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THE CONTENT OF BIOLOGICALLY ACTIVE SUBSTANCES AND TOTAL ANTIOXIDANT ACTIVITY OF BLACK TEA DRINKS AND DECOCTIONS OF MEDICINAL PLANTS FERMENTED WITH KOMBUCHA

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Abstract: Kombucha is a healthy-fermented beverage traditionally made by fermentation of sweetened tea with a symbiotic culture of different yeasts and acetic acid bacteria, named also SCOBY – a symbiotic culture of bacteria and yeast. During the fermentation of glucose, yeast produces ethanol, which is used by acetic acid bacteria to produce acetic and other acids. The resulting ethanol and acids, as well as fermentation by-products, provide the original flavor and aroma of the beverage. In this study, we compared the growth and chemical composition of beverages when growing tea mushroom on a typical substrate, black tea, and decoctions of two medicinal plants, rose hips and yellow gentian rhizomes. To obtain the fermented drinks, the "tea mushroom" was grown on 1.3% (w/v) Ahmad black tea, and plant decoctions from rose hips and gentian roots with the addition of different concentrations of sucrose (0.5%, 1%, 5%, 10% and 20%) for 14 days. At day 0, 7 and 14 of cultivation, we determined body mass of the Kombucha and characteristics of fermented beverages (pH, total acid levels, ethanol content, sucrose concentration, total polyphenols and flavonoids, total antioxidant capacity). The results indicate that Kombucha grew well on both, its usual substrate -black tea drink and decoctions of medicinal herbs, rose hips and roots of yellow gentian. According to the growth rate of SCOBY on the tested media, the samples can be ranked in the following order: rose hips > yellow gentian roots > Ahmad black tea. The optimal concentration of sucrose in the medium for the development of Kombucha was 5%. During cultivation, sucrose was gradually fermented by the Kombucha yeast to ethanol, which in turn was used by acetic acid bacteria to synthesize acetic acid. As a result, an acidification of the pH of the culture medium was observed. The content of polyphenolic substances in the infusions increased slightly during cultivation, indicating that Kombucha has a limited ability to synthesize these compounds. The total antioxidant activity of the infusions decreased during the cultivation on the tea made of rose hips, but it increased on the decoctions of the roots of yellow gentian on the 14th day of the growth. Thus, not only phenolic substances determine the antioxidant properties of fermented kombucha beverages.

Keywords: Kombucha, fermentation, yeast, acetic acid bacteria, polyphenols, antioxidant activity, beverages.

1. INTRODUCTION

Thanks to the development of biotechnology, it is possible to obtain large quantities of substances useful to humans that are synthesized by living organisms. Natural compounds such as hormones, enzymes and vitamins are widely used in pharmacology, cosmetics, chemicals, etc. An important step in biotechnological production is the cultivation of an organism capable of synthesizing biologically active substances. One of the biotechnologically important objects is Kombucha. Kombucha (named also SCOBY - symbiotic culture of bacteria and yeast) is a symbiotic combination of two different types of microorganisms - yeasts (*Shizosaccharomyces pombe, Zygosaccharomyces rouxii, Saccharomyces ludwigii, genera Candida, Torulopsis Psevdosaccharomyces, Hanseniaspora*, etc.,) and acetic acid bacteria (*Acetobacter xylinum, Acetobacter suboxydans, Acetobacteraceti, Acetobacter xylinoides, Bacterium gluconicum*) (Villarreal-Soto et al., 2018, Kapp & Sumner, 2019; Laureys et al., 2020). This symbiotic interaction results in the production of a number

of useful and essential substances for the human body (Kapp & Sumner, 2019). The fermentation of tea by Kombucha is a combination of three types: alcoholic, lactic and acetic ones, due to the presence of several yeasts and bacteria that coexist in the medium (Kapp & Sumner, 2019). The fermented drink contains vitamins, ascorbic acid, nicotinic acid and pantothenic acid. It also produces ethanol in the same amount as kefir, as well as caffeine, catechins and carbon dioxide, which prevents the fungus from sinking (Bishop et al., 2022) This is a very refreshing and thirst-quenching drink in the hot weather.

The tea mushroom infusion is not only tasty. It is also healthy because it has antibacterial properties. Thanks to SCOBY, the growth of pathogenic bacteria slows down and some species die off completely. Kombucha is considered a medicine created by nature itself and is therefore widely used in alternative medicine (Mousavi et al., 2020). The fermented beverage obtained by cultivating kombucha has antioxidant and anti-inflammatory potential, and is a very effective treatment for many diseases of the gastrointestinal tract, atherosclerosis, hypertension, rheumatism, etc. (Mousavi et al., 2020; Bishop et al., 2022).

The production technology, the composition of the microbiota and the production of biologically active substances are important factors for industrial production of Kombucha. The aim of this study was to compare the levels of bioactive compounds and antioxidant activity of fermented beverages when Kombucha was grown in different culture media based on tea and plant decoctions.

2. MATERIALS AND METHODS

Preparation of media and selection of conditions for cultivation Kombucha

To obtain the fermented drink, the "tea mushroom" was grown on Ahmad black tea, and plant decoctions from rose hip and gentian root with the addition of different concentrations of sucrose (0.5%, 1%, 5%, 10% and 20%). To prepare the infusion, one Ahmad tea bag (2 g) was used per 1.5 l of boiled water (1.3% tea infusion).

Rose hips and gentian rhizomes were crushed before the infusions were prepared. The crushed raw materials (9 g) were placed in an enamel dish, poured over 0.45 l of boiled water, covered with a lid and boiled for 30 minutes. The vessel was then removed and allowed to cool to room temperature. The infusions were then filtered and water was added to the finished filtrate to the required volume to obtain a 1.3% tea decoction. Sucrose was added to the resulting infusion to achieve the appropriate concentrations (0.5-20%).

A layer of Kombucha mushroom was transferred to the prepared culture media. After that, for 7-14 days, the SCOBY (symbiotic culture of bacteria and yeast) was cultivated on tea or decoctions of various medicinal plants. On the seventh and fourteenth day of cultivation, we measured the mass of the "fruiting body" formed by the fungus, the pH of the medium, the sucrose concentration, the ethanol concentration, the total polyphenol content, the flavonoid content, the vitamin C content, and the total antioxidant activity of the infusions.

