drink. To determine the antioxidant capacity of FVFD, DPPH* and ABTS- were used as stable free radicals in the free radical scavenging assay. The DPPH* assay was used to study the ability of the investigated FVFD to donate a donor of hydrogen atoms or electrons in the transformation of the DPPH* into its reduced from, DPPH−H. The performed radical monocation of ABTS+ is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of the hydrogen-donating antioxidants. Results showed that FVFD scavenged DPPH* and ABTS+ free radicals in a dose-dependent manner. As presented in Figure 1A, DPPH* radical scavenging activity by FVFD of 1, 5 and 10% were 3.5, 37.7 and 81.9%, respectively. ABTS+ radical scavenging activity by FVFD of 1, 5 and 10% were 30.0, 74.3 and 91.7%, respectively (Figure 1B). Over 80% scavenging rate of DPPH* and ABTS+ were achieved after treatment with 10% FVFD. Results showed that FVFD exhibited significant antioxidant activity.
Figure 1. Antioxidant activity of FVFD. (A) DPPH∙ and (B) ABTS∙+ free radical scavenging activities of FVFD or placebo drinks (1, 5 and 10%). Data are presented as mean ± SD from three independent experiments. C, vehicle-treated control; AA, L-ascorbic acid; ***, p < 0.001 vs. vehicle-treated control; ###, p < 0.001 vs. placebo samples.

Figure 2. Promotion of skin cell proliferation and collagen synthesis of FVFD. (A) Cell proliferation of skin fibroblast Hs68 cells in the presence of FVFD or placebo drink (1, 5 and 10%) for 72 h, measured using a MTT assay. (B) Collagen synthesis of skin fibroblast Hs68 cells following treatment with FVFD or placebo drink (1, 5 and 10%) for 72 h, and the percentage synthesized collagen contents was determined using a Sircol soluble collagen assay. Each value is presented as a mean ± SD from independent triplicate experiments. C, vehicle-treated control; *, p < 0.05; ***, p < 0.001 vs. vehicle-treated control; #, p < 0.05; ##, p < 0.01 vs. placebo samples.

3.2. Cell Viability of FVFD

Numerous studies have established that natural substances can be used in health food or cosmeceutics. However, safety concerns in vitro must be determined before food and cosmetic manufacture. Therefore, this investigation determines the cell viability of FVFD in human skin Hs68 cells, using MTT assay. The cell viability is 100.3, 105.8 and 108.9% after FVFD treatment for 72 h at 1, 5 and 10% in Hs68 cells (Figure 2A), suggesting that FVFD was non-toxic and enhanced the survival of Hs68 cells at a dose of 5-10%. In morphology observation, FVFD did not cause significant morphological change of Hs68 cells. The study demonstrated that FVFD is non-toxic to human skin fibroblast Hs68 cells.

3.3. Collagen Synthesis of FVFD

Antioxidants help the body create collagen, a building and support protein found in the connective tissue of the skin. Collagen is an essential part of skin health, and new collagen formation leads to improved skin firmness and elasticity. Therefore, FVFD was tested for the effect on collagen expression in human dermal fibroblasts. The fibroblasts were exposed to FVFD at 1, 5 and 10% for 72 h, and the level of total collagen in the medium was determined by Sirius Red-based colorimetric assay. As shown in Figure 2B, FVFD exerted various effects on the amount of collagen in the fibroblast medium. FVFD significantly enhanced the amount of collagen secreted into the medium. FVFD increased collagen level in a dose-dependent manner. At concentrations of 1, 5 and 10%, collagen content in the medium was significantly increased to 104.8, 125.1 and 149.9%, respectively, compared with the untreated control (100.0%).

3.4. Clinical Antioxidant Activity of FVFD

Superoxide dismutase and catalase are antioxidant enzymes which do not only play fundamental but
indispensable role in the antioxidant protective capacity of biological systems against free radical attack. Evidence from clinical studies supports the claim that the daily oral consumption of FVFD antioxidants and improves the antioxidant enzymes. The clinical characteristics and the serum levels of the markers of antioxidant enzymes in the FVFD and the control subjects have been shown in Table 1. After the 8 week FVFD intervention, the mean levels of SOD and catalase activities of subjects were improved by 10.2% and 105.0%, respectively, as compared with the baseline results. Especially, FVFD facilitated the remarkable increase in the catalase activity of subjects after the study. Thus, there was a decrease in the oxidants and an increase in the antioxidants after the FVFD treatment, thereby leading to an overall improvement in the oxidative stress.

