I. MAN AND BIOSPHERE

PRODUCTION OF LACTIC ACID WORT-BASED BEVERAGES WITH MINT ESSENTIAL OIL ADDITION

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Abstract. Lactic acid wort-based beverages are functional, non-alcoholic, with low pH value and produced by the fermentation of wort by lactic acid bacteria. They are not well accepted by consumers because of their poor sensory characteristics. Therefore, 0.025 and 0.05 % (v/v) mint (*Mentha piperita*) essential oil was used as a tool for improvement of lactic acid wort-based beverages organoleptic profile. Wort was produced by 60% Pilsen malt, 20% Vienna malt, and 20% Caramel Munich II malt. It was inoculated with probiotic lactic acid bacteria *Lactobacillus casei* ssp. *rhannosus* LBRC11 at a concentration of 10^7 cells/ml and fermentation was carried out at constant temperature of 25°C. The dynamics of pH, concentration of viable cells, phenolic compounds and antioxidant activity were monitored and the beverages obtained were evaluated by a tasting panel. The results showed that addition of mint essential oil in concentration of 0.025 and 0.05 % (v/v) inhibited lactic acid fermentation but improved the sensory profile of the beverage obtained only when 0.025% mint essential oil was added. Mint essential oil addition led to an increase in the total phenolic compounds concentration, phenolic acids and flavonoid phenolic compounds, measured by Folin–Ciocalteu and modified Glories method but resulted in a decrease in the antioxidant activity, measured by the DPPH radical scavenging assay, cupric reducing antioxidant power (CUPRAC) and ferric reducing antioxidant power (FRAP). The antioxidant activity measured by the ABTS radical scavenging assay was almost equal for the beverages with and without mint essential oil addition. The results obtained will be used for modeling of lactic acids fermentation with addition of mint essential oil for the production of functional wort-based beverages.

Keywords: lactic acid fermentation, wort, mint essential oil, phenolic compounds, antioxidant activity

INTRODUCTION

The increasing society demand for healthier and natural beverages is oriented towards functional beverages [1]. European Commission stated that “a food product can only be considered functional if together with the basic nutritional impact it has beneficial effects on one or more functions of the human organism thus either improving the general and physical conditions or/and decreasing the risk of the evolution of diseases” [2]. Beverages can be described as functional if they do not contain more than 1.2% v/v ethanol [3]. Lactic acid fermented wort-based beverages are non-alcoholic, with low pH value (3.5–4.5) and produced by the fermentation of wort by lactic acid bacterial (LAB) strains [4]. They can be classified as functional because of wort and LAB. Wort consists of many substances that are beneficial for human health such as: fibres (β -glucan and arabinoxylan), antioxidants (phenolic compounds and melanoidins), and vitamins (folate, riboflavin, pantothenic acid, pyridoxine and niacin) [5,6]. The beverages can be defined as probiotic if LAB are maintained alive in it until the time of consumption and present in significant numbers, at levels of at least 10^7 viable cells *per* milliliter [2]. Despite the acknowledged health benefits of lactic acid fermented wort beverages, they are poorly accepted by consumers

because of their sour or warty-like taste and aroma. Therefore, the main challenge for producers is to improve the sensorial characteristics of lactic acid fermented wort-based beverages [4].

Mint (*Mentha piperita*) is used for flavoring different food and beverages. In addition, *Mentha* spp. are also used in folk remedy for treatment of nausea, bronchitis, flatulence, anorexia, ulcerative colitis, and liver complaints due to its antiinflammatory, carminative, antiemetic, diaphoretic, antispasmodic, analgesic, stimulant, emmenagogue, and anticatharrhal activities [7,8]. Therefore, mint essential oil is suitable for use in functional beverages production.

The aim of this study was to investigate the influence of mint essential oil addition on the production of functional lactic acid wort-based beverages. The dynamics of the concentration of viable LAB, phenolic compounds and antioxidant activity was determined in order to estimate the biological value of the beverages produced. The sensorial characteristics of the beverages were also described.