Mass determination

The mass of the Kombucha mushroom was measured on the seventh and fourteenth day. Kombucha was carefully removed from the infusion, placed on filter paper to absorb excess liquid, transferred to a pre-weighed petri dish, and weighed on an electronic balance.

Determination of pH and total acidity of the beverages

The acidity of the medium was measured with a pH meter at the beginning of cultivation (day 0) and then at day 7 and day 14.

Total acidity was determined by titrating the tea infusion with 1 n NaOH in the presence of phenolphthalein (Spaska, 2022). Calculations were made considering that 1 ml of 1 n NaOH corresponds to 0.06 g of acetic acid in 10 ml of the medium.

Determination of sucrose Concentration

To determine the sugar concentration, we used the refractometric method based on the refractive index of sucrose solutions (Popov, 1971).

Determination of ethanol Concentration

The concentration of ethanol in the solutions was determined by the colorimetric method based on the interaction with potassium bichromate according to the method described in (Sumbhate et al., 2012). The method is based on the interaction of ethanol with potassium bichromate in the presence of sulfuric acid and acetate buffer (pH 4.3) to form a green complex with an absorbance maximum at 578 nm.

Determination of total polyphenols

The polyphenol content was determined by the titrimetric method based on the oxidation of tannins by potassium permanganate in the presence of indigocarmine (Mahdavi et al., 2010). The titration results were compared with the standard Leventhal titration coefficient of 6.4 (1 ml of 0.1 n $KMnO_4$ oxidizes 6.4 μg of rutin).

Determination of flavonoids

The principle of the method is based on the fact that $AlCl_3$ forms stable orange complexes with the C-4 keto group or the C-3 or C-5 hydroxyl group of flavones and flavanols, which have a maximum absorbance at 510 nm (Ogura et al., 1968; Bayliak et al., 2016). The experimental samples were processed simultaneously with the calibration curve samples prepared with a standard solution of quercetin in 96% alcohol.

Determination of total antioxidant activity

ABTS+ cation radical scavenging was used to measure the total antioxidant capacity (TAC) (Erel, 2004). In brief, the ABTS+- cation radical was formed by the interaction of ABTS (2,2-azino-bis-3-ethylene-benzthiazoline-6-sulfonic acid) with hydrogen peroxide at a low pH (3.6). The antioxidant molecules are able to reduce the dark green ABTS+ cation radical to the colorless ABTS. Decreased ABTS+ absorbance was recorded at 414 nm. The standard solution of the antioxidant was Trolox, a water-soluble analog of vitamin E.

Statistical analysis

Statistical processing of the data was performed using the Mynova computer program. The mean of each sample was obtained from three replicates and used for further analysis. The mean of each sample (M) and the standard error mean (m) were used as statistical indicators. The comparison of means and the determination of a significant difference between them were performed using Dunnett's and Student's t-tests.

3. RESULTS AND DISCUSSION

The main substrate for cultivation of Kombucha is tea. It is also known that Kombucha can grow on different plant decoctions. Therefore, the content of biological substances in the fermented beverage can vary depending on the composition of the growth medium. Considering that medicinal plants have a number of biologically active substances, fermented beverages made on their basis may have special flavors and differ in the content of biological substances. In this work, we focused on studying the growth characteristics of Kombucha on Ahmad tea and decoctions from medicinal plants - rose hips and yellow gentian roots.

In the first stage of the work, we analyzed the growth rate of Kombucha on classic black tea of the Ahmad brand and decoctions of rose hips and yellow gentian roots.

Both decoctions we have chosen are considered quite useful in alternative and evidence-based medicine. Rose hips are used to make tea, infusions, and decoctions that are taken for medicinal purposes. Rose hips contain a large number of nutrients, including vitamins C, A, K, E, P, and B. The fruits are rich in essential oils, pectin's, and organic acids. Rosehip improves metabolic processes, strengthens the immune system and increases the body's resistance to various infections, absorbs and removes heavy metal salts and toxins from our body (Mármol et al., 2017).

Yellow gentian is a medicinal plant found only in the Carpathian Mountains. The substances present in the roots (especially genziopicrin) have a bitter taste and thus increase sensitivity to taste stimuli, stimulate secretion and motility of the gastrointestinal tract. In medicine, gentian preparations in the form of infusions, herbs and bitter tincture are used as a means of stimulating and improving digestion and assimilation of food in cases of loss of appetite, dyspepsia, and as a choleretic drug (Nicolic et al., 2021).

We measured body mass of the tea mushroom on the seventh and tenth four days of cultivation. The initial mass of Kombucha was approximately the same in all experiments and was about 3 g. In parallel, we determined the dependence of the Kombucha mass dynamics on the concentration of sucrose in the studied infusions (Fig 1).

As shown in Figure 1, the mass of the fungus increased during cultivation on all media. The optimum mass, regardless of the medium composition, was in the presence of sucrose at a concentration of 5%. Kombucha growth was slower on media with 0.5 and 1% sucrose. This may indicate a lack of energy source. Interestingly, the "growth" of the fruiting body also slowed down at relatively high concentrations of the carbon source (20%). We hypothesize that under these conditions, Kombucha yeast uses sucrose only through fermentation. Fermentation gives a lower biomass yield per unit of carbohydrate consumed than using of carbohydrates as energy source under aerobic conditions. At the same time, fermentation apparently produces a large amount of ethanol, which cannot be metabolized quickly by the acetic acid bacteria of Kombucha, and in this case ethanol acts as an inhibitor of Kombucha growth. In addition, an excessive amount of acids formed during the oxidation of ethanol by acetic acid bacteria can inhibit the development of Kombucha.

In order to determine whether the growth of Kombucha was related to the change of concentration of ethanol and acids in the infusions, we measured the change of pH of the medium, the content of total acids and the content of ethanol in the medium of Kombucha during cultivation on different substrates. Sucrose concentration "5%" was accepted as the optimal growing conditions.

First, we determined the pH changes in the media during the cultivation of Kombucha on Ahmat tea, rosehip decoctions, and yellow gentian roots. As can be seen in Fig. 2, the initial pH of the medium was closely to neutral for Ahmat tea and decoction from yellow gentian root, and slightly acidic (~pH 5.5) for decoction from rose hip medium. The lower initial pH in the rosehip-based medium can be due to the presence of more acids, particularly ascorbic acid. As the incubation time increased, the medium became more uniformly acidic.

