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Publication date: 2014

Document version: Early version, also known as pre-print

Citation for published version (APA):
Prolonged Shelf Life in Fermented Milk Products Investigated by Bioassay-Guided Chemometrics

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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 intends to investigate which antimicrobial compounds are produced.

In order to undertake a targeted chemical analysis, a yeast metabonomics study has been developed and used in a bioassay-guided NMR-based metabolomics study. The bioassay revealed significant differences between the reference samples and the samples containing bioprotective cultures. 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.

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. Aim was for the largest difference between BiOP mixture 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

Results
Bioassay

The bioassay shows a clear and reproducible differentiation between BiOP mixture and reference samples as exemplified in figure 2. Two BiOP mixture extracts (red curves) were found to have a significantly lower antimicrobial activity probably ascribed to the sample preparation or intramolecularities 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 among the sugars is identified. This led to a sub-spectral analysis revealing the most pronounced grouping upon evaluation of δ(H) = 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 1.1 ppm, where the concentrations of acetic 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 attributed to a compound present at a sub-M concentration not visible in the proton NMR data.

Conclusions and future work

A well functioning yeast-growth bioassay which clearly distinguishes between fermented milk products with and without bioprotective lactic acid bacteria has been developed. The bioassay was successfully applied in the 24-samples metabolomics study. The PCA of the NMR spectra of the samples revealed only minor unspecific concentration differences around δ(H) = 3.5 attributed to sugars. However, sub-spectral PCA revealed differences between BiOP mixture and reference samples regarding acetic acid, acetic and an unidentified compound with δ(H) = 8.35. All compounds showed a slightly higher concentration in the BiOP mixture. 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-M concentration and not visible in the NMR data.

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

Acknowledgements

NMR equipment used in this work was purchased via grant: 10-085364 from The Danish Research Council for Independent Research | Nature and Universe.

Yeast-growth bioassay and bioassay-guided NMR-based metabolomics

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 metabolomics study. The bioassay revealed significant differences between the reference samples and the samples containing bioprotective cultures. 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.

Sample preparation

Yeast

Yeast analysis, a yeast metabonomics study. All extracts were prepared in triplicates and the bioassay used two separate yeast inocula, 6 replicates and biological triplicates.