Additionally, subjects reported no adverse effects, and the values of biochemical factors associated with liver (the levels of cholesterol, SGOT and SGPT) (Table S2) and kidney (the levels of BUN and uric acid) (Table S3) functions were in the normal reference ranges (Table S4). The levels of cholesterol, SGOT, SGPT, BUN, uric acid and creatinine showed no significant differences among the treatment group at week 0 (p > 0.05). After 8 weeks consumption, a reduction of triglyceride level by 42.8 mg/dl was found in FVFD group (p < 0.05) (Table S2).

3.5. Efficacy of FVFD in Improving Skin Moisture, Brightness and Elasticity

Most dermatologists agree that antioxidants help fight free radical damage and can help maintain healthy skin. They do so by affecting intracellular signaling pathways involved in skin damage and protecting against photodamage, as well as preventing wrinkles and inflammation. Moreover, the direct free radical scavenging, by FVFD, of clinical studies to improve the collagen network, hydration, and elastic properties of skin were examined to confirm its ability to antioxidant activity in daily oral FVFD. Table 2 presents the significant improvements effects of FVFD on skin moisture, brightness, and elasticity appeared at 4 weeks after the intervention and extended to the end of the study. In comparison with the baseline results, the mean levels of skin moisture, brightness, and elasticity of FVFD group at 8 weeks were improved by 6.1%, 4.6%, 11.2%, respectively (Table 3). Nonetheless, placebo group also exhibited the similar degree of improvement in skin moisture and elasticity, which might be due to the placebo effect and individual variation.

| Item     | Group  | Weeks | Mean (mean ± SD) | Improving rate (%) (mean ± SD) |
|----------|--------|-------|------------------|---------------------------------|
| SOD      | FVFD   | 0     | 15.7 ± 2.6       | 100.0 ± 16.5                    |
|          |        | 8     | 17.3 ± 2.3       | 110.2 ± 13.1                    |
|          | Placebo| 0     | 12.9 ± 3.6       | 100.0 ± 27.5                    |
|          |        | 8     | 12.3 ± 3.8       | 95.0 ± 31.3                     |
| Catalase | FVFD   | 0     | 38.6 ± 26.6      | 100.0 ± 69.1                    |
|          |        | 8     | 79.1 ± 32.5      | 205.0 ± 41.0                    |
|          | Placebo| 0     | 30.1 ± 10.6      | 100.0 ± 36.5                    |
|          |        | 8     | 35.7 ± 11.9      | 118.5 ± 33.4                    |

Recommended: SOD, U/mL; Catalase, nmol/min/mL.

Table 2. Changes of skin moisture, brightness and elasticity for FVFD group. Mean and improvements of skin moisture, brightness and elasticity levels in FVFD and placebo drink subjects at 4 and 8 weeks. SD, standard deviation; n = 30; significant difference relative to baseline: ***, p < 0.01; ***, p < 0.001; significant difference relative to placebo group: ###, p < 0.01.

