Proceedings of Engineering and Technology Innovation, vol. 8, 2018, pp. 23 - 31

The Study of Tibicos Fermentation Product as Pilot Model of Healthy Drinks

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Received 31 July 2017; received in revised form 25 August 2017; accepted 10 September 2017

Abstract

Sugar kefir grains (Tibicos) are a symbiotic culture of bacteria and yeasts which were assembled by various strains of microbes attached polysaccharide bracket composed of white transparent particles. Initially we used chemical colorimetric method to measure that of carbohydrate concentrations, and Brad ford method for that of protein concentrations. We utilized black sugar as carbohydrate source and medium to cultivate Tibicos, and collected media after 0 hr., 24 hr., and 48 hr. different time points. These collected Tibicos grains and fermentation broth were measured with the above methods.

In addition, we used Gram stain to observe the inside microbial populations of Tibicos. The results showed that the weight of cultured grains with brown sugar solution at 25 ℃ and sealed for 48 hr increased to 45% as that of original grains. The components of Tibicos fermentation broth were analyzed by HPLC for ions as following: Lactic acid, Chloride ion, Malate, Sulfate, Oxalic acid, Phosphate, Citrate, and other minor ingredients.

Furthermore, we used the Gram stain to observe the microbial composition within Tibicos grains. The more detail identification of microbial population in Tibicos was done with bacteria and yeasts. The bacteria parts used PCR amplification with the 16S rDNA primer sets (533R and 341Fgc), gel purification, fragments sequencing, and alignment with 16S ribosomal RNA sequences in NCBI database (16S rDNA of Bacteria and Archaea). The result sequences were assigned as Bacillus strain as following: (a) *Bacillus circulans*, (b) *Bacillus eiseniae*, (c) *Bacillus oceanisediminis*, (d) *Bacillus atrophaeus*, (e) *Bacillus siralis*, (f) *Bacillus massiliosenegalensis*. The Yeast parts were first isolated as single colony, purified the chromosomal DNA, and amplified by PCR method with 18 S rDNA primer set (FR1 and NS1), then gel purified, DNA sequenced, and aligned in NCBI database (Nucleotide collection). The identified yeast strains as following: (a) *Sporobolomyces koalae*, (b) *Meyerozyma guilliermondii*, (c) *Aureobasidium pullulans*.

Keywords: tibicos, health drink, fermentation broth, 18S rDNA sequence, 16S rDNA sequence

1. Introduction

Sugar kefir grains (Tibicos) are assembled by various strains of microbes attached polysaccharide bracket composed of white transparent particles. These microbes included bacteria and yeast, which generated the organic acids and some intergradient that could improve the heath state of people [1]. There are Tibicos all over the world, but because of the weather

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and climate in different regions of the latitude of their differentiated; however, Tibicos are not all the same, either which found that there are two of the same area with the same culture conditions. Tibicos could adapt to the environment and become diverse that mixed bacteria were highly viable bacteria. There are some folktales that Tibicos fermentation broth (brown sugar kefir) can prevent insomnia and reduce high blood pressure, high fat, weight, but we try to investigate these legends. And there is little literature to investigate Tibicos; we found that there were some literatures about western kefir cultured in milk that could be a good parallel model to explore.

For example: some western kefir strain are a combination of *Lactobacillus, Pediococcus, Streptococcus, Leuconostoc* bacteria, and from yeast, *Candida, Kloeckera* [2]. And other possible yeast strain also exist in water kefir, such as *Candida*, *Kloeckera*. So, it is interesting whether that Taiwan common Tibicos strains are like the combination of western kefir strains or not? Generally, Tibicos fermentation broth are made from yeast, lactic acid bacteria, brewer's yeast etc., and Tibicos from different Taiwan area can be made from many fermentation broth which contain different sugar-containing liquids (such as brown sugar, sucrose, glucose, etc.), and these different carbohydrates are used to supply nutrients to feed it to produce lactic acid, alcohol (ethanol) and carbon dioxide gas as a carbonate-containing drink.

With the change of the times, the more developed of science and technology, people's lives are more and more comfortable, and have multiple choice of food and drinks, but have the more risk of choice with nutrient imbalance. The health drinks may be the good solution for this imbalance of food choice [3-4]. Furthermore, could we find the same effect on Tibicos' drink? To develop a pilot model for health drink with Tibicos worth our attention to this novel and tradition health drink.