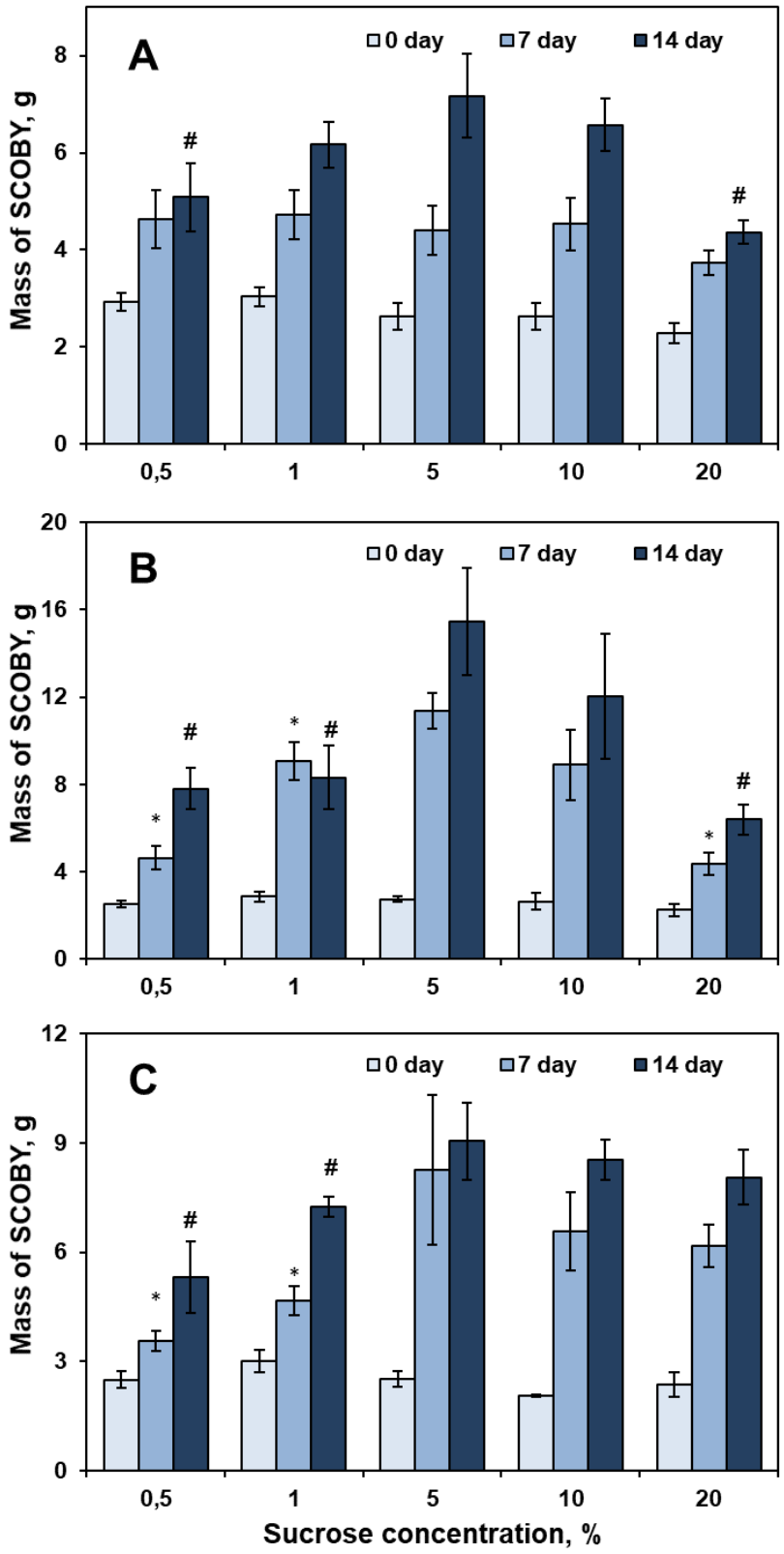

Fig. 1. Effects of different sucrose concentration on mass gain of the Kombucha SCOBY (symbiotic culture of bacteria and yeast) in media prepared from black tea Ahmat drink (A), decoctions of rose hips (B) and roots of yellow gentian (C). Data are presented as means ± S.E.M. (n = 4). Significantly different from the control group "5% sucrose" on days 7 (*) and 14 (#) with P<0.05.

