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Characterization and quantification of folates produced by yeast strains isolated from kefir granules

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Abstract For the first time to our knowledge, distribution and content of individual folate forms in kefir yeast strains were investigated. This was done using a validated method based on reversed-phase high performance liquid chromatography (HPLC) with fluorescence and diode array (DAD) detection. Eight kefir yeast strains, belonging to different *Candida* and *Saccharomyces* species, were isolated from Russian kefir granules. They were grown in synthetic media at standardized conditions before analysis. The average folate content for these yeast strains was $10,780 \pm 550 \mu\text{g}/100 \text{ g}$ dry matter. In all yeast strains tested, the most abundant folate forms as percentages were 5-methyltetrahydrofolate (43–59%), and 5-formyltetrahydrofolate (23–38%), whereas tetrahydrofolate occurred in a lesser proportion (19–23%).

Keywords Folate · Kefir granule · Yeast strains · HPLC

Introduction

Kefir is regarded as a health promoting product that acts as a natural probiotic, i.e., a food with live bacteria which are beneficial to health [1]. It is produced by the fermentation of milk with kefir granules (grains). Such granules contain a mixture of lactic acid bacteria, acetic acid bacteria (producing lactic acid and acetic acid), and yeast (produc-

ing ethanol and carbon dioxide), which exist in a complex symbiotic relationship [2, 3]. Recent investigations of the microbiological composition of different types of kefir granules have shown that lactic acid bacteria are present as the largest portion (65–80%), whereas yeasts and other microbial species are present in smaller proportion [4]. In addition to these beneficial microorganisms, kefir contains minerals, e.g., calcium, essential amino acids, and several vitamins, e.g., folate [5]. Folate is relative to the nutritional needs of humans, frequently among the most limiting of all vitamins [6]. It is required for the synthesis of DNA and RNA, which are primary events for cellular replication and growth [7, 8]. Folate occurs in different forms in nature which vary significantly in stability and bioavailability [6]. A sufficient intake of folate gives an increased protection against megaloblastic anemia and child birth defects such as neural tube defects [9–11]. However, a substantial part of the population in European countries does not get enough folate from foods [12]. There is therefore an interest both from a general health perspective, as well as an opportunity for industry, to develop new healthy products to increase the folate content in foods.

Important sources of folate in the Swedish diet are dairy products, and according to a dietary survey performed in 1997–98 these provide around 15% of the daily folate intake [13]. Fermented dairy products have considerably higher folate levels compared to pasteurized milk products due to the ability of starter cultures to synthesize folate [14].

It is known that baker's yeast (*Saccharomyces cerevisiae*) is a rich dietary source of native folate, containing around 3 mg folate/100 g dry matter [15, 16]. It has earlier been shown that the high folate content in kefir is primarily due to the yeast and not the lactic acid bacteria [14]. When the yeast content relative to lactic acid bacteria was increased in kefir grains (from ratio 1:15 to 1:5.6) the total folate content in fresh kefir was increased from 4.3 to 6.4 μg folate/100 g. Kefir yeasts might thus potentially contribute to the total folate in kefir. However, the differences in folate-producing capabilities among yeast strains from kefir grains are poorly investigated. Furthermore, the data

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on the distribution of the different folate forms in yeast strains from kefir grains are lacking.

The objective of this work was therefore to evaluate the potential of yeasts of dairy origin as folate sources and to provide new data regarding distribution of individual folate forms in these yeast strains. Eight yeast strains isolated from Russian kefir granules were cultivated in a synthetic medium at standardized conditions and analyzed for individual folate forms by using a validated method based on reversed-phase high performance liquid chromatography (HPLC) with fluorescence and diode array (DAD) detection.

Materials and methods

Media and reagents

Acetonitrile was of isocratic grade for HPLC; the other chemicals were of analytical quality. If not otherwise stated, the chemicals were purchased from VWR International (Darmstadt, Germany). Water was purified using a Milli-Q system (Millipore, USA).

Rat serum was obtained from Scanbur (Sollentuna, Sweden). It was dialysed at 4 °C in three steps by using 50 mM phosphate buffer pH 6.1 containing 0.1% 2-mercaptoethanol as described in details earlier [15]. The dialysed rat serum was stored in small portions (0.5 ml) at -20 °C for a maximum of 1 month. Folate conjugase activity was checked using pteroyltri- γ -L-glutamic acid as substrate in 0.1 M phosphate buffer pH 6.1 containing 1% sodium ascorbate at 37 °C as described earlier [15].