Since ancient times, Tibicos has been fermented to drink for a long time, so far, some scholars have been suspected that Tibicos ingredients on the human body has no practical help, as well as the spread by Internet and the effect on the street stalls. However, we try to explore with scientific experiments to confirm the results with the Tibicos fermentation broth composition analysis and identification of microbes. We can have better understanding of Tibicos and the scientific data that prove the value of Tibicos. It is worthy our attention that the future development of health drinks with Tibicos will be a great mission for us to investigate further functions of these healthy drinks.

2. Method

2.1. The culture, preparation and Gram stain of Tibicos

To prepare 15 gram brown sugar and to add this distilled water 200 ml into a wide open glass bottle, the brown sugar water must be mixed evenly, and put 35 gram of Tibicos grains into glass bottle; do not stir. In further experiment, it fermented 0h, 24h, 48h, and collected metabolic solution. Change the brown sugar water every day; to stop the proliferation of Tibicos, just to rinse this grains with the distilled water, and placed in the container into the refrigerator. Use the mesh cloth to keep this Tibicos with air. Do not use metal containers.

To crush Tibicos grains after smear on the slides, air dry and fix the microbes. Stain with crystal purple staining, add to the slide with appropriate (covered with bacteria coated) crystal violet dye solution for 1 minute. Wash and Dip the dyeing solution and rinse carefully with water. Drop iodine solution for 1min. Wash the iodine solution with water. Decolor the slide with 95% ethanol for 20-25s to the effluent without color, wash immediately. Drop the safranin dye 5min. And wash the dirt on the smear with water. Dry the stained smear slides and put the air to dry or dry with absorbent paper. Observe under microscopic examination with low magnification, and then high-power, and finally observed with oil mirror, and determine the morphology of the microbes [5].

2.2. Gel electrophoresis of the microbes' genomic DNA PCR fragment

Weigh out the appropriate mass of agarose into an Erlenmeyer flask. Agarose gels are prepared using a w/v percentage solution. The concentration of agarose in a gel will depend on the sizes of the DNA fragments to be separated, with most gels ranging between 0.5%-2%. The volume of the buffer should not be greater than 1/3 of the capacity of the flask. Add running buffer to the agarose-containing flask. Swirl to mix. The most common gel running buffers are TAE (40 mM Tris-acetate, 1 mM EDTA). Melt the agarose/buffer mixture. This is most commonly done by heating in a microwave, but can also be done over a Bunsen flame. At 30 s intervals, remove the flask and swirl the contents to mix well. Repeat until the agarose has completely dissolved. Add ethidium bromide (EtBr) to a concentration of 0.5 μg/ml. Alternatively, the gel may also be stained after electrophoresis in running buffer containing 0.5 μg/ml EtBr for 15-30 min, followed by de-staining in running buffer for an equal length of time. Allow the agarose to cool either on the benchtop or by incubation in a 65 ℃ water bath. Place the gel tray into the casting apparatus. Alternatively, one may also tape the open edges of a gel tray to create a mold. Place an appropriate comb into the gel mold to create the wells. Pour the molten agarose into the gel mold. Allow the agarose to set at room temperature. Remove the comb and place the gel in the gel box. Then setup the apparatus and electrophoresis, and observe the result with A gel documentation system [6].

2.3. The molecular analysis of bacterial and yeast DNA in Tibicos

The 16 S rDNA of the Tibicos' bacteria was amplified by PCR using 533R and 341Fgc primer set. The PCR results were separated by the DGGE method and DNA gel electrophoresis. After sequencing, the target gene was amplified by PCR using 533R and 341F again. Send the PCR results to the sequence. Compare the results in the NCBI database [7].

Isolate and purify fungal colonies in Tibicos' grain. Stain colonies and identification. Isolate single colony and extract chromosomal DNA. The 18S rDNA of the cells was amplified by PCR using FR1 and NS1 primers. Collect and purify the PCR DNA products from the DNA gel electrophoresis. After sequencing, compare the results in the NCBI database [8].

2.4. The carbohydrate and protein analysis of Tibicos' metabolite in time course

Dilute the Tibicos solution to 1% (0 hr., 24 hr., and 48 hr.). Take 50μl of the test solution and add 500μl of 95% sulfuric acid, shake and mix well. Add 50 μl of 5% phenol, shake and mix well. Take 200μl, measured OD492. Take the value into the equation; get the value from the standard curve. These samples were measured and converted into accurate units [9].