| Item     | Group  | Weeks | Mean (mean ± SD) | Improving rate (%) (mean ± SD) |
|----------|--------|-------|------------------|---------------------------------|
| Moisture | FVFD   | 0     | 74.7 ± 7.3       | 100.0 ± 9.7                     |
|          |        | 4     | 78.3 ± 7.5       | 104.9 ± 9.6***                   |
|          |        | 8     | 78.3 ± 7.7       | 106.1 ± 9.7***                   |
|          | Placebo| 0     | 73.2 ± 6.0       | 100.0 ± 8.2                     |
|          |        | 4     | 75.9 ± 3.6       | 103.7 ± 4.8                     |
|          |        | 8     | 78.1 ± 3.4       | 106.7 ± 4.4**                    |
| Brightness| FVFD   | 0     | 65.3 ± 3.1       | 100.0 ± 4.8                     |
|          |        | 4     | 67.7 ± 3.7       | 103.7 ± 5.5***,***              |
|          |        | 8     | 68.3 ± 3.9       | 104.6 ± 5.7***,***              |
|          | Placebo| 0     | 65.9 ± 2.9       | 100.0 ± 4.4                     |
|          |        | 4     | 65.5 ± 2.5       | 99.3 ± 3.8                      |
|          |        | 8     | 65.7 ± 2.8       | 99.7 ± 4.2                      |
| Elasticity| FVFD   | 0     | 41.1 ± 2.9       | 100.0 ± 7.1                     |
|          |        | 4     | 44.7 ± 1.9       | 108.8 ± 4.2***                  |
|          |        | 8     | 45.7 ± 1.9       | 111.2 ± 4.1***                  |
|          | Placebo| 0     | 40.6 ± 3.0       | 100.0 ± 7.5                     |
|          |        | 4     | 43.7 ± 2.3       | 107.7 ± 5.4**                   |
|          |        | 8     | 44.7 ± 2.2       | 110.0 ± 4.9**                   |
Table 3. Improvements of skin characteristics (moisture, brightness, elasticity, crow’s feet, texture, wrinkle, pores, spots and collagen content) of FVFD and placebo drink subjects at 4 and 8 weeks

| Sample                  | FVFD       | Placebo    |
|-------------------------|------------|------------|
| Test time (weeks)       | 4          | 8          | 4          | 8          |
| Moisture                | +4.9%      | +6.1%      | +3.7%      | +6.7%      |
| Brightness              | +3.7%      | +4.6%      | -0.7%      | -0.3%      |
| Elasticity              | +8.8%      | +11.2%     | +7.7%      | +10.0%     |
| Crow’s Feet             | -11.1%     | -15.5%     | -8.4%      | -12.4%     |
| Texture                 | -8.9%      | -24.9%     | -7.3%      | -6.1%      |
| Wrinkle                 | -1.5%      | -21.1%     | +19.9%     | +33.2%     |
| Pores                   | -9.5%      | -6.3%      | +3.2%      | -1.5%      |
| Spots                   | -17.6%     | -22.5%     | +4.8%      | +8.0%      |
| Collagen Content        | +10.2%     | +13.5%     | +4.5%      | +7.7%      |

3.6. Efficacy of FVFD in Reducing Depth of Crow’s Feet, Skin Texture, Wrinkle, Pores and Spots

The mean levels of crow’s feet of FVFD group at 4 and 8 weeks were improved by 11.1% and 15.5%, respectively; those of placebo group at 4 and 8 weeks were decreased by 8.4% and 12.4%, respectively (Table 3 and Table 4). Although FVFC could obviously lighten crow’s feet, placebo drink also displayed a similar degree of improvement of crow’s feet. The disparaging result might be influenced by the factor of individual variation. The results of skin textures (Table S5), wrinkles (Table S6), pores (Table S7) and spots (Table S8) of subjects at 0, 4 and 8 weeks; FVFD was able to implement the consecutive amelioration to the skin parameters. FVFD reduced the mean levels of skin textures, wrinkles, pores and spots at 8 weeks by 24.9%, 21.1%, 6.3% and 22.5% as compared with the baseline results (Table 3). Noticeably, FVFC endorsed the better progress of improvement in these indexes than placebo drink.

Table 4. Changes of crow’s feet for FVFD group. Mean and improvements of crow’s feet levels in FVFD and placebo drink subjects at 4 and 8 weeks. SD, standard deviation; n = 30; significant difference relative to baseline: ***, p < 0.001; significant difference relative to placebo group: ###, p < 0.01

| Item       | Group   | Weeks | Mean (mean ± SD) | Improving rate (%) (mean ± SD) |
|------------|---------|-------|------------------|---------------------------------|
| Crow’s Feet| FVFD    | 4     | 24.2 ± 3.5       | 88.9 ± 14.4*                   |
|            |         | 8     | 23.0 ± 3.6       | 84.5 ± 15.8*                   |
|            | Placebo | 4     | 20.7 ± 3.9       | 91.6 ± 18.8*                   |
|            |         | 8     | 19.8 ± 3.9       | 87.6 ± 19.8*                   |