Yeast strains were maintained on YPD (yeast extract 10 g l⁻¹, peptone 20 g l⁻¹, dextrose 20 g l⁻¹, agar 20 g l⁻¹) agar slants at +4 °C and long-term stored at -80 °C in 15% (w/v) glycerol. Yeast extract and peptone were obtained from Becton Dickinson and Company, (Franklin Lakes NJ, USA). The synthetic medium used for experiments was a modified version of CBS (Centraalbureau voor Schimmeltculturen) with 2% glucose as described by Albers et al. [17]. All media components were purchased from Sigma-Aldrich (Stockholm, Sweden).

Folic acid, (6S)-5,6,7,8-tetrahydrofolate, sodium salt (H₄folate), (6S)-5-formyl-5,6,7,8-tetrahydrofolate, sodium salt (5-HCO-H₄folate) and (6S)-5-methyl-5,6,7,8-tetrahydrofolate, sodium salt (5-CH₃-H₄folate) were all a kind gift from Merck Eprova AG, Schaffhausen, Switzerland. Pteroyltri- γ -L-glutamic acid (PteGlu₃) and 10-formylfolic acid, sodium salt (10-HCO-folic acid) were obtained from Dr. Schirck's Laboratories (Jona, Switzerland). The folate standards were stored at -80 °C until use. The purity of all standards was checked according to the procedure of van den Berg et al. [18] using molar extinction coefficients reported by Eitenmiller and Landen [19]. The standard stock solutions of folates of 200 μ g ml⁻¹ (purity corrected) were prepared under subdued light in 0.1 M phosphate buffer pH 6.1 containing 1% sodium ascorbate and 0.1% MCE. Aliquots of the standard stock solutions were placed in separate tubes,

flushed with nitrogen, and stored below -80 °C at most 3 months. Because of folate susceptibility to oxidation [20], the calibration solutions were prepared immediately before use by dilution of the stock solution with extraction buffer (0.1 M phosphate buffer pH 6.1 with 2% sodium ascorbate (w/v) and 0.1% MCE (v/v)).

Yeast strains from kefir granules

The yeast strains were originally isolated from Russian kefir grains as described by Pettersson et al. [21]. They have been identified by API 20 AUX as belonging to different *Candida* and *Saccharomyces* species. The strains are available on the market in Kefir cultures produced by Medipharm AB, Kågeröd, Sweden.

Culturing conditions and sampling procedure

Yeast precultures were grown overnight at 30 °C in rotating tubes (Falcon®, Becton Dickinson and Company, Franklin Lakes NJ, USA) containing 5 ml of the synthetic medium. Precultures were inoculated to E-flasks, containing 100 ml of the same synthetic medium, in amounts to obtain a start optical density (OD₆₁₀) of 0.2. Strains were grown on an orbital shaker (30 °C, 220 RPM) until OD₆₁₀ reached 8–10 (approximately 12 h). At this stage the cells were harvested by centrifugation (3,000×g, 4 °C) for 15 min. Thereafter the cells were washed once with 5 ml cold 0.9% NaCl and again centrifuged (3,000×g, 4 °C) for 5 min. The cell pellet was put in the freezer (-80 °C). When deeply frozen the cells were freeze dried for 4 days. The lyophilized cells were packed in glass tubes, flushed with nitrogen for 5 min, and stored at -20 °C until folate extraction and analysis.

Sample pretreatment

The extraction, deconjugation, and determination of folates in kefir yeast was performed in accordance with an earlier validated HPLC method for folate analysis in baker's yeast (recovery 97–98% and intra-day and interday-precision 1.9–4.0 (RSD%) for the different folate forms) [15]. Extraction of folates from yeast samples was performed by dissolving 50 mg of yeast in 25 ml of extraction buffer (0.1 M phosphate buffer pH 6.1 with 2% sodium ascorbate (w/v) and 0.1% MCE (v/v)) and boiling for 12 min. Deconjugation of folate polyglutamates to monoglutamates was always done on the same day as the extraction. This was done by adding 50 μ l of rat serum to 1 ml of the yeast extract in a glass tube and incubating it on a shaking water bath at 37 °C for 3 h. The completion of deconjugation was controlled by spiking yeast samples with PteGlu₃ prior to deconjugation step and by checking chromatograms for presence of folate polyglutamates as described in details earlier [15]. The obtained yeast extracts containing folate monoglutamates were always directly analyzed by HPLC on the same day without any intermediate freezing step.

The exclusion of intermediate freezing step was important to prevent possible losses of folates because of degradation during freezing/thawing procedure as we have shown earlier [20].