Take 200 μl of the Tibicos solution (0 hr., 24 hr., 48 hr) with 200 μl Bradford reagent for 10 min. Observe under OD595 absorbance and convert the OD value with the protein standard curve to the protein concentration [10].

2.5. The HPLC analysis of Tibicos' metabolite

Follow the DionexIonPac® AS11 Manual 5.7 for Gradient Separation of Krebs cycle Acids. The time course samples were injected using the autosampling system (SPECTRA SYSTEM AS3500, Thermo, Finnigan) by means of an ion liquid chromatography IonPac® AS11 2 × 250 mm column, Dionex. The organic acid was separated using a 5-100 mM NaOH gradient eluent, and the anion concentration in the culture medium was finally analyzed with conductivity analysis [11].

3. Results and Discussion

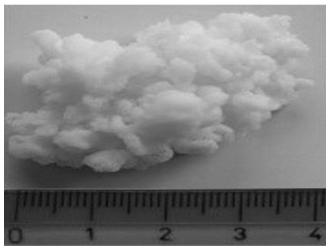
Since Tbicos were traditional drinks long time ago[1], we established the platform to develop it into health drinks. In Fig. 1, we can observe the Tibicos drinks in Fig. 1(c) and kefir grains in Figs. 1(a), (b), and (d). We test the Tibicos under different temperature and carbohydrates, for example: glucose, sugar and black sugar (data not shown). To compare the Tibicos and kefer

grains, we can find similar morphology of grains. However, the black sugar and room temperature were the best condition for the culture of the Tibicos.

(a) General Tibicos grains

(b) The grains under microscope

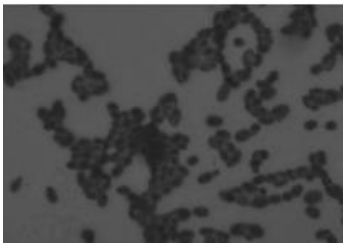
(c) A tradition Tibicos drinks



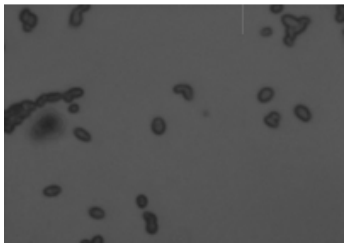
(d) The general kefir grains which were grown in milk

Fig. 1 The Tibicos, drinks and kefir grains

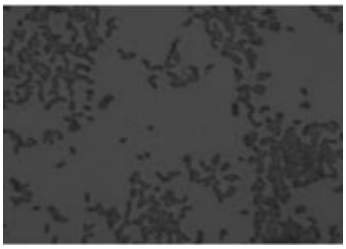
From the observation the smearing of the grain under microscope and Gram stain, we can find that the grains form the Tibicos were Gram positive cocci (Figs. 2(a), (b), and (c)), Gram negative rod-like bacilli (Fig. 2(d)), Gram negative cocci (Fig. 2(e)), and Gram positive bacilli (Fig. 2(f)). These results will be the first observation of the Tibicos grains by Gram stain, and there were Gram positive cocci and bacilli, and Gram negative cocci and bacilli. We can compare the similar results in kerfir grains [12].