MATERIALS AND METHODS

Microorganisms

Wort fermentation was carried out using the probiotic LAB strain *Lactobacillus casei* ssp. *rhannosus* LBRC11, which was isolated from home-made cheese.

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Essential oil

Mint essential oil was purchased by Bulgarian rose AD - Karlovo.

Media

1. MRS – broth medium Composition (g/dm³): peptone from casein - 10; yeast extract - 4; meat extract - 8; glucose - 20; K₂HPO₄ - 2; sodium acetate - 5; diammonium citrate - 2; MgSO₄ - 0.2; MnSO₄ - 0.04; Tween 80 - 1 cm³/dm³; pH = 6.5. Sterilization - 15 minutes at 118°C.

2. LAPTg10 – agar medium Composition (g/dm³): peptone - 15; yeast extract - 10; tryptone - 10; glucose - 10; Tween 80 - 1 cm³/dm³; agar - 15. pH=6.6 - 6.8. Sterilization - 20 minutes at 121°C.

Reagents

Folin-Ciocalteu reagent, gallic acid, caffeic acid, quercetin, DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-tripyridyl-s-triazine), 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, neocuproine, FeCl₃·6H₂O, and Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were purchased by Sigma-Aldrich. Hydrochloric acid was purchased by Merck, Germany. All the other reagents were of analytical grade.

Wort production

Wort was produced with 60% Pilsen malt, 20% Vienna malt and 20% Caramel Munich II malt, which was purchased from Bestmaltz (Germany). The malt was milled using a hand disc mill (Corona, Germany). The wort was produced in a 20 L laboratory scale brewery (Braumeister, Germany). 4.5 kg of malt was mixed with water at a ratio of 1:5. Mashing was conducted by increasing the temperature by 1°C/min and by maintaining rests at the following temperatures: 30 min at 50°C and 60 min at 77°C. Lautering and boiling were also conducted in the same Braumeister. Boiling duration was approximately 30 minutes without hop addition. After hot trub removal, the wort was autoclaved at 121 °C for 30 minutes for preserving it until experiments. It was filtered aseptically and used for lactic acid fermentation. The wort extract was 11 % (w/w) and the pH was 5.30.

Fermentation

Wort was divided into 3 equal parts: one was mixed with 0.025 % (v/v) mint essential oil, the other was mixed with 0.05 % (v/v) mint essential oil and the last one was used as a reference sample. The fermentations were carried out in plastic bottles with a volume of 330 cm³. LAB suspensions for inoculations were obtained by pre-inoculation in MRS broth and incubation at 37±1°C for 24 hours. 250 cm³ of the wort variants were inoculated with 2% of the obtained

LAB suspensions to obtain initial concentration of 10⁷ cells/cm³ in the beverage variants immediately after inoculation. Bottles were incubated at a constant temperature of 25±1°C.

Analytical methods and procedures

Fermentation parameters. The extract and pH (methods 8.2.2, 8.3, 8.17) were measured according to EBC standard methods [9].

Determination of the number of viable LAB cells. Pour plate method on LAPTg10–agar medium medium. Appropriate tenfold dilutions of the samples were prepared using sterile saline solution. 1 cm³ of the last three dilutions was used for pour plating in Petri dishes, then melted and cooled LAPTg10–agar medium was poured, the Petri dishes were shaken well to mix the corresponding dilution with the medium and were left at room temperature to solidify. The inoculated and solidified Petri dishes were incubated for 48-72 hours at 37 ±1°C until the appearance of countable single LAB colonies. The number of single colonies was used to estimate the concentration of viable LAB cells in the sample.

Determination of Phenolic Compound Content

The wort and lactic acid wort-based beverages were diluted at a ratio of 1:5 with methanol, rested for 30 minutes and filtered using Whatman No. 1 filter paper. The filtrate was used to analyse the phenolic compounds and the antioxidant activity of the wort. If necessary, further dilution of the methanol extracts with deionized water was performed before the analysis.