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|            |         | 8     | 19.8 ± 3.9       | 87.6 ± 19.8*                   |
Table 5. Changes of skin collagen content for FVFD group. Mean and improvements of skin collagen density in FVFD and placebo drink subjects at 4 and 8 weeks. SD, standard deviation; n = 30; significant difference relative to baseline: ***, p < 0.001; significant difference relative to placebo group: ##, p < 0.01; ###, p < 0.01

| Item        | Group | Weeks | Mean (mean ± SD)        | Improving rate (%) (mean ± SD) |
|-------------|-------|-------|-------------------------|-------------------------------|
| Collagen Content | FVFD  | 0     | 67.5 ± 13.4             | 100.0 ± 20.3***               |
|             |       | 4     | 71.0 ± 13.5             | 110.2 ± 19.0***              |
|             |       | 8     | 73.2 ± 13.5             | 113.5 ± 18.5###              |
|             | Placebo | 0     | 65.6 ± 11.2             | 100.0 ± 17.0***              |
|             |       | 4     | 68.5 ± 11.2             | 104.5 ± 16.3***              |
|             |       | 8     | 70.6 ± 10.9             | 107.7 ± 15.5***              |

3.7. Efficacy of FVFD in Promoting Skin Collagen Synthesis

Collagen is an elemental structure protein in the extracellular matrix, so that the integrity and content of collagen fabric are associated with a certain degree of skin aging. As compared with the baseline results, FVFD increased the mean levels of collagen of subjects at 4 and 8 weeks by 10.2% and 13.5%, respectively (Table 3). Additionally, the results of FVFD groups were significantly different from those of placebo group, meaning that the efficacy of FVFD for stimulating collagen production was superior to placebo drink (Table 5).

4. Discussions

Skin is a unique organ directly exposed to the outside environment, so the health conditions and aging process of skin are prone to be affected by the external factors, such as pollutants and UV irradiation [14]. The characteristics of skin aging are manifested through wrinkles, laxity, brown spots, thickening, and coarseness [15]. Although there is mounting evidence that a plethora of bioactive compounds from vegetables and fruits enable to retard the aging process of skin and improve skin properties, the clinical efficacy of FVFD for improvement of skin aging is not being thoroughly disclosed at present. In this preliminary clinical evaluation, we tried to look into the utility of a FVFD for bettering skin health. Moreover, the skin cycle is around 28 days [16,17]. In 8 weeks of skin condition inspection, we can record around 2 times skin renewal for examining the efficacy of skin ameliorates of FVFD intervention.

According to the analysis of antioxidant content, lactic fermentation with yeast could enrich the contents of antioxidants in vegetable-fruit juices as evidenced by the elevated SOD level in the fermented juice. It suggests that our lactic fermentation process may allow an increase in antioxidant capacity of vegetable-fruit juice, but the bioavailability of antioxidants produced by the lactic fermentation requires further investigation. Moreover, we also used the in vitro methodologies to assess the antioxidant ability of FVFD and its influence on fibroblasts before the clinical evaluation in an effort to conceptualize the benefits of FVFD for skin cells. The free radical scavenging activity of FVFD was able to be competitive with the reducing power of ascorbic acid based on the DPPH• and ABTS•+ scavenging results.
the concern of cytotoxicity. Not only do these results advocate the legitimacy of the clinical study, but they pave the path to the exploration of advanced cellular and molecular research for skin aging. For example, a fermented plant extract was possible to suppress elastase and collagenase activities, increase type I collagen expression, and restore the antioxidant enzyme activities in a UVB-induced cellular model [18].