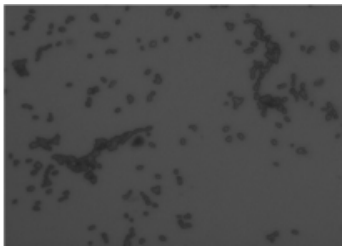
(a) Gram positive cocci



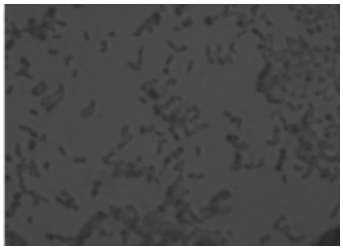
(b) Gram positive cocci



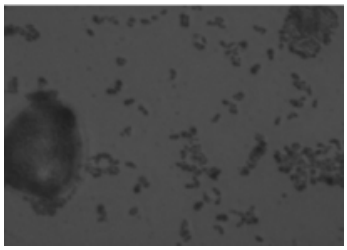
(c) Gram positive cocci



(d) Gram negative rod-like bacilli



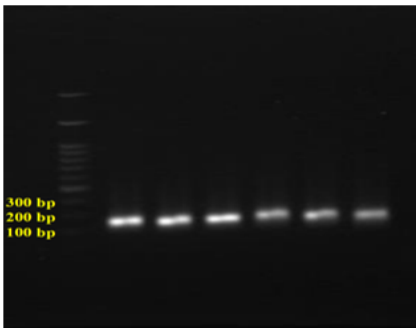
(e) Gram negative cocci



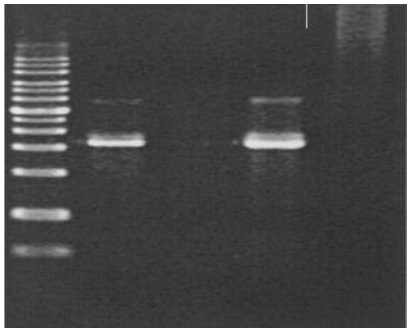
(f) Gram positive bacilli

Fig. 2 By Gram staining, the Tibicos grains are showed both Gram negative and positive stain mix population

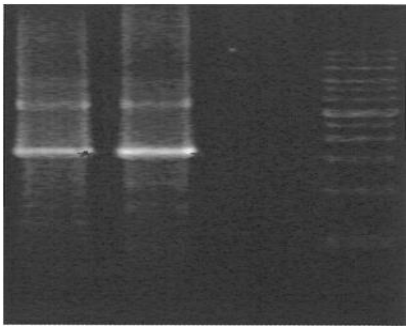
We used molecular method to identify the different strains of bacteria (Fig. 3(a)) and yeast (Fig. 3(b), (c)), and the primer sets used in this experiment were 341 to 533R for bacteria 16S rDNA sequences, NS-1 to FR1, FR3 for yeast 18S rDNA sequences. The gel electrophoresis for PCR results show that near 200 base pairs fragment in all six bacteria strains (Fig. 3(a)), and yeast 1.6 kb fragments of T1, T2 (Fig. 3(b)), T3 (Fig. 3(c)). The specific sites of sequences in primer sets were shown in Fig. 3(d).



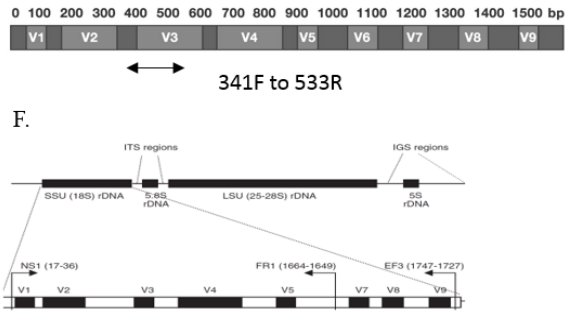
(a) PCR fragment of 6 bacteria strains with 553R to 341F primer set



(b) Yeast NS1 to FR1 PCR fragments with DNA Marker, isolate T3, T2,T1



(c) Isolate T3, T2, blank, DNA Marker



(d) The 16S rRNA bacteria genome map and PCR primer 553R to 341F sites and The yeast 18S rRNA NS1 (17-36), FR1 (1664-1649), FR2 (1747-1727) primers

Fig. 3 Results of gel electrophoresis

The results of molecular analysis for bacteria (Table 1) and yeast (Table 2) were compared with the NCBI databank of 16S rDNA of Bacteria and Archaea and NCBI database (Nucleotide collection) respectively. The bacteria parts used PCR amplification with the 16S rDNA primer sets (533R and 341Fgc), gel purification, fragments sequencing, and alignment with 16S ribosomal RNA sequences. The result sequences were assigned as *Bacillus* strain as following: (a) *Bacillus circulans*, (b) *Bacillus eiseniae*, (c) *Bacillus oceanisediminis*, (d) *Bacillus atrophaeus*, (e) *Bacillus siralis*, (f) *Bacillus massiliosenegalensis*. The Yeast parts were first isolated as single colony, purified the chromosomal DNA, and amplified by PCR method with 18 S rDNA primer set (FR1 and NS1), then gel purified, DNA sequenced, and aligned in NCBI database. The identified yeast strains as following: (a) *Sporobolomyces koalae*, (b) *Meyerozyma guilliermondii*, (c) *Aureobasidium pullulans.*