A. Content of Total Phenolic Compounds with FC-reagent

The content of total phenolic compounds was determined by the Folin-Ciocalteu (FC) method with the following modifications [10]. 1 cm³ of beverage, 4 cm³ of FC working solution, 5 cm³ of sodium carbonate (7.5%, w/v) were mixed in a test tube. The mixture thus obtained was stirred and allowed to stand for 1 h, after which the absorbance (A) of the sample at 765 nm was determined on a Shimadzu UV-VIS1800 spectrophotometer (Kyoto, Japan) against a blank prepared with distilled water. The results were presented as gallic acid equivalent in mg GAE/dm³ wort by using a calibration curve.

B. Content of Phenolic Compounds by the Glories Method

The content of total phenolic compounds, phenolic acids and flavonoid phenolic compounds was determined by a modified method of Glories [11]. 1 cm³ of 0.1% HCl in 95% ethanol (v/v), 18.2 cm³ of 2% HCl (v/v) and 1 cm³ of beverage were

mixed in a tube. The solution was stirred thoroughly and allowed to stand for 15 min, after which the absorbance was measured against a blank prepared with distilled water. A 280 nm was used to evaluate the total phenolic content, A320 nm was used to evaluate the content of phenolic acids and A360 nm was used to evaluate the content of flavonoid phenolic compounds. The calibration curves for the total phenolic compounds, phenolic acids and flavonoid phenolic compounds were constructed using gallic acid, caffeic acid and quercetin, respectively.

Determination of Antioxidant Potential - The antioxidant activity was determined by a standard curve using Trolox as standard, and the results were expressed as μM Trolox equivalents per 1 dm^3 .

A. Antioxidant Activity Against the DPPH Radical

The antioxidant activity of malt and wort was measured by the DPPH method [12] with some modifications. 0.25 cm^3 of the methanol extract was added to 2.25 cm^3 of DPPH solution in methanol ($6 \times 10^{-5}\text{ M}$); the mixture was left for 15 min (kept in the dark at room temperature) to allow the reaction to proceed and then the A 517 nm against a blank prepared with purified water was determined. The control sample was prepared with methanol.

B. Antioxidant Activity by the FRAP (ferric reducing ability of plasma) method

The FRAP analysis was performed according to [13] with the following modifications. First, 300 mM acetate buffer (3.1 g of $\text{C}_2\text{H}_3\text{NaO}_2 \cdot 3\text{H}_2\text{O}$ and 16 cm^3 of $\text{C}_2\text{H}_4\text{O}_2$) with a pH of 3.6, 10 mM TPTZ solution prepared in 40 mM HCl and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution were made. The working solution was prepared by mixing the acetate buffer, TPTZ solution and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution in a ratio of 10:1:1. 0.15 cm^3 of the methanol extract was allowed to react with 2.85 cm^3 of FRAP solution for 4 min in the dark. A593 nm against a blank prepared with methanol was measured.

C. Antioxidant Activity by the ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)) method

The ABTS analysis was performed according to [14] with the following modifications. $7 \times 10^{-3}\text{ M}$ ABTS solution and $2.45 \times 10^{-3}\text{ M}$ potassium persulfate solution were prepared. The two solutions were mixed in a ratio of 1:1 and the mixture was left in the dark for 12–16 h. The resulting solution was

stable for 2 days. It was then diluted with methanol in a ratio of 1:30 until an absorbance of 1.1 ± 0.1 was reached, which was measured against methanol at $\lambda = 734\text{ nm}$. 0.15 cm^3 of beverage and 2.85 cm^3 ABTS were measured against methanol at aforementioned wavelength. The control sample was prepped with methanol and ABTS as described before.

D. Antioxidant Activity by the CUPRAC (cupric reducing antioxidant capacity) method

The CUPRAC analysis was performed according to [15]. The following solutions were prepared— 0.01 M solution of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, ammonium acetate buffer with pH = 7 and $7.5 \times 10^{-3}\text{ M}$ solution of neocuproine. The following samples were prepared: a working sample— 1 cm^3 of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ + 1 cm^3 of acetate buffer + 1 cm^3 of neocuproine + 0.5 cm^3 of extract + 0.6 cm^3 of distilled water; a blank sample – 1 cm^3 of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ + 1 cm^3 of acetate buffer + 1 cm^3 of neocuproine + 1.1 cm^3 of distilled water. The samples were homogenized and allowed to stand for 30 min at room temperature. Measurement of absorbance was made at $\lambda = 450\text{ nm}$ against the blank sample.

