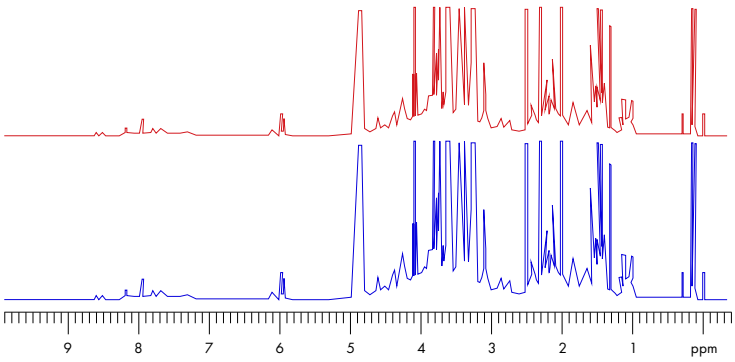
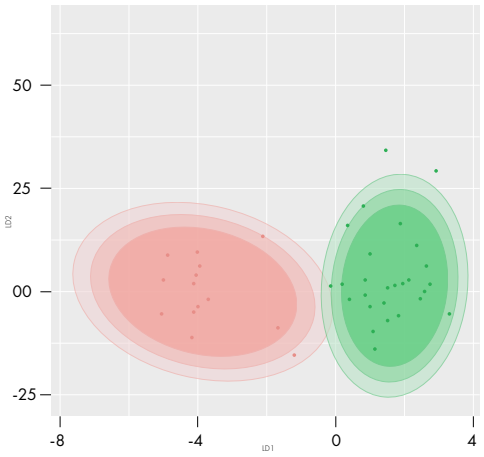
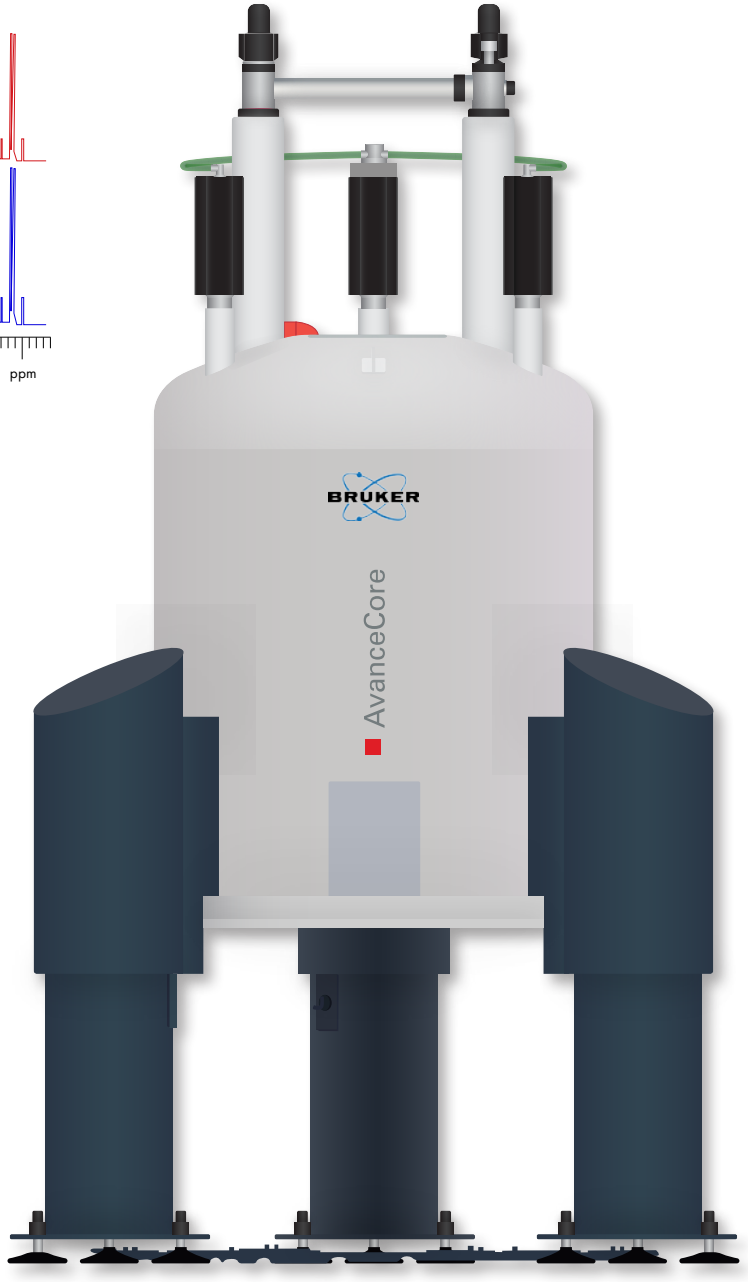
PROBIOTIC CONSISTENCY PRODUCT ANALYSIS

BACTERIAL STRAIN DIFFERENTIATION USING NMR METABOLOMICS







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Background

Nuclear Magnetic Resonance (NMR) spectroscopy-based metabolomics [1], a quantitative study of primary and secondary metabolites of a system, is a widely recognized standard analytical method for biological systems with a special emphasis on probing metabolic changes occurring in body fluid, tissues, and mammalian cells due to several condition and disorder-dependent physiological changes [2,3]. Recently, the metabolomics approach has been extended to study the consistency of natural botanical products and identify the species present [4–10], supporting quality control and quality assurance requirements in food and nutraceutical sectors. Amidst dietary products in the market targeting health benefits, probiotic products have seen tremendous growth [11] as they harness a multitude of health benefits such as restoring & maintaining the gut microbiota, supporting of the immune system, reducing the risk and duration of antibiotic-associated and infectious diarrhoea, supporting healthy cholesterol, and many other health benefits [12–14].