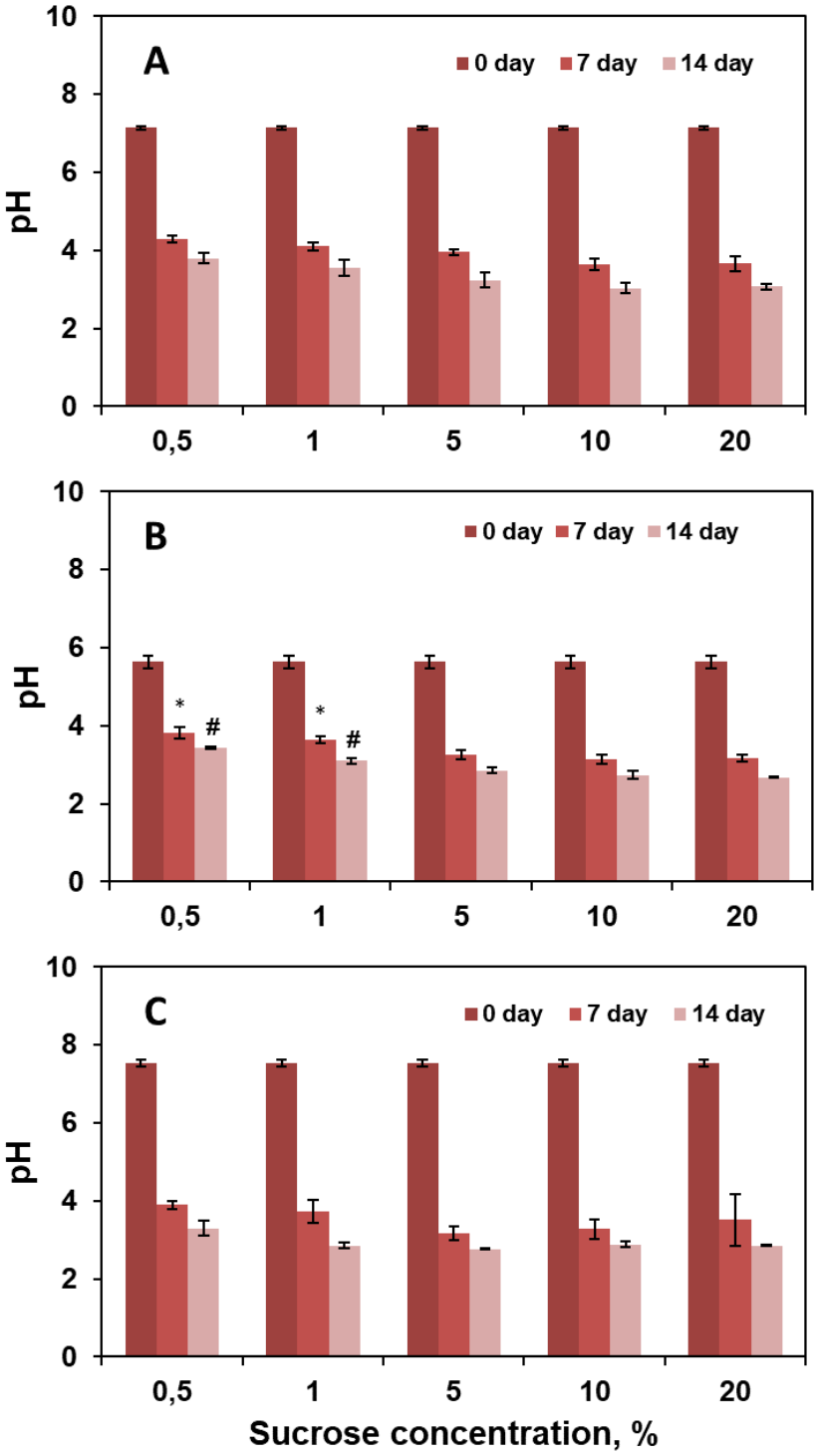

Fig. 2. Changes in the pH value of the culture media during the cultivation of Kombucha on black tea Ahmat (A), decoctions of rose hips (B) and roots of yellow gentian (C). Data are presented as means ± S.E.M. (n = 4). Significantly different from the control group "5% sucrose" on days 7 (*) and 14 (#) with P<0.05.

Fig. 3 shows the total acid concentration in the media where Kombucha was cultivated. As can be seen, the acid content in fermented "Ahmad" tea did not depend on the concentration of sucrose in the medium. At the same time, an increase in acidity was observed in the decoctions of rose hips and roots of yellow gentian both during cultivation and with an increase in sucrose in the medium. In the case of rose hip decoction, such dependence was more evident.

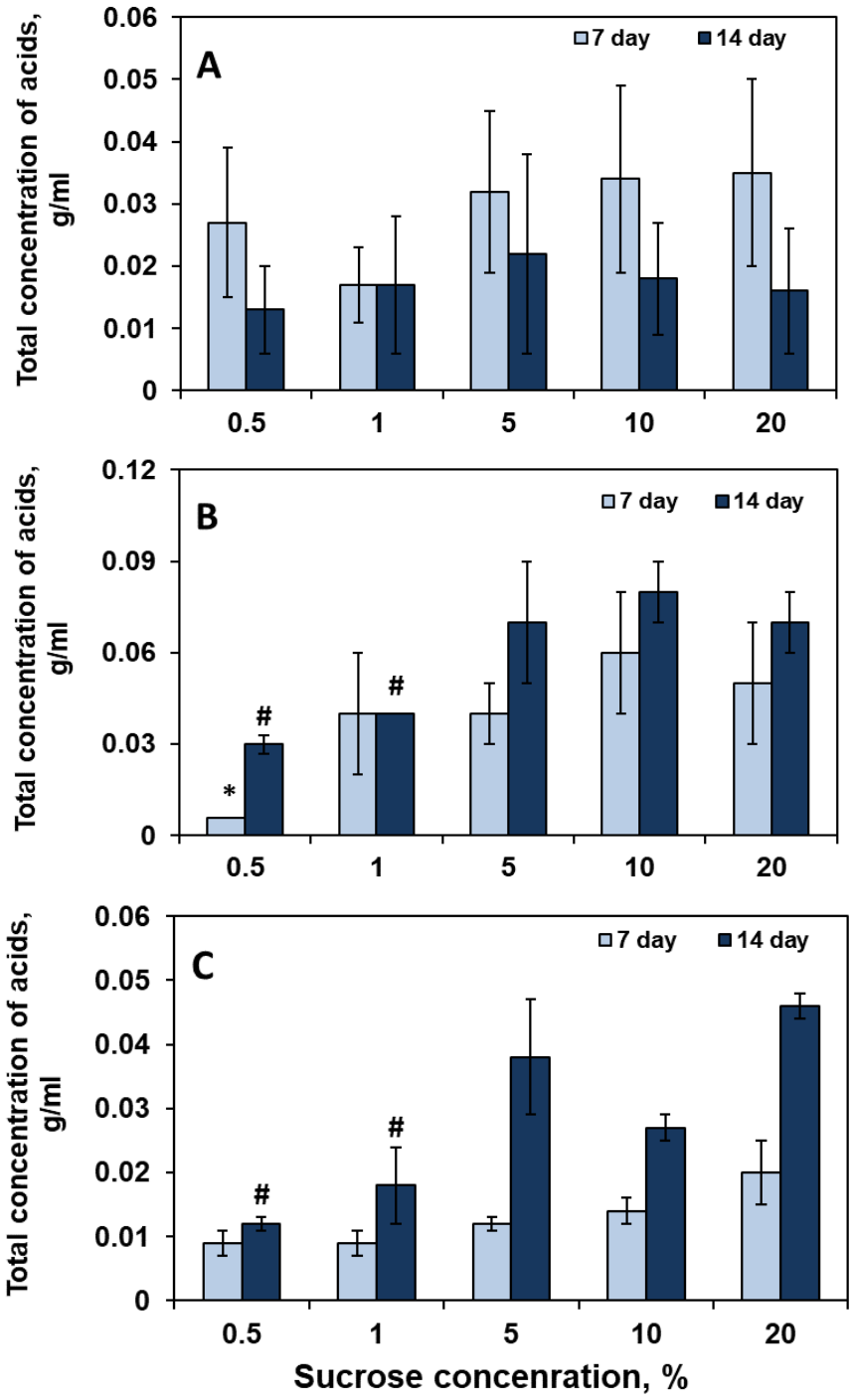

Fig. 3. Total concentration of acids in the medium during the cultivation of Kombucha on black tea Ahmat (A), decoctions of rose hips (B) and roots of yellow gentian (C). Data are presented as means ± S.E.M. (n = 4). Significantly different from the control group "5% sucrose" on days 7 (*) and 14 (#) with P<0.05.