Sensory analysis

A sensory evaluation of the beverages was carried out by a trained, 6-member tasting panel. The samples were evaluated by their taste and aroma using descriptive analysis and ranking method (methods 13.10 and 13.11) [9].

Statistical analysis

The results of all the analyses were expressed as the mean values $\pm$ standard deviation of three replicates using Microsoft Excel 2013.

RESULTS AND DISCUSSION

In our preliminary studies, the antimicrobial activity of mint essential oil against *Lactobacillus casei* ssp. *rhannosus* LBRC11 was investigated using the disc-diffusion method. It was found that it did not show antimicrobial activity against the tested microorganism in concentrations of up to 1% (v/v) [unpublished data]. Because of the oil strong taste and aroma, it was decided that it should be used in concentrations of 0.05% (v/v). The selected LAB strain has demonstrated a number of probiotic properties [16], which made it suitable for functional beverages production.

Fig. 1. Changes in the viable cell concentration during fermentation.

Fig. 2 Changes in the pH during fermentation.

Fermentation dynamics

During the fermentation the extract dynamics was negligible. At the end of the fermentation the extract decreased by 0.1% w/w for all the variants. It can be explained by the fact that wort contained approximately 30-35% fermentable sugars, the predominant of which was maltose.

The changes in the viable cell concentration and pH are shown in Figure 1 and Figure 2, respectively. The data in Fig. 1 show a steady increase from 10^7 cells/cm³ to 10^{11} cells/cm³ in the number of viable LAB cells during reference fermentation. The addition of mint essential oil led to a slight increase in the biomass concentration during the first 12 hours, as the oil inhibited LAB growth to some extent. Then the cells entered the exponential growth phase and by the 24th hour their number increased by 2log N - from 10^7 cells/cm³ to 10^9 cells/cm³. At the end of the fermentation the amount of LAB viable cells decreased to 10^8 cells/cm³. It can be explained by the joint influence of mint essential oil inhibition and the entry of LAB in a stationary growth phase. Nevertheless, all beverages can be classified as probiotic [2].

In all beverage variants, the pH decreased gradually from the beginning of the fermentation process (Fig. 2). At the end of the fermentation the pH of the beverage with 0.05% mint oil addition was 0.2 units lower, the pH of the beverage with 0.025% mint oil addition was 0.5 units lower and the pH of the reference sample was 1.4 units lower than the initial pH values of the corresponding beverage variants. The results confirmed the hypothesis for the inhibiting action of mint essential oil even at these low concentrations.

Biological value

It is known that LAB and essential oils add a certain biological value to beverages. All the beverages produced were firstly evaluated in terms of total phenols and then their antioxidant activities were

compared. The concentration of total phenolic compounds, measured by the FC method (Fig. 3a), decreased almost 3 times between the 6th and 12th hour and remained constant till the end of fermentation for all the beverages produced. The results for total phenolic compounds, measured by the modified Glories method (Fig. 3b) were very different. In general, their quantity was kept relatively constant during the whole fermentation process. At the end of the fermentation the lowest content of phenolic compounds was determined in the reference sample, while the beverages with the addition of essential oil showed similar results. The observed differences are due to the fact that the modified Glories method is based on the characteristic absorption of the benzene cycles of the majority of phenols at 280 nm and is less influenced by the oxidative status of the analyzed molecules [17]. Peaks in the phenolic acids (Fig. 3c) and flavonoids (Fig. 3d) were observed at the 36th hour in all the studied variants. At the end of the fermentation the content of phenolic acids (Fig. 3c) was the lowest in the reference sample and almost equal in beverages with mint essential oil addition. During the fermentation process, the tendency of flavonoid phenolic compounds was the same as for phenolic acids with a peak at the 36th hour. Flavonoid phenolic compounds (Fig. 3d) were between 77 mg/dm³ and 84 mg/dm³ in the finished beverages.

