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Prolonged Shelf Life in Fermented Milk Products Investigated by Bioassay-Guided Chemometrics

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Yeast-growth bioassay and bioassay-guided NMR-based metabonomics
A yeast-growth bioassay for evaluation of the antimicrobial effect of aqueous extracts of fermented milk products has been developed and used in a bioassay-guided NMR-based metabonomics study. The bioassay revealed significant differences between the reference samples and the samples containing bioprotective culture. The NMR-study, however, revealed only minor concentration changes. It is therefore concluded that the antimicrobial compound is potent at sub-mM concentrations. Further studies using other analytical techniques are being conducted.

Introduction

Specific strains of lactic acid bacteria have been found to prolong shelf life without significantly changing taste or texture of fermented milk products if present during fermentation. The ***hypothesis*** is that the bacteria produce small amounts of antimicrobial compounds. The present study intents to investigate which antimicrobial compound(s) is produced.
In order to undertake a targeted chemical analysis, a yeast-growth bioassay has been developed for fermented milk products (Reference) or with bioprotective culture (BioP). A 24-sample metabonomics study including the bioassay and NMR spectra has been conducted.

Yeast-growth bioassay

Development of assay

The yeast-growth bioassay was developed as a microplate assay of 200 μL aqueous extracts of the fermented milk products mixed with yeast. Aiming for the largest difference between BioP and reference samples, the following parameters were optimised:

- choice of contaminant (yeast species)
- yeast harvested at different phase of growth
- growth temperature
- growth medium
- incubation time
- initial yeast concentration

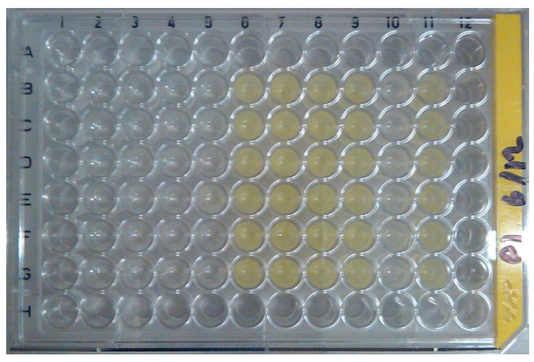


Figure 1: Results from microplate-based yeast growth assay (Microplate after incubation: Column 2-5 BioP samples, 6-9 reference samples, 10 & 11 sterile and growth tests).

Metabonomics study

Experimental details

Sample preparation

The aqueous extracts of fermented milk were prepared as follows:
1.Centrifugation for 2 hours at 20.000g
2.Aqueous partition transferred to centrifugal filter unit (10 kDa, 15 mL).
3.Centrifugation at 5.000g for 2 hours.
4.Filtrate was used as aqueous sample.

Bioassay

The optimised bioassay was used in the metabonomics study. All extracts were prepared in triplicate and the bioassay used two separate yeast inocula, 6 replicates and biological triplicates.

NMR spectra

The NMR-spectra were acquired using a 600 MHz Bruker Avance III HD spectrometer equipped with a cryogenically cooled 5 mm dual-channel probe optimized for ^{13}C and ^{1}H. Samples consisted of 90% extract, 10 % D_2O and 10 mM phosphate buffer (pH 4.7) and were analyzed at 300 K. Proton spectra, at 600.13 MHz, were acquired using presaturation and spoil gradients to remove water signal, 90º-pulses, a spectral width of 12 kHz, collecting 512 scans with a length of 32768 data points with a relaxation delay of 2.0 sec. FID's were zero-filled to twice the size and exponentially multiplied with a line broadening factor of 0.3 Hz before Fourier transform. All spectra were manually phase corrected and automatically baseline corrected. Two independently optimized spectra were acquired for each sample. Four samples were removed due to lack of concentration.

Principal Component Analysis

The PCA was performed using the Matlab based PLS_toolbox v.5.8 combined with in-house developed scripts by Nils Nyberg. The data were calibrated using the acetoin peak at $\delta(^1H) = 2.13$, interpolated to a common axis, binned (width of 0.015 ppm) and bins were adjusted to fit peaks.