The concentration of ethanol (Fig. 4) in the medium of the fermented "Ahmad" tea and gentian roots was higher than that of decoction of rose hips. At the same time, the content of ethanol in the gentian-based medium depended on the content of sucrose concentration.

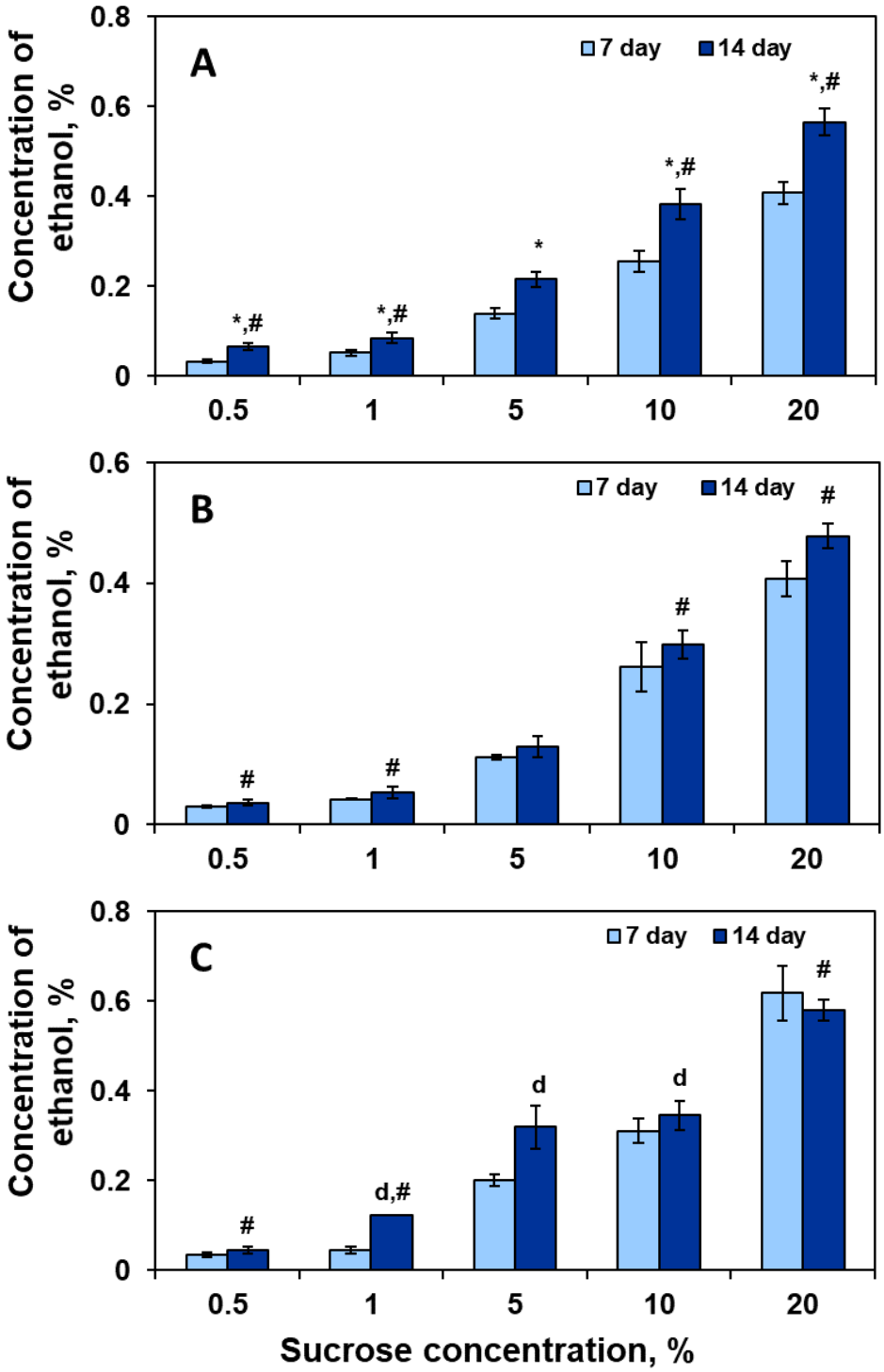

Fig. 4. Total concentration of ethanol in the medium during the cultivation of Kombucha on black tea Ahmat (A), decoctions of rose hips (B) and roots of yellow gentian (C). Data are presented as means ± S.E.M. (n = 4). Significantly different from the control group "5% sucrose" on days 7(*) and 14 ($^{\#}$), from the corresponding value at the same concentration of sucrose on the day 7 (^{d}) with P<0.05.

Phenolic compounds, most of which are water soluble, are abundant in tea and rose hips. With a view to this, we were interested in investigating whether the total polyphenol content and the content of one of their most abundant groups - flavonoids - vary with the media composition.

On day 7 of growth, the total content of polyphenols remained unchanged when Kombucha was cultivated on Akhmat tea and rose hip decoctions (Fig. 5A-B).