It can be assumed that the higher phenol content of beverages with mint essential oil addition was due to the mint essential oil [18]. The antioxidant potential was assessed by 4 different methods and the results are shown in Figure 4. At the end of the fermentation the antioxidant activity against the DPPH radical (Fig. 4a) was the highest for the reference sample, while in the drinks with the addition of mint essential oil the results were almost equal. Nevertheless, the antioxidant activity increased between 200 and 500 μ mol Trolox/dm³ compared to the beginning of the fermentation.

Fig. 3a. Changes in the total phenolic compound content, measured using the FC method.

Fig. 3b. Changes in the total phenolic compound content, measured using the modified Glories method.

Fig. 3c. Changes in the phenolic acid content, measured using the modified Glories method.

Fig. 3d. Changes in the flavonoid content, measured using the modified Glories method.

Fig. 4a. Changes in the antioxidant activity, measured by DPPH method.

Fig. 4b. Changes in the antioxidant activity, measured by FRAP method.

Fig. 4c. Changes in the antioxidant activity, measured by ABTS method.

Fig. 4d. Changes in the antioxidant activity, measured by CUPRAC method.

The results of the measured total antioxidant activity against Fe^{3+} (FRAP) are presented in Fig. 4b. In the course of the fermentation process, a gradual increase in the antioxidant activity of all tested samples was observed, as it was the highest between the 12th and 24th hour, after which it started gradually to decrease. At the end of the process, it was the highest in the reference sample.

It is noteworthy that the activity against the the ABTS radical (Fig. 4c) changed to a very small extent during fermentation. The antioxidant activity against the ABTS radical at the end of the fermentation was the lowest in the reference sample.

In the course of the fermentation, a drastic decrease in the activity measured by the CUPRAC method (Fig. 4d) was observed, and 6 hours after the beginning of the process it decreased almost 4 times in all studied variants. Until the end of the process, there was a continuous increase in the antioxidant activity only in the control, while in the variants with mint essential oil fluctuations in the values were observed.

The differences between the results obtained using the four methods for evaluation of the antioxidant activity were based on the distinct reaction mechanisms and take into account different proportions of the multiple substances with antioxidant properties in the beverages produced.

Sensorial evaluations

Sensorial evaluation of all beverages obtained were made at the end of the fermentation process. The results are presented in Fig. 5. The reference sample was very viscous compared to the beverages with mint essential oil addition. It can be assumed that the high viscosity was due to the polysaccharides synthesized by LAB [19]. Enhancement of the antioxidant propensity during fermentation is mainly ascribed to the release of bioactive compounds such as polysaccharides by LAB [20]. The reference sample was lighter because of more viable cells in it compared to the beverages with mint essential oil. Nevertheless, the panel preferred the beverage with 0.025% mint essential oil addition because of its pleasant taste and aroma. The beverage with 0.05% mint essential oil had too strong mint taste and aroma.

Fig. 5 Sensorial evaluation of the beverages produced

CONCLUSION

Lactic acid wort-based beverages with 0.025% (v/v) or 0.05% (v/v) mint essential oil addition were produced. Fermentation without mint essential oil addition was used as a reference. The results showed that the addition of mint essential oil in concentration of 0.025 and 0.05 % (v/v) inhibited lactic acid fermentation but improved the sensory profile of the beverage obtained only when 0.025% mint essential oil was added. The reference was characterized by the lowest content of total phenolic compounds, phenolic acids and flavonoid phenolic compounds at the end of the study process, but it showed the highest antioxidant activity against the DPPH radical, against Cu^{2+} and Fe^{3+} . The addition of

mint essential oil was the main reason for the higher phenolic compounds content in beverages with mint essential oil, but exopolysaccharides were the main reason for the higher antioxidants activity of reference. The results obtained will be used for modelling of lactic acids fermentation with addition of mint essential oil for the production of functional wort-based beverages.

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