Chromatographic equipment and conditions

Analyses were performed using an HPLC system (Agilent 1100) consisting of a gradient quaternary pump, a thermostated autosampler, a thermostated column compartment, a diode array detector (DAD), and a fluorescence detector. The HPLC system was controlled by a personal computer running Agilent Chemstation software. The separation of folates was performed on Aquasil C₁₈ column, 150×4.6 mm; 3 μm (Thermo Electron Corporation, USA) with a guard column Opti-guard C₁₈, 1 mm (Optimize Technologies, INC, USA) at 23 °C. The flow-rate was 0.4 ml min⁻¹; the injection volume 20 μl; the temperature in the thermostated autosampler 8 °C. For the detection and quantification of H₄folate, 5-CH₃-H₄folate and 5-CHO-H₄folate a fluorescence detector was used (excitation at 290 nm and emission at 360 nm), and for 10-CHO-folic acid and folic acid a DAD detector was used (the DAD channel was set at 290 nm). The mobile phase used was acetonitrile-30 mM phosphate buffer (pH 2.3) under linear gradient elution conditions. The gradient started at 6% (v/v) acetonitrile with a lag of 5 min, then the gradient was raised linearly to 25% acetonitrile during 20 min, and was kept constant for 2 min; thereafter it was decreased linearly to 6% acetonitrile during 1 min and was applied for 14 min in order to re-equilibrate the column. Retention times of folate standards were used for peak identification; comparison of ratio of sample peak heights and areas from fluorescence and diode array detectors to ratio of standard peak heights and areas as well as fluorescence and diode array spectra were used for verifying of peaks if necessary.

Quantification

Quantification was based on an external standard method in which the peak area was plotted against concentration. A multilevel calibration curve was used ($n=7$) and least-squares regression analysis was used to fit lines to the data. The amount of each folate form was calculated in its free acid form. The detector response was linear up to concentration 100 ng/ml for 5-CH₃-H₄folate and H₄folate and 600 ng/ml for 5-HCO-H₄folate, 10-HCO-folic acid, and folic acid. The calibration curves had a correlation coefficient higher than 0.9998 for all folate forms. The limits of quantification were 0.3, 0.5, 8.0, 15.0, and 4.0 ng ml⁻¹ for 5-CH₃-H₄folate, H₄folate, 5-HCO-H₄folate, 10-HCO-folic acid, and folic acid, respectively [15]. The repeatability of a measurement (RSD for three successive injections of the same work solution at concentration 100 ng/ml for 5-CH₃-H₄folate and H₄folate and 600 ng/ml for 5-HCO-H₄folate, 10-HCO-folic acid, and folic acid) was better than 0.01% for retention time and better than 6% for peak area for all folate forms.

The results were presented as means of duplicates. The difference between two separate values of a duplicate was within 0.3–13% for all samples.

Results and discussion

Identification of individual folate forms by HPLC

The folate forms found in all the analyzed yeast strains were H₄folate, 5-CH₃-H₄folate, and 5-HCO-H₄folate. Representative chromatograms, comparing the folate peaks in yeast extract with folate standards when using fluorescence detection, are shown in Fig. 1. A detailed chromatogram sequence, comparing the 5-HCO-H₄folate peak detected