Results

Bioassay

The bioassay shows a clear and reproducible differentiation between BioP and reference samples as exemplified in *figure 2*. Two BioP extracts (red curves) were found to have a significantly lower antimicrobial activity probably ascribed to the sample preparation or inhomogeneities in the samples.

Principal Component Analysis

An overall analysis resulted in the data shown in the *top row figure 3* and did not point to a differentiating factor between BioP and reference samples: only a minor concentration difference

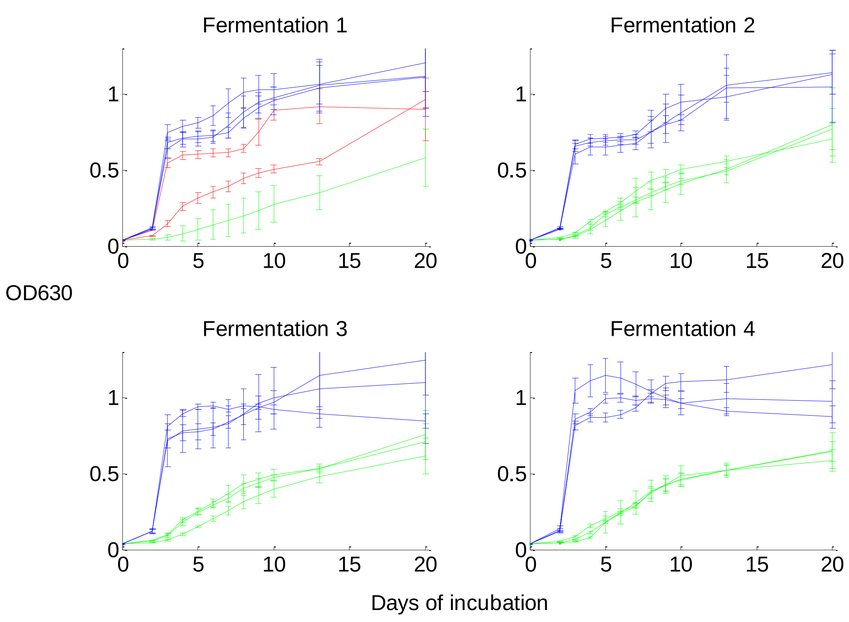
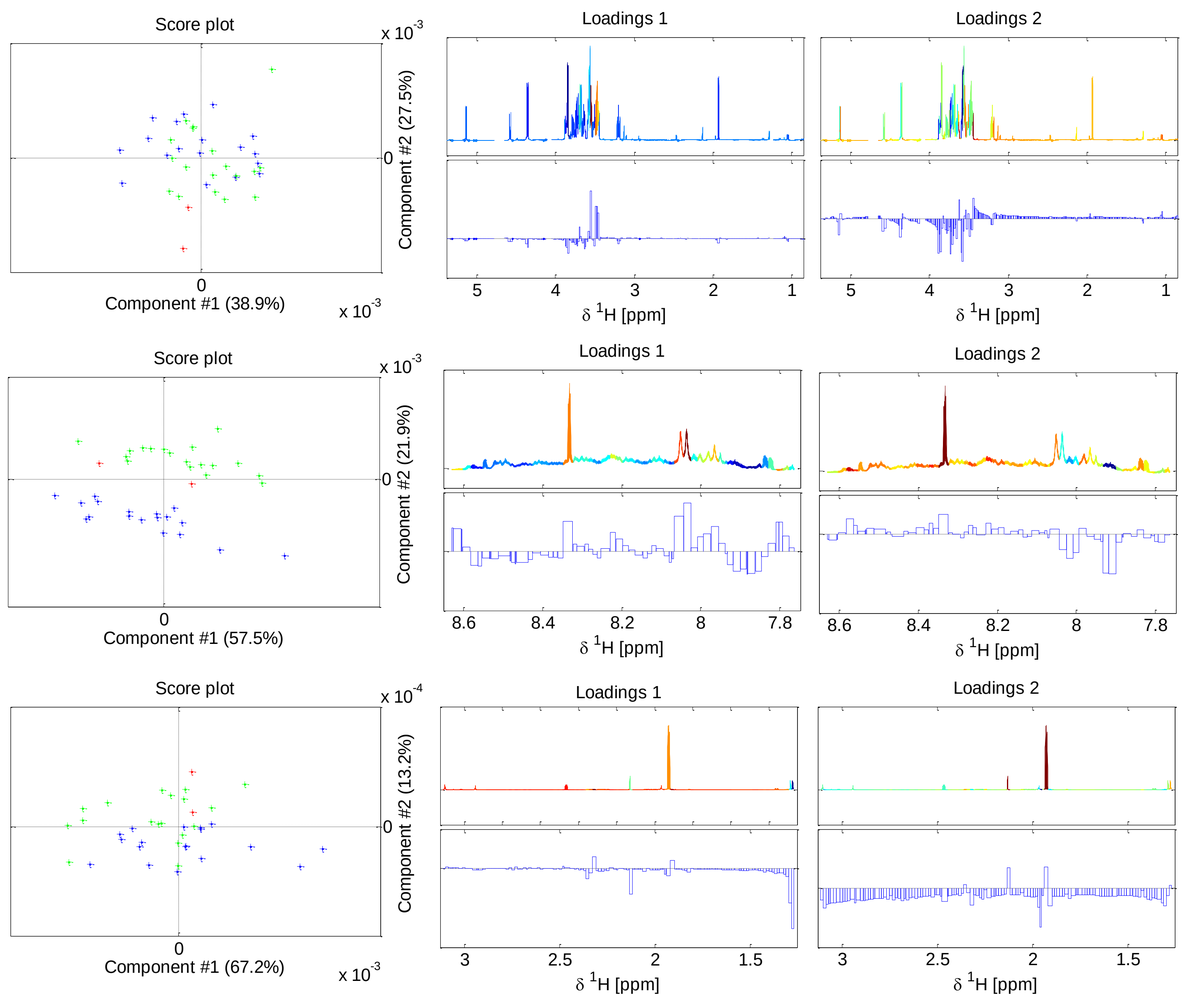