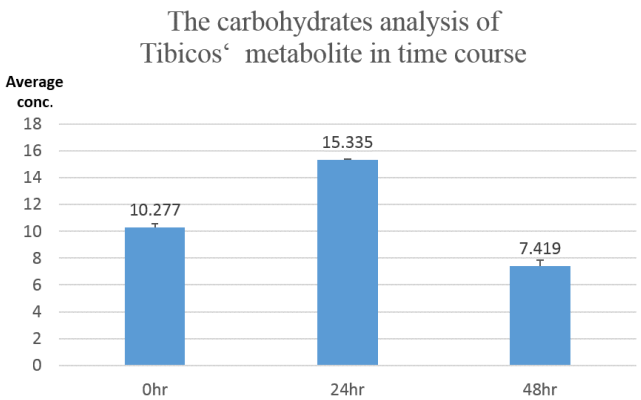
Table 1

	Descript	Max score	Total score	Query cover	Ident
a	Bacillus circulans strain ATCC 4513 16S ribosomal RNA gene, partial Sequence	268	268	96%	96%
b	Bacillus eiseniae strain A12 16S ribosomal RNA gene, partial sequence	281	281	97%	98%
c	Bacillus oceanisediminis strain H2 16S ribosomal RNA gene, partial sequence	281	281	97%	98%
d	Bacillus atrophaeus 1942 strain 1942 16S ribosomal RNA gene, partial sequence	283	283	97%	98%
e	Bacillus siralis strain 171544 16S ribosomal RNA gene, partial sequence	287	287	95%	98%
f	Bacillus massiliosenegalensis strain JC6 16S ribosomal RNA gene, partial sequence	281	281	90%	100%

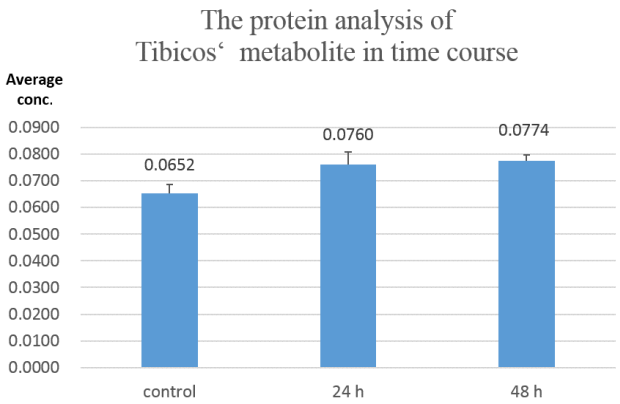
Table 2

	Descript	Max score	Total score	Query cover	Ident
a	Sporidiobolus salmonicolor gene for 18S ribosomal RNA, partial sequence	217	217	60%	95%
b	Meyerozyma guilliermondii strain 15H4-PO-P5-2 18s ribosomal RNA, gene, partial sequence	710	710	100%	97%
c	Aureobasidium pullulans gene for 18S rRNA, partial sequence, strain	486	486	99%	99%

The results of Tibico metabolites' carbohydrate and protein analysis in time course were shown in Fig. 4. From these results, we could notice that after 24 hours, the carbohydrate in metabolite increased about 49% to that of 0 hr and decreased 28 % to that of 0 hr after 48 hours. The protein content in metabolite increased 16.6% (24 hr) and 18.7% (48 hr) to that of 0 hr.



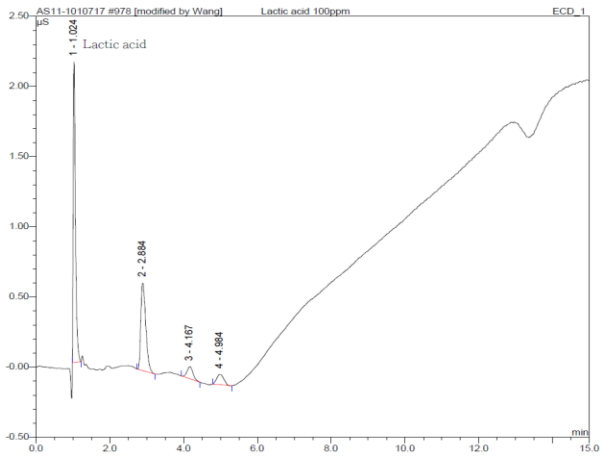
(a) The carbohydrate content change in Tibicos' metabolite with time course