After the 8 week FVFD intervention, all skin parameters of subjects received positive amelioration; in particular, the levels of skin moisture, brightness, elasticity, crow’s feet, texture, pores, spots, and collagen content were significantly improved in comparison with the baseline results. Notwithstanding placebo drink also achieved the similar improvement effects on skin moisture, elasticity, and crow’s feet, FVFD still gained more advantages for skin care in terms of the comprehensive improvement effect. The dispersing results may be correlated with randomized allocation, individual variety, the influence of outdoor activities, and placebo effect. Moreover, the improvement of skin parameters is probably attributed to reduction of oxidative stress (e.g., decrease of SOD level), enhancement of anti-inflammatory response (e.g., down-regulation of nitric oxide, proinflammatory cytokines), and up-regulation of the expression of collagen, fibronectin, and water channel proteins in skin cells [19,20]. Regarding the efficacy of pasteurized lactic acid bacteria, the comparison between live and pasteurized S. thermophilus TCI125 for skin health will require further efforts. However, some studies have proved that heat killed L. lactis H61 is beneficial for skin health by modulation of immunostimulation and the balance of intestinal microbiota in rodents [21]. In brief, through the cellular models and clinical investigation, we have confirmed that the conventional vegetable-fruit juice after our lactic fermentation with yeast could substantially improve skin conditions of subjects due to the synergistic effect of bacterial metabolites, vitamins, and phytochemicals.

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Declaration of Interest

None of the authors are in conflict of interest with this research.

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Supplemental Data

Table S1. The contents of SOD of vegetable-fruit juice before and after fermentation (TCI vegetable and fruit Enzyme). VFD, vegetable-fruit drink; FVFD, fermented vegetable-fruit drink

| Sample          | VFD            | FVFD          | Normalization (fold) |
|-----------------|----------------|---------------|----------------------|
| SOD (U/ml)      | 893.8          | 1544.1        | 1.7                  |

Table S2. Changes of serum biochemical parameters of liver functions of FVFD group. Mean and improvements of cholesterol, triglyceride, SGOT and SGPT levels in FVFD and placebo drink subjects at 8 weeks. SD, standard deviation; n = 30; significant difference relative to baseline: **, p < 0.01

| Item           | Group   | Weeks | Mean (mean ± SD) | Improving rate (%) (mean ± SD) |
|----------------|---------|-------|------------------|--------------------------------|
| Cholesterol    | FVFD    | 0     | 185.5 ± 31.2     | 100.0 ± 16.8                   |
|                |         | 8     | 190.9 ± 33.3     | 102.9 ± 17.4                   |
|                | Placebo | 0     | 189.5 ± 33.3     | 100.0 ± 17.6                   |
|                |         | 8     | 197.3 ± 36.4     | 104.1 ± 18.5                   |
| Triglyceride   | FVFD    | 0     | 146.5 ± 71.7     | 100.0 ± 48.9                   |
|                |         | 8     | 103.7 ± 66.0     | 70.8 ± 63.6**                  |
|                | Placebo | 0     | 133.6 ± 94.1     | 100.0 ± 70.4                   |
|                |         | 8     | 103.1 ± 60.5     | 77.1 ± 60.5                    |
| SGOT           | FVFD    | 0     | 28.3 ± 18.6      | 100.0 ± 65.8                   |
|                |         | 8     | 25.7 ± 18.2      | 91.0 ± 70.8                    |
|                | Placebo | 0     | 25.8 ± 7.3       | 100.0 ± 28.1                   |
|                |         | 8     | 24.4 ± 6.6       | 94.6 ± 27.1                    |
| SGPT           | FVFD    | 0     | 28.7 ± 27.2      | 100.0 ± 95.0                   |
|                |         | 8     | 33.5 ± 31.4      | 117.0 ± 93.7                   |
|                | Placebo | 0     | 24.5 ± 17.0      | 100.0 ± 69.5                   |
|                |         | 8     | 25.7 ± 14.5      | 104.9 ± 56.5                   |

Recommended: Cholesterol, <200 mg/dL; Triglyceride, <150 mg/dL; SGOT and SGPT, 0-40 U/L.