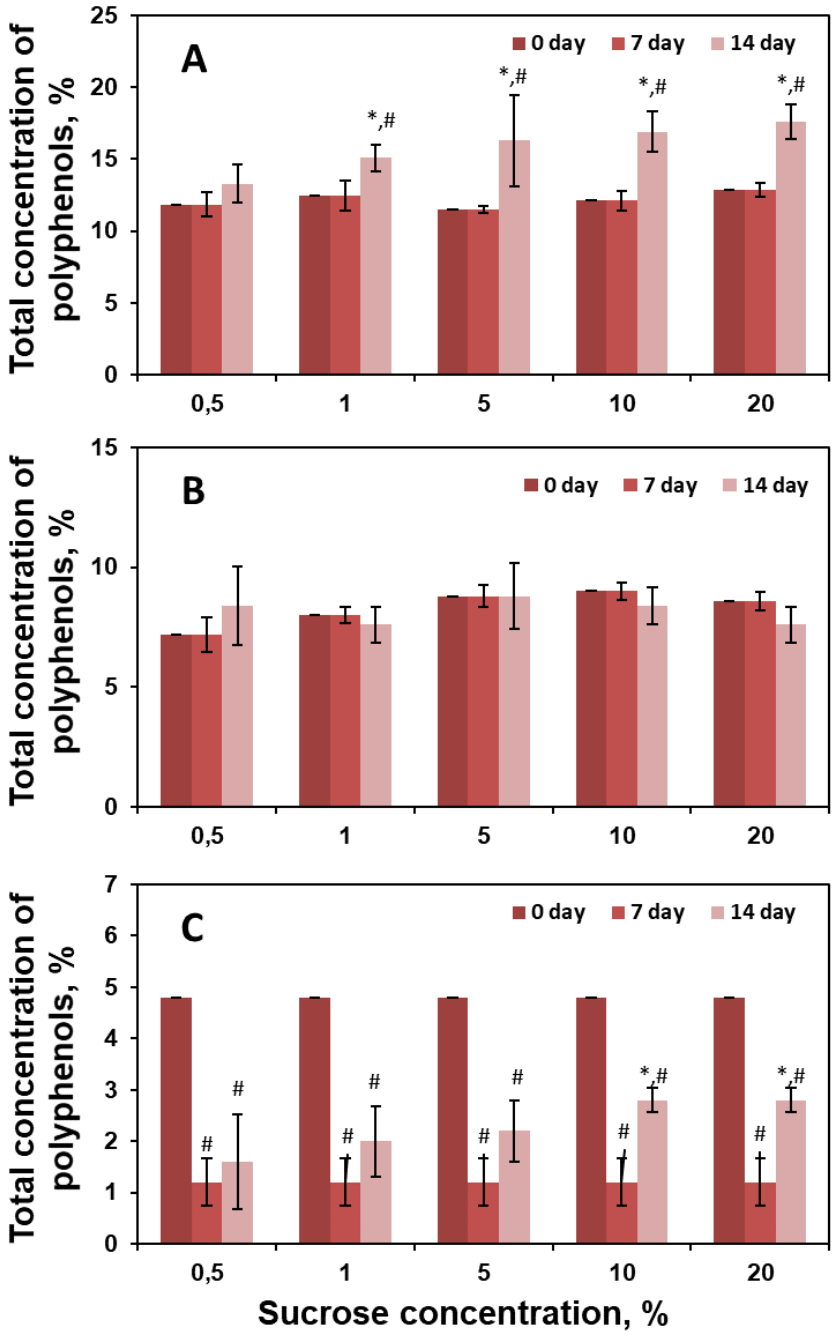

*Fig. 5. Total concentration of polyphenols in the medium during the cultivation of Kombucha on black tea Ahmat (A), decoctions of rose hips (B) and roots of yellow gentian (C). Data are presented as means ± S.E.M. (n = 4). *Significantly different from the corresponding value at the same concentration of sucrose on day 0(#) and 7(*) with P<0.05.*

At the same time, during the cultivation of SCOBY on yellow gentian decoctions (Fig. 5C), we observed a sharp (about 4-fold) decrease in polyphenols at all sucrose concentrations on day 7 of growth. On the 14th day of growth, there was an increase in the content of polyphenols compared to the 7th day on media with Akhmat tea and decoctions of roots of yellow gentian (Fig. 5A, 5C).

The flavonoid content in Ahmad tea on day 14 of growth was lower compared to day 7 on medium containing 5, 10 to 20% sucrose (Fig. 6A).

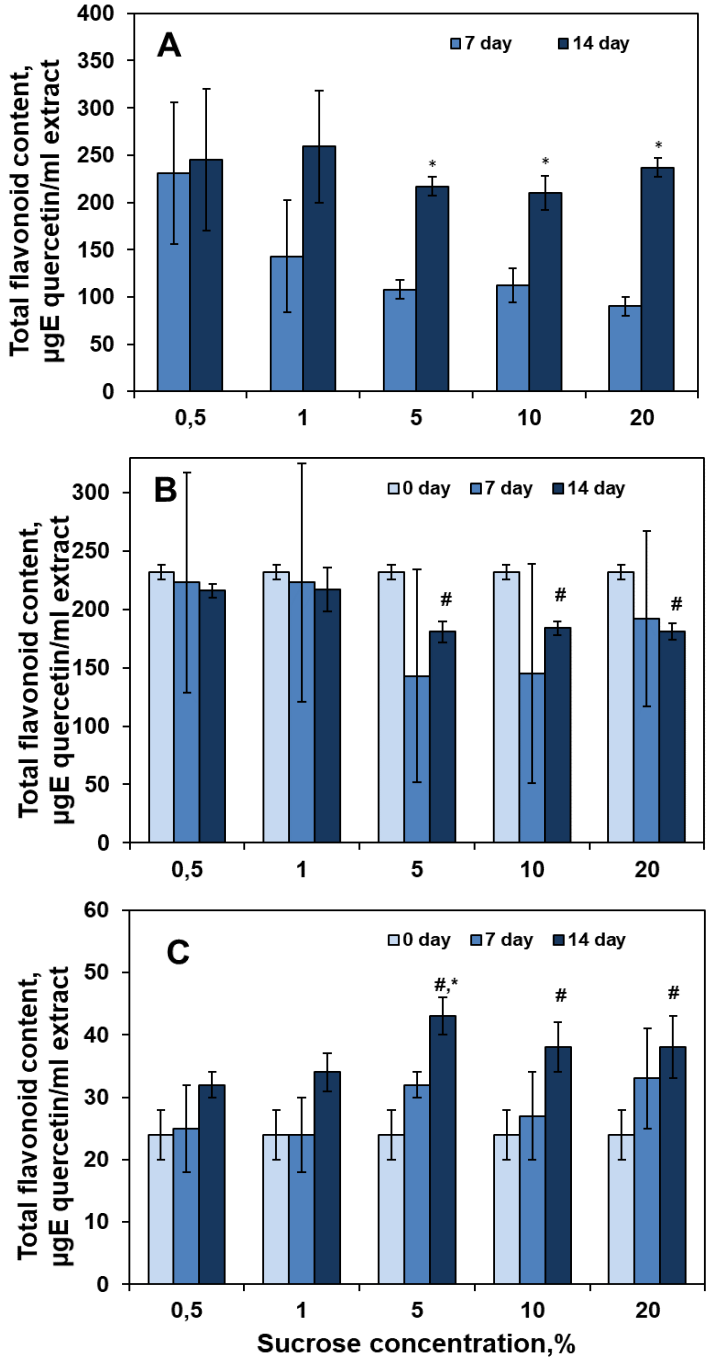

*Fig. 6. Total flavonoid content in the medium during the cultivation of Kombucha on black tea Ahmat (A), decoctions of rose hips (B) and roots of yellow gentian (C). Data are presented as means ± S.E.M. (n = 4). *Significantly different from the corresponding value at the same concentration of sucrose on day 0(#) and 7(*) with P<0.05.*

The concentration of sucrose in the medium prepared for Ahmad tea, rose hip decoctions, and yellow gentian roots was similar at day 7 and 14 of kombucha (Fig. 7).