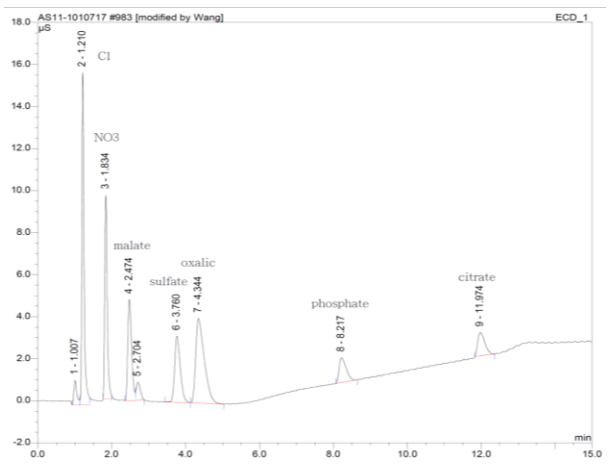
(b) The protein content change in Tibicos' metabolite with time course

Fig. 4 Results of the carbohydrate and protein analysis in Tibicos' metabolite with time course

The results of the HPLC analysis in Tibicos' metabolite with time course were shown respectively in following: the profile of lactic acid concentration in Fig. 5(a), the profile of Cl, NO_3, malate, sulfate, oxalic, phosphate, citrate concentration in Fig. 5(b), the profile of lactic acid, Cl, sulphate concentration in Fig. 5(c), the profile of Tibicos' metabolite in 24 hr. with lactic, Cl, malate, sulfate, oxalic, phosphate, citrate concentration in Fig. 5(d), the profile of Tibicos' metabolite in 48 hr. with lactic, Cl, malate, sulfate, oxalic, phosphate, citrate concentration in Fig. 5(e). The final time courses were listed in the table of Fig. 5(f). We find that the concentration of lactic acid increased 4.1 fold after 24 hours, and 4.3 fold after 48 hours to that of 0 hr, the concentration of chloride ion increased 2.4 fold after 24 hours, and 2.5 fold after 48 hours to that of 0 hr, the concentration of malate, oxalic acid, citrate and phosphate was newly produced after 24 and 48 hours, the concentration of sulphate has a little change.