Table S3. Changes of serum biochemical parameters of kidney functions of FVFD group. Mean and improvements of BUN, uric acid and creatinine levels in FVFD and placebo drink subjects at 8 weeks. SD, standard deviation; n = 30

| Item          | Group | Weeks | Mean (mean ± SD) | Improving rate (%) (mean ± SD) |
|---------------|-------|-------|------------------|--------------------------------|
| BUN           | FVFD  | 0     | 11.3 ± 1.9       | 100.0 ± 16.3                   |
|               |       | 8     | 11.1 ± 2.7       | 97.5 ± 24.3                    |
|               | Placebo | 0    | 11.4 ± 2.5       | 100.0 ± 21.4                   |
|               |       | 8     | 12.3 ± 4.0       | 107.7 ± 32.1                   |
| Uric acid     | FVFD  | 0     | 5.2 ± 1.7        | 100.0 ± 32.3                   |
|               |       | 8     | 5.2 ± 1.8        | 98.5 ± 34.4                    |
|               | Placebo | 0    | 5.5 ± 1.4        | 100.0 ± 24.9                   |
|               |       | 8     | 5.6 ± 1.8        | 101.6 ± 31.6                   |
| Creatinine    | FVFD  | 0     | 0.9 ± 0.2        | 100.0 ± 19.1                   |
|               |       | 8     | 0.8 ± 0.2        | 96.1 ± 18.3                    |
|               | Placebo | 0   | 0.9 ± 0.1        | 100.0 ± 15.8                   |
|               |       | 8     | 0.9 ± 0.2        | 96.9 ± 18.9                    |

Recommended: BUN, 7-23 mg/dL; Uric acid, M: 4.8-8.7 mg/dL, F: 2.6-8.0 mg/dL; Creatinine, M: 0.64-1.27 mg/dL, F: 0.4-1.03 mg/dL.

Table S4. Improvements in serum biochemical parameters (SOD, catalase, liver and kidney functions) of FVFD and placebo drink subjects at 8 weeks

| Sample          | FVFD | Placebo |
|-----------------|------|---------|
| Test time (weeks) | 8    | 8       |
| SOD             | +10.2% | -5.0%   |
| Catalase        | +105.0% | +18.5% |
| Cholesterol     | +2.9%  | +4.1%   |
| Triglyceride    | -29.2% | -22.9%  |
| SGOT            | -9.0%  | -5.4%   |
| SGPT            | +11.7% | +4.9%   |
| BUN             | -2.5%  | +7.7%   |
| Uric acid       | -1.5%  | +1.6%   |
| Creatinine      | -3.9%  | -3.1%   |
Table S5. Changes of skin texture for FVFD group. Mean and improvements of skin texture levels in FVFD and placebo drink subjects at 4 and 8 weeks. SD, standard deviation; \( n = 30 \); significant difference relative to baseline: *, \( p < 0.05 \); **, \( p < 0.01 \); significant difference relative to placebo group: #, \( p < 0.05 \)

| Item     | Group | Weeks | Mean (mean ± SD) | Improving rate (%) (mean ± SD) |
|----------|-------|-------|------------------|-------------------------------|
| Texture  | FVFD  | 0     | 300.3 ± 304.3    | 100.0 ± 101.3                 |
|          |       | 4     | 273.5 ± 312.6    | 91.1 ± 114.3*                 |
|          |       | 8     | 225.6 ± 242.3    | 75.1 ± 107.4**                |
|          | Placebo | 0     | 358.2 ± 322.7    | 100.0 ± 90.1                  |
|          |       | 4     | 332.1 ± 248.0    | 92.7 ± 74.7                   |
|          |       | 8     | 336.3 ± 239.1    | 93.9 ± 71.1                   |

Table S6. Changes of skin wrinkles for FVFD group. Mean and improvements of skin wrinkles levels in FVFD and placebo drink subjects at 4 and 8 weeks. SD, standard deviation; \( n = 30 \)

| Item     | Group | Weeks | Mean (mean ± SD) | Improving rate (%) (mean ± SD) |
|----------|-------|-------|------------------|-------------------------------|
| Wrinkle  | FVFD  | 0     | 17.7 ± 12.1      | 100.0 ± 68.7                  |
|          |       | 4     | 17.4 ± 15.1      | 98.5 ± 86.9                   |
|          |       | 8     | 13.9 ± 11.5      | 78.9 ± 82.7                   |
|          | Placebo | 0    | 15.1 ± 7.7       | 100.0 ± 51.3                  |
|          |       | 4     | 18.1 ± 9.3       | 119.9 ± 51.5                  |
|          |       | 8     | 20.1 ± 8.7       | 133.2 ± 43.5                  |