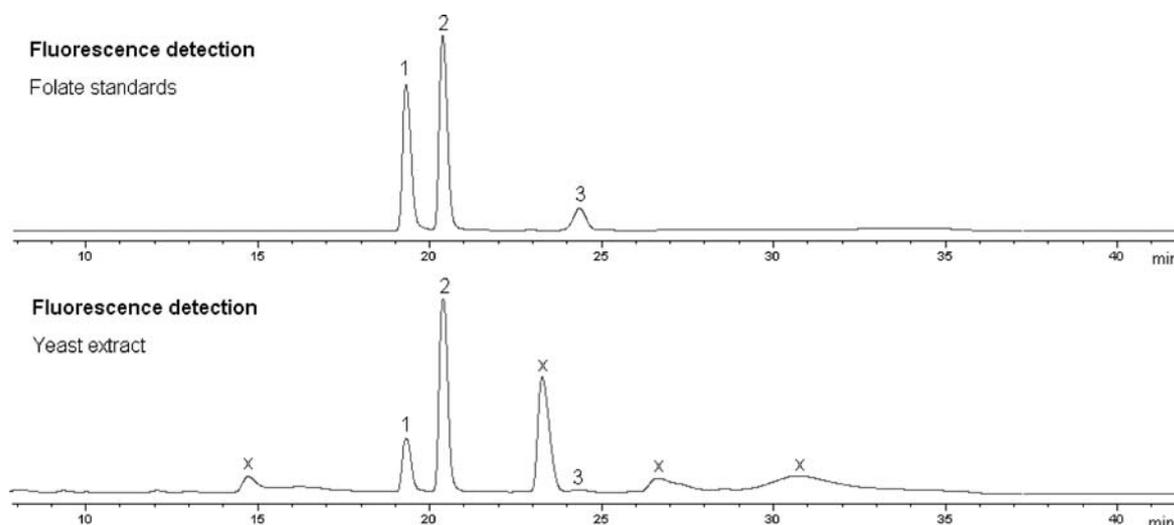


Fig. 1 Chromatograms of folates in a standard mixture (100 ng/ml for H₄folate and 5-CH₃-H₄folate and 600 ng/ml for 5-HCO-H₄folate) and yeast extract containing 42 ng/ml of H₄folate, 115 ng/ml of 5-CH₃-H₄folate, and 71 ng/ml of 5-HCO-H₄folate detected by

fluorescence ($\lambda_{\text{ex}}=290$ nm, $\lambda_{\text{em}}=360$ nm). Peaks: 1=H₄folate; 2=5-CH₃-H₄folate; 3=5-HCO-H₄folate; x=interfering compounds from the yeast matrix. For chromatographic parameters and conditions, see section Chromatographic equipment and conditions

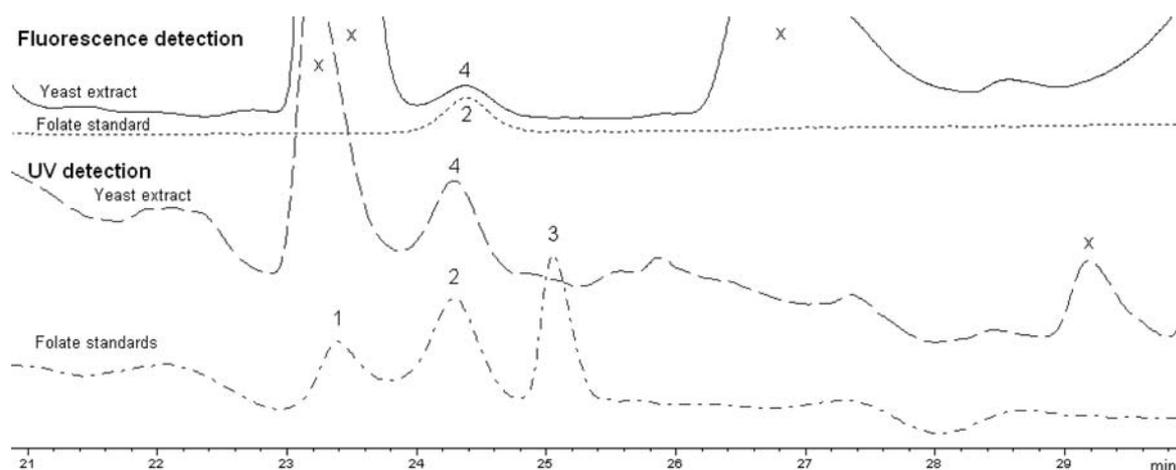


Fig. 2 Identification of 5-HCO-H₄folate peak by comparing chromatograms of standard mixture (80 ng/ml for all folate forms) and yeast extract containing 42 ng/ml of H₄folate, 115 ng/ml of 5-CH₃-H₄folate, and 71 ng/ml of 5-HCO-H₄folate detected by fluorescence ($\lambda_{\text{ex}}=290$ nm, $\lambda_{\text{em}}=360$ nm) and DAD (set at 290 nm). Peaks shown on chromatograms of the standard mixture: 1=10-HCO-

folic acid; 2=5-HCO-H₄folate; 3=folic acid. Peaks shown on chromatograms of the yeast extract: 4=5-HCO-H₄folate; x=interfering compounds from yeast matrix. Early-eluting folates (H₄folate and 5-CH₃-H₄folate) are not shown on these chromatograms. For chromatographic parameters and conditions, see section Chromatographic equipment and conditions

in yeast with the corresponding standard when using both DAD and fluorescence detection is shown in Fig. 2. To confirm the found folate peaks in the yeast extract, the retention times and peak shapes were compared to those of folate standards and were found to be similar. Furthermore, for 5-HCO-H₄folate the ratio of the folate standard peak height from fluorescence and diode array chromatograms was compared to the corresponding folate peak height ratio of yeast extract and was found to be very similar. The identification of 10-HCO-folic acid was, however, hindered by a disturbing peak eluted close to the peak of 10-HCO-folic acid (Fig. 2). Because of this, the detection and determination of 10-HCO-folic acid in yeast samples could not be performed. The use of more selective mass spectrometric detection might be necessary for this folate form. However, this folate form naturally occurs in much lower amounts than main folate forms (H₄folate, 5-CH₃-H₄folate, and 5-HCO-H₄folate) and is of secondary importance in folate analysis [6].