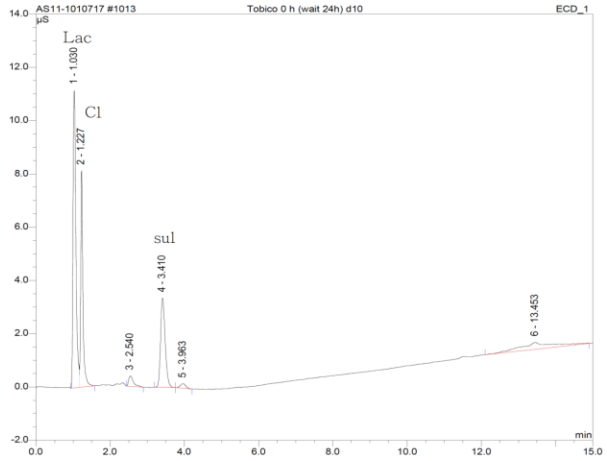


(a) Profile of Lactic acid

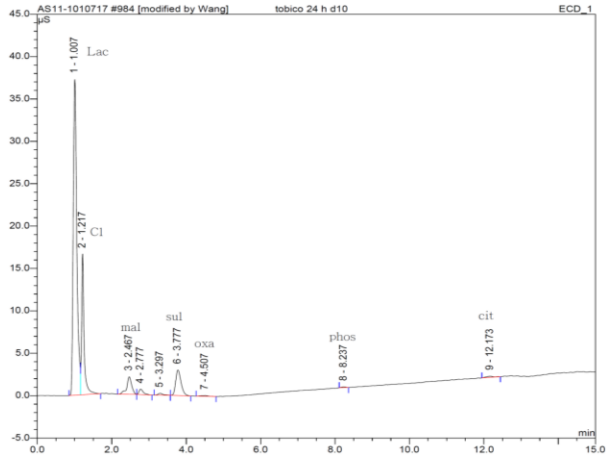


(b) Profile of Cl, NO_3, malate, sulfate, oxalic, phosphate, citrate

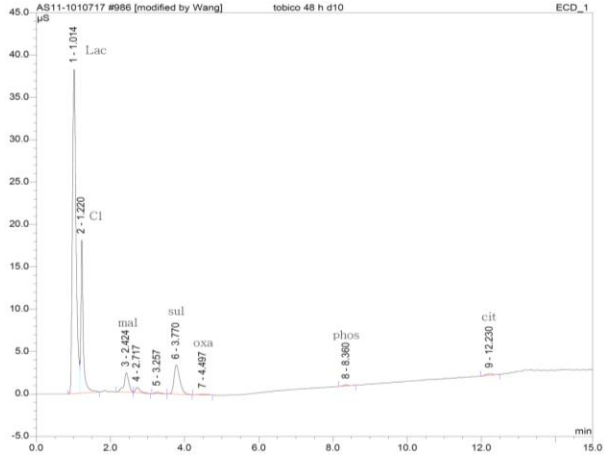
Fig. 5 Results of the HPLC analysis in Tibicos' metabolite with time course



(c) Profile of lactic acid, Cl, sulfate



(d) Profile of Tibicos' metabolite in 24 hr with lactic, Cl, malate, sulfate, oxalic, phosphate, citrate



(e) Profile of Tibicos' metabolite in 48 hr. with lactic, Cl, malate, sulfate, oxalic, phosphate, citrate

The Quantity of Anions in Sample (mg/uL)	Tobicos 0 h (wait 24 h)	Tobicos 24 h	Tobicos 48 h
Lactic acid	4.123	17.044	17.728
Cl^-	0.049	0.115	0.124
malate	-	0.052	0.056
sulfate	0.107	0.09	0.111
oxalic acid	-	0.002	0.002
phosphate	-	0.004	0.007
citrate	-	0.011	0.013

(f) Table of organic acid contents with time course

Fig. 5 Results of the HPLC analysis in Tibicos' metabolite with time course (continued)

4. Conclusions

In this paper, the content of lactic acid, malate, phosphate and citrate in the metabolic solution of Tibicos was discussed. The concentration of protein and organic acid was increased obviously, which may play an important role in the health drink development. In addition to the Kefir culture study, Kefir was found to be cultured in milk or cultured in cocoa, while the environment of Kefir was cultivated in milk [12], and compared to our Tibicos for environmental testing. In this study of sucrose, brown sugar, and glucose, it is found that Tibicos is suitable for culturing in brown sugar, and this environmental optimization would be related to local different species of Tibicos or Kefir.

The original brown sugar water contained only 4.123 mg / μl lactic acid, while adding Tibicos in this solution, after 24hr. this solution increased to 17.044 mg / μl lactic acid. And after 48 hr., the concentration of lactic acid in Tibicos metabolic solution was 17.728 mg / μl. The long-term drinking of Tibicos metabolic solution can allow the human body to ingest some lactic acid, this could give our digestive system with benefits [13]. No citric acid was detected in original brown sugar solution, but after adding Tibicos grains for 24 hr., this solution contained 0.011 mg / μl citric acid, and after 48 hr. of fermentation, this citric concentration of Tibicos solution increased to 0.013 mg /μl. Furthermore, literature showed that the body intake of the citric acid would help the body to degrade the body fat by inhibiting the accumulation of tissue fat. This probiotics solution can also help in the growth of the body which would promote the body metabolism [14]. On the market, there are also a lot of citric acid derived health food, and stimulate the human body with this nutritional content [15]. No malic acid was found in original brown sugar solution, after

adding Tibicos grains for 24 hr., this metabolites contained 0.052 mg/μl malic acid, after fermentation for 48 hr., the concentration of malic acid in Tibicos metabolites increased to 0.056 mg/μl. Malic acid would activate human pulmonary artery and improve to lower the body high blood pressure [16].

In this study, the *Aureobasidium pullulans* were isolated from yeast in the Tibicos grains. This strain was isolated from the postharvest pathogens and was belonged to valuable fungi [17]. We isolated *Bacillus circulans* from Tibicos grains, however, this *Bacillus circulans* would increase the plasmin production and help to prevent thrombosis-related diseases [18]. We also isolated *Bacillus oceanisediminis* from Tibicos, and this bacillus belonged to the Gram-positive *bacilli oceanisediminis*, which was first discovered in the South Korean coastal marine sediments [19]. *Meyerozyma guilliermondii* was also isolated form Tibicos, and this yeast has the spore germination which would have antibacterial activity, and often used in bread preservatives that will extend the shelf life of bread [20]. This yeast was also added in the soy sauce which extend the freshness of its flavor [21].

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