Table S7. Changes of skin dryness for FVFD group. Mean and improvements of skin dryness levels in FVFD and placebo drink subjects at 4 and 8 weeks. SD, standard deviation; \( n = 30 \); significant difference relative to baseline: *, \( p < 0.05 \); **, \( p < 0.01 \); significant difference relative to placebo group: #, \( p < 0.05 \)

| Item     | Group | Weeks | Mean (mean ± SD) | Improving rate (%) (mean ± SD) |
|----------|-------|-------|------------------|-------------------------------|
| Dryness  | FVFD  | 0     | 300.3 ± 304.3    | 100.0 ± 101.3                 |
|          |       | 4     | 273.5 ± 312.6    | 91.1 ± 114.3*                 |
|          |       | 8     | 225.6 ± 242.3    | 75.1 ± 107.4**                |
|          | Placebo | 0    | 358.2 ± 322.7    | 100.0 ± 90.1                  |
|          |       | 4     | 332.1 ± 248.0    | 92.7 ± 74.7                   |
|          |       | 8     | 336.3 ± 239.1    | 93.9 ± 71.1                   |

Table S8. Changes of skin shine for FVFD group. Mean and improvements of skin shine levels in FVFD and placebo drink subjects at 4 and 8 weeks. SD, standard deviation; \( n = 30 \); significant difference relative to baseline: *, \( p < 0.05 \); **, \( p < 0.01 \); significant difference relative to placebo group: #, \( p < 0.05 \)

| Item     | Group | Weeks | Mean (mean ± SD) | Improving rate (%) (mean ± SD) |
|----------|-------|-------|------------------|-------------------------------|
| Shine    | FVFD  | 0     | 300.3 ± 304.3    | 100.0 ± 101.3                 |
|          |       | 4     | 273.5 ± 312.6    | 91.1 ± 114.3*                 |
|          |       | 8     | 225.6 ± 242.3    | 75.1 ± 107.4**                |
|          | Placebo | 0    | 358.2 ± 322.7    | 100.0 ± 90.1                  |
|          |       | 4     | 332.1 ± 248.0    | 92.7 ± 74.7                   |
|          |       | 8     | 336.3 ± 239.1    | 93.9 ± 71.1                   |
Table S7. Changes of skin pores for FVFD group. Mean and improvements of skin pores levels in FVFD and placebo drink subjects at 4 and 8 weeks. SD, standard deviation; *n* = 30

| Item | Group | Weeks | Mean (mean ± SD) | Improving rate (%) (mean ± SD) |
|------|-------|-------|------------------|---------------------------------|
| Pores | FVFD  | 0     | 745.9 ± 416.3    | 100.0 ± 55.8                    |
|       |       | 4     | 675.3 ± 366.5    | 90.5 ± 54.3                     |
|       |       | 8     | 698.9 ± 462.6    | 93.7 ± 66.2                     |
|       | Placebo | 0    | 729.1 ± 417.2    | 100.0 ± 57.2                    |
|       |       | 4     | 752.5 ± 434.2    | 103.2 ± 57.7                    |
|       |       | 8     | 718.0 ± 397.6    | 98.5 ± 55.4                     |

Table S8. Changes of skin spots for FVFD group. Mean and improvements of skin spots levels in FVFD and placebo drink subjects at 4 and 8 weeks. SD, standard deviation; *n* = 30; significant difference relative to baseline: *, *p* < 0.05; ***, *p* < 0.001; significant difference relative to placebo group: ###, *p* < 0.01

| Item | Group | Weeks | Mean (mean ± SD) | Improving rate (%) (mean ± SD) |
|------|-------|-------|------------------|---------------------------------|
| Spots | FVFD  | 0     | 91.9 ± 34.5      | 100.0 ± 37.6                    |
|       |       | 4     | 75.7 ± 32.6      | 82.4 ± 43.0***                  |
|       |       | 8     | 71.2 ± 30.1      | 77.5 ± 42.3***                  |
|       | Placebo | 0    | 73.1 ± 33.5      | 100.0 ± 45.8                    |
|       |       | 4     | 76.7 ± 30.3      | 104.8 ± 39.6*                   |
|       |       | 8     | 79.0 ± 34.0      | 108.0 ± 43.1**                  |