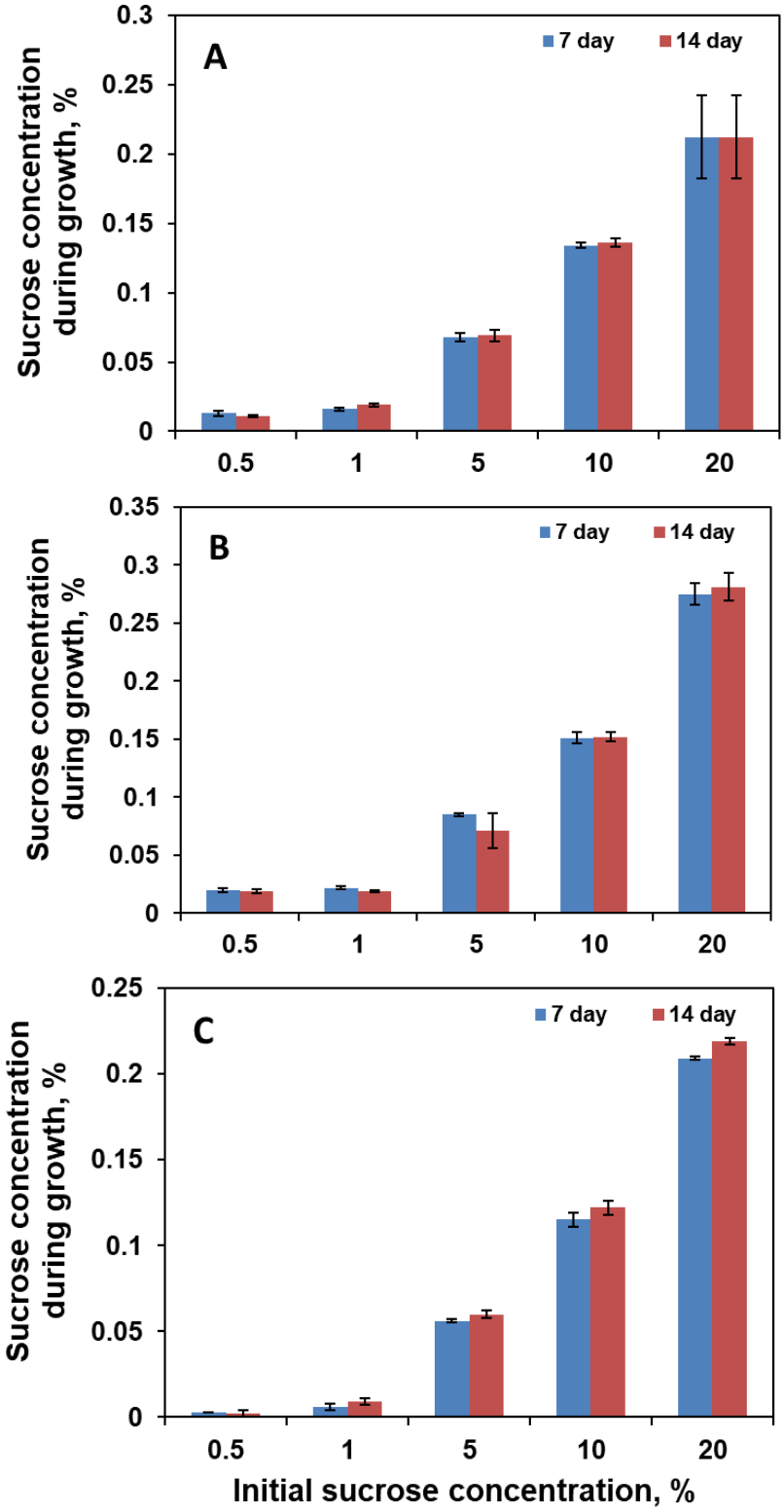

Fig. 7. Sucrose concentration in the medium during the cultivation of Kombucha on black tea Ahmat (A), decoctions of rose hips (B) and roots of yellow gentian (C). Data are presented as means ± S.E.M. (n = 4).

An important feature of plant polyphenols is their ability to exhibit antioxidant properties. Since the infusions studied varied in their polyphenol and flavonoid content, we were interested in

comparing the total antioxidant activity of kombucha infusions prepared on black tea and decoctions of rosehip fruit and gentian root. Total antioxidant capacity (TAC) reflects the ability of all antioxidant molecules in the infusion to neutralize free radicals. TAC was determined by the ability to neutralize the ABTS-$^{+\bullet}$radical cation. The obtained results are shown in Fig. 8.