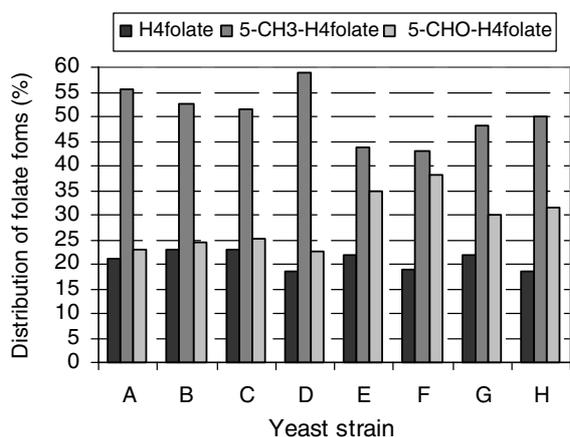


Fig. 3 Distribution of the various folate forms found in kefir yeast strains expressed as percent

Table 1 Folate content ($\mu\text{g}/100$ g dry matter)^a in different yeast strains isolated from kefir granules

Strain	H ₄ folate	5-CH ₃ -H ₄ folate	5-HCO-H ₄ folate	Total folate
A	2,310	6,070	2,520	10,900
B	2,360	5,410	2,520	10,290
C	2,410	5,380	2,630	10,420
D	2,110	6,640	2,560	11,310
E	2,340	4,700	3,750	10,790
F	2,050	4,620	4,130	10,800
G	2,170	4,820	3,020	10,010
H	2,170	5,850	3,680	11,700

^aValues are means of duplicates. The difference between two separate values of a duplicate was within 0.3–13% for all samples

Folate contents in kefir yeast strains

Total folate content in the yeast strains isolated from Russian kefir granules varied slightly, ranging from 10,010 to 11,700 $\mu\text{g}/100$ g dry matter with an average mean of $10,780 \pm 550$ $\mu\text{g}/100$ g (Table 1). According to our earlier screening study of 44 yeast strains isolated from different sources, the total folate content in yeasts varied considerably, from 4,000 to 14,500 μg total folate/100 g dry matter, which showed great differences between yeast strains regarding folate-producing capability [22]. On the basis of these data, kefir yeast strains might be placed into the group of yeasts with high folate-producing capability.

However, the distribution of the individual folate forms differed clearly in different kefir yeast strains (Table 1, Fig. 3) as well as in yeast strains from other sources analyzed earlier [22]. Kefir yeast strains could be separated into two groups depending on their capability to produce different folate forms. The first group (strains A–D) produced high amounts of 5-CH₃-H₄folate (52–59% of total folate content), but much lower amounts of 5-HCO-H₄folate

and H₄folate (19–25% of total folate content). The second group (strains E–H) produced similarly high amounts of both 5-CH₃-H₄folate and 5-HCO-H₄folate (43–50%, respectively, 30–38% of total folate content) but considerably lower amounts of H₄folate (19–22% of total folate content). The detection of considerable amounts of 5-HCO-H₄folate in kefir yeast strains distinguished these strains from earlier analyzed yeast strains, which contained only two dominating folate forms (5-CH₃-H₄folate and H₄folate) in different proportions [22].

The knowledge about the distribution of individual folate forms in kefir yeast strains is of practical significance for development of new kefir products. By choosing kefir granules with yeast strains that produce a higher proportion of the most stable folate forms such as 5-HCO-H₄folate and 5-CH₃-H₄folate [6] it is possible to improve the stability of folates during fermentation and storage and thus to increase the folate content in kefir products.

Conclusion

This is the first study to our knowledge where the individual folate forms were characterized and quantified in yeast strains isolated from kefir granules. The most abundant folate forms were 5-methyltetrahydrofolate (43–59% of total folate) and 5-formyltetrahydrofolate (23–38% of total folate), whereas tetrahydrofolate occurred in a lesser proportion (19–23% of total folate). The average folate content for the eight yeast strains was 10,780±550 µg/100 g dry matter. Our results indicate that yeasts of dairy origin are rich folate sources and can be used for developing novel fermented foods with higher folate content.

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