Figure 2: Growth curves obtained for the samples in the metabonomics study
(Growth monitored by the developed bioassay as optical density at 630 nm (turbidity) as a function of incubation time. Green: BioP samples, Blue: Reference samples, Red: BioP samples with atypical low antimicrobial activity).

Figure 3: Score and loading plots from the proton NMR data (Green: BioP samples, Blue: Reference samples, Red: BioP samples with low biological activity. First row: All spectral region. Second and third rows: Sub-spectral regions showing some degree of separation. The loadings are depicted in colour code on the spectra (blue-yellow-red) and as bins).



among the sugars is identified. This led to a sub-spectral analysis revealing the most pronounced grouping upon evaluation of $\delta(^1H) = 7.8 - 8.6$ as shown in the *middle row figure 3*. The major difference is attributed to the resonance at 8.35 ppm.
Another differentiating spectral region is found between 1.3 and 3.1 ppm, where the concentrations of acetoin and acetic acid are seen to be indicative, however, with a large variation between samples (*lower row figure 3*).
The BioP samples with low antimicrobial efficacy are in all cases seen to group with the other BioP samples.

Discussion

With the significant differentiation of the samples in the bioassay, it is surprisingly small differences which are found in the NMR data. The data does not show any new compounds in the BioP samples and the differences in concentrations are clearly too small to explain the large and consistent biological differences. Moreover, the BioP samples with low activity (red) group with the active BioP samples. As such, it is most likely that the antimicrobial activity must be ascribed to a compound present at a sub-mM concentration not visible in the proton NMR data.

Conclusions and future work

A well functioning yeast-growth bioassay which clearly distinguishes between fermented milk samples with and without bioprotective lactic acid bacteria has been developed. The bioassay was successfully applied in the 24-samples metabonomics study. The PCA of the NMR spectra of the samples revealed only minor unspecific concentration differences around $\delta(^1H) = 3.5$ attributed to sugars. However, sub-spectral PCA revealed differences between BioP and reference samples regarding acetic acid, acetoin and an unidentified compound with $\delta(^1H) = 8.35$. All compounds showed a slightly higher concentration in the BioP samples. These trends, however, do not explain the very distinctive difference observed in the bioassay and we therefore conclude that the antimicrobial compound must be present at a sub-mM concentration and therefore not visible in the NMR data.

With this conclusion, the further analytical work will rely primarily on the use of MS-based techniques.

Acknowledgements

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