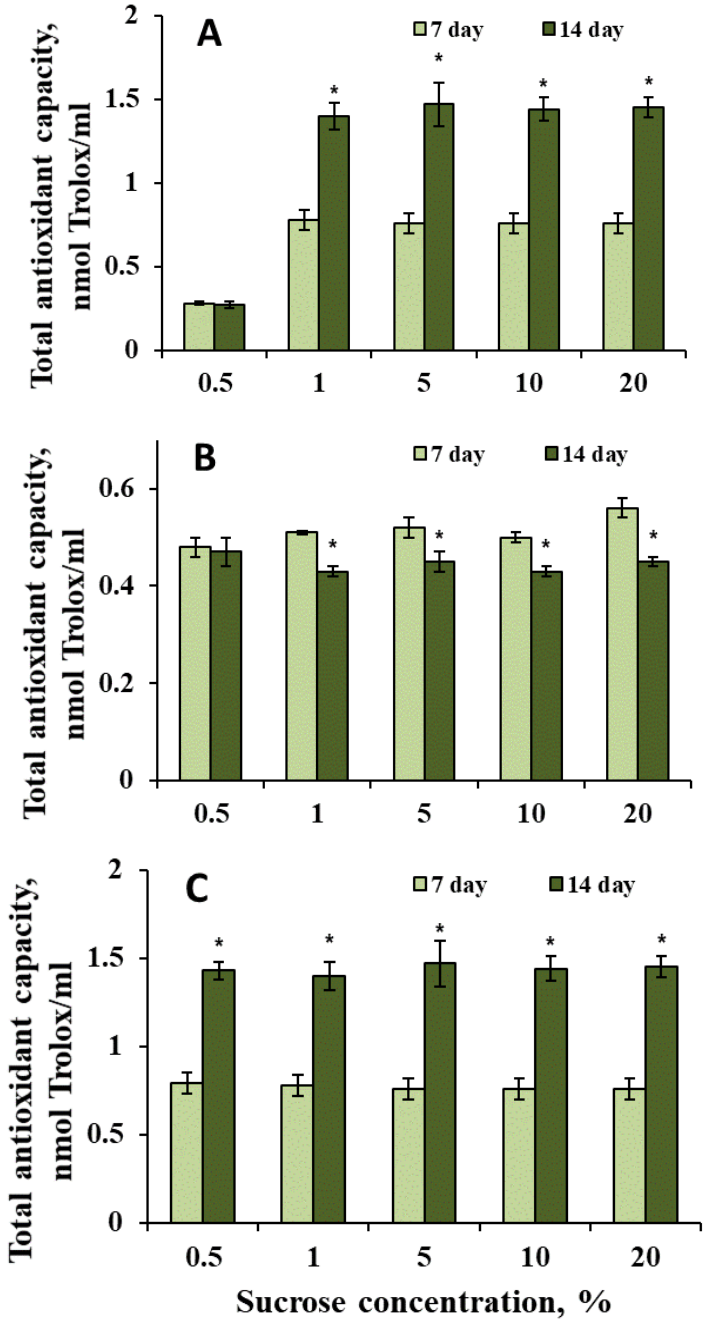

*Fig. 8. Total antioxidant capacity (TAC) in the medium during the cultivation of Kombucha on black tea Ahmat (A), decoctions of rose hips (B) and roots of yellow gentian (C). Data are presented as means ± S.E.M. (n = 4). *Significantly different from the corresponding value at the same concentration of sucrose on the 7th day with P<0.05.*

As we can see, the TAC of infusions on black tea was similar on the 7th and 14th day of cultivation, but it was significantly lower than the TAC of infusions on rose hip decoctions. The infusions on yellow gentian root showed the highest antioxidant activity, which is obviously due to the presence of substances other than polyphenols in the infusion, since infusions based on yellow gentian root were characterized by the lowest content of these substances.

4. CONCLUSIONS

The results obtained indicate that Kombucha grows well on both, its usual substrate -black tea and decoctions of medicinal herbs, rose hips and roots of yellow gentian. According to the rate of growth of SCOBY (symbiotic culture of bacteria and yeast) on the studied media, the samples studied can be placed in the following order: rose hips > yellow gentian roots > Ahmad black tea. The optimal concentration of sucrose in the medium for the development of Kombucha is 5%. During cultivation, sucrose is gradually fermented by the Kombucha yeast to ethanol, which in turn is used by acetic acid bacteria to synthesize acetic acid. As a result, an acidification of the pH of the medium is observed. The content of polyphenolic substances in the infusions increased slightly during cultivation, indicating that Kombucha has a limited ability to synthesize these compounds. The total antioxidant activity of the infusions decreased during the cultivation on the tea made of rose hips, but it increased on the decoctions of the roots of yellow gentian on the 14th day of the growth.

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Байляк Марія, Абрат Олександра, Ворошило Катерина. Вміст біологічно активних речовин та сумарна антиоксидантна активність напоїв з чорного чаю та відварів лікарських рослин, ферментованих чайним грибом. *Журнал Прикарпатського університету імені Василя Стефаника*, 10 (2023), 20-33.

Комбуча - це корисний ферментований напій, який традиційно виготовляється шляхом ферментації підсолодженого чаю симбіотичною культурою різних дріжджів та оцтовокислих бактерій, яку також називають SCOBY - симбіотичною культурою бактерій та дріжджів. Під час зброджування глюкози дріжджі виробляють етанол, який використовується оцтовокислими бактеріями для виробництва оцтової та інших кислот. Отриманий етанол і кислоти, а також побічні продукти бродіння забезпечують оригінальний смак і аромат напою. У цьому дослідженні ми порівняли ріст і хімічний склад напоїв при вирощуванні чайного гриба на типовому субстраті, чорному чаї, і на відварах двох лікарських рослин - плодах шипшини і кореневищах тирличу жовтого. Для отримання ферментованих напоїв "чайний гриб" вирощували на 1,3% (маса/об'єм) чорному чаї Ahmad та рослинних відварах з плодів шипшини і коріння тирличу з додаванням різних концентрацій сахарози (0,5%, 1%, 5%, 10% і 20%) протягом 14 днів. На 0-й, 7-й та 14-й день культивування ми визначали масу тіла чайного гриба та характеристики ферментованих напоїв (pH, загальний рівень кислотності, вміст етанолу, концентрацію сахарози, загальний вміст поліфенолів та флавоноїдів, загальну антиоксидантну здатність). Результати показують, що чайний гриб добре росте як на звичайному субстраті - напої з чорного чаю, так і на відварах лікарських трав, плодах шипшини та коренях жовтої тирличу. За швидкістю росту SCOBY на досліджуваних середовищах зразки можна розташувати в такому порядку: плоди шипшини > коріння тирличу жовтого > чорний чай Ахмад. Оптимальна концентрація сахарози в середовищі для розвитку чайного гриба становила 5%. Під час культивування сахароза поступово зброджувалася дріжджами чайного гриба до етанолу, який, у свою чергу, використовувався оцтовокислими бактеріями для синтезу оцтової кислоти. В результаті спостерігалося підкислення pH живильного середовища. Вміст поліфенольних речовин у настоях дещо збільшувався під час культивування, що свідчить про обмежену здатність чайного гриба до синтезу цих сполук. Загальна антиоксидантна активність настоїв знижувалася під час культивування на чаї з плодів шипшини, але зростала на відварах коренів тирличу жовтого на 14-й день росту. Таким чином, не тільки фенольні речовини визначають антиоксидантні властивості ферментованих напоїв з чайного гриба.

Ключові слова: Чайний гриб, ферментація, дріжджі, оцтовокислі бактерії, поліфеноли, антиоксидантна активність, напої.