The health claims of probiotic products are strain-specific, therefore demonstrating successful verification of probiotic identity must be performed in support of regulatory requirements. For example, *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*) and *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) supplements help maintain urogenital health, *Lacticaseibacillus casei*, (formerly *Lactobacillus casei*) *Lactobacillus acidophilus*, and *Bifidobacterium longum* are used for weight management since they help lower total and LDL cholesterol, and *L. plantarum* 06CC2 has shown potential to help alleviate allergy-associated symptoms [12], among many others. In addition, the compositional variation in the products must be negligible for them to pass quality constraints and deliver satisfactory product performance, i.e., the products must be consistent in their chemistry. NMR spectroscopy, in conjunction with multivariate statistical analysis, can differentiate between products of similar strains produced by different manufacturers, as well as assess their consistency. In this report, a comprehensive account of how NMR-based metabolomics can be used to differentiate *Lactobacillus acidophilus* strains and establish sample consistency is provided. Also, a promising case of using NMR as a strain-identification tool is presented.

Materials and Methods

Freeze-dried single strain probiotic samples with three different strains of *Lactobacillus acidophilus*, henceforth referred to as strains A, B, and C were procured from different manufacturers. The cells were lysed both chemically and mechanically and the cellular metabolites alongside the excipients were extracted using a combination of organic solvents (methanol, chloroform, acetone, ethanol, hexane, and their mixtures) and water. In a separate study, two strains (strains A and B) were cultured, the cells were harvested from the media, washed, lysed, and the cellular metabolites extracted to test the efficacy of NMR in identifying strains. One-dimensional NMR spectra were collected on a 400 MHz Bruker Avance III spectrometer with tetramethyl silane (TMS, 0 ppm) as an internal chemical shift reference. The collected spectra were processed, baselined and phase corrected, and binned using Bruker Amix program for multivariate statistical analysis, performed on the ***R*** platform.

Results and Discussion

The ^{1}H NMR spectra of three *Lactobacillus acidophilus* strains are stack-plotted in **Figure 1**, which show prominent differences, attributed to the cellular metabolite makeup, excipients, and processing aids present in the samples. The ensemble of peaks in these spectra represent different classes of molecules ranging from amino acids to sugars, contributed by the cells as well as processing aids and excipients added for standardization, cryo-protection, and other functions. The peak intensities directly correspond to the concentration of molecules, underscoring the inherent quantitative nature of the technique. Despite differences in the spectral profile being visually evident, statistical treatment of the data is required to differentiate the samples categorically and establish their consistency by amplifying the differences in the profiles. NMR spectra of multiple samples containing these *L. acidophilus* strains from different batches were collected and subjected to a linear discriminant analysis (**LDA, Figure 1**), a type of dimension-reducing multivariate statistical analysis, which amplifies the spectroscopic differences between samples of different classes. Each point on the score plot represents the individual sample, more specifically its metabolite profile. The closer the points are located on the score plot, the more similar the metabolite profile of the samples will be and in the case of samples of same strain, the data points cluster with high precision. Along the same lines, the data points on the score plot that are far away from each other have different metabolite profiles. Each colour-coded cluster in the LDA score plot (**Figure 1**) represents individual strains of Lactobacillus acidophilus, and the clear distinction between them underscores the efficacy of NMR metabolomics in differentiating samples of different strains produced by different manufacturers.

These individual clusters encircled in three ellipsoids, collectively referred to as ordination plots, offer a great deal of insight into the consistency of the tested samples as these are compared with compositionally similar samples from different batches and lots. The ordination plot consists of data points from samples of the same bacterial strain which are clustered within three ellipsoids marking different confidence intervals. The innermost ellipsoid marks ≥98% confidence level between the samples, i.e., a ca. 2% variation in their metabolite makeup. The second and third ellipsoids mark ≥95-<98%, and ≥90-<95% confidence intervals, respectively. The data points representing the probiotic samples of three strains from different batches fall within the innermost ellipsoid, inferring the spectral similarity to be of the highest degree (≥98%), therefore, inferring that the studied samples are highly consistent in their composition and chemistry. No ordination plot is presented here for strain C as the number of samples tested at the time of writing this report was low and didn't support the development of such a plot. Nevertheless, the data points of samples with strain C stand out from the other two clusters, underscoring its chemical uniqueness compared to the other two. An important aspect of the metabolomic assay of probiotic samples is to capture the compositional and chemical variation in a sample as a spectral database, which is used in the statistical analysis and functions as a reference for strain differentiation and consistency testing. Therefore, it is prudent to have a large spectral database, which is accomplished by collecting spectral data for a large volume of samples of a particular probiotic bacterial strain of a specific chemistry. The developed spectral database and the statistical model can then be used in routine differentiation and consistency testing of the samples.

The strain-identification capability of NMR was tested by analyzing the pure cell cultures of two strains A and B. Pure cell cultures facilitate the removal of all excipients from the extraction matrix. The extraction renders only the cellular metabolites which are key for identifying the bacterial strains as the metabolic pathways differ between the strains resulting in distinct classes of secondary metabolites. The ^{1}H NMR spectra of these two *L. acidophilus* strains are plotted along side the LDA plot in **Figure 2**, which successfully discriminates the collected spectral data into two different clusters representing the two studied strains. The data points fall within the first consistency ellipsoid (the other two ellipsoids, ≥95-<98%, and ≥90-<95%, are not shown for easy visualization of the plot) which marks >98% of similarity in the metabolite profile of the cells constituting products from different batches. The results are promising, and more studies are underway to establish NMR as a tool for routine strain identification testing and explore its strengths and weaknesses for this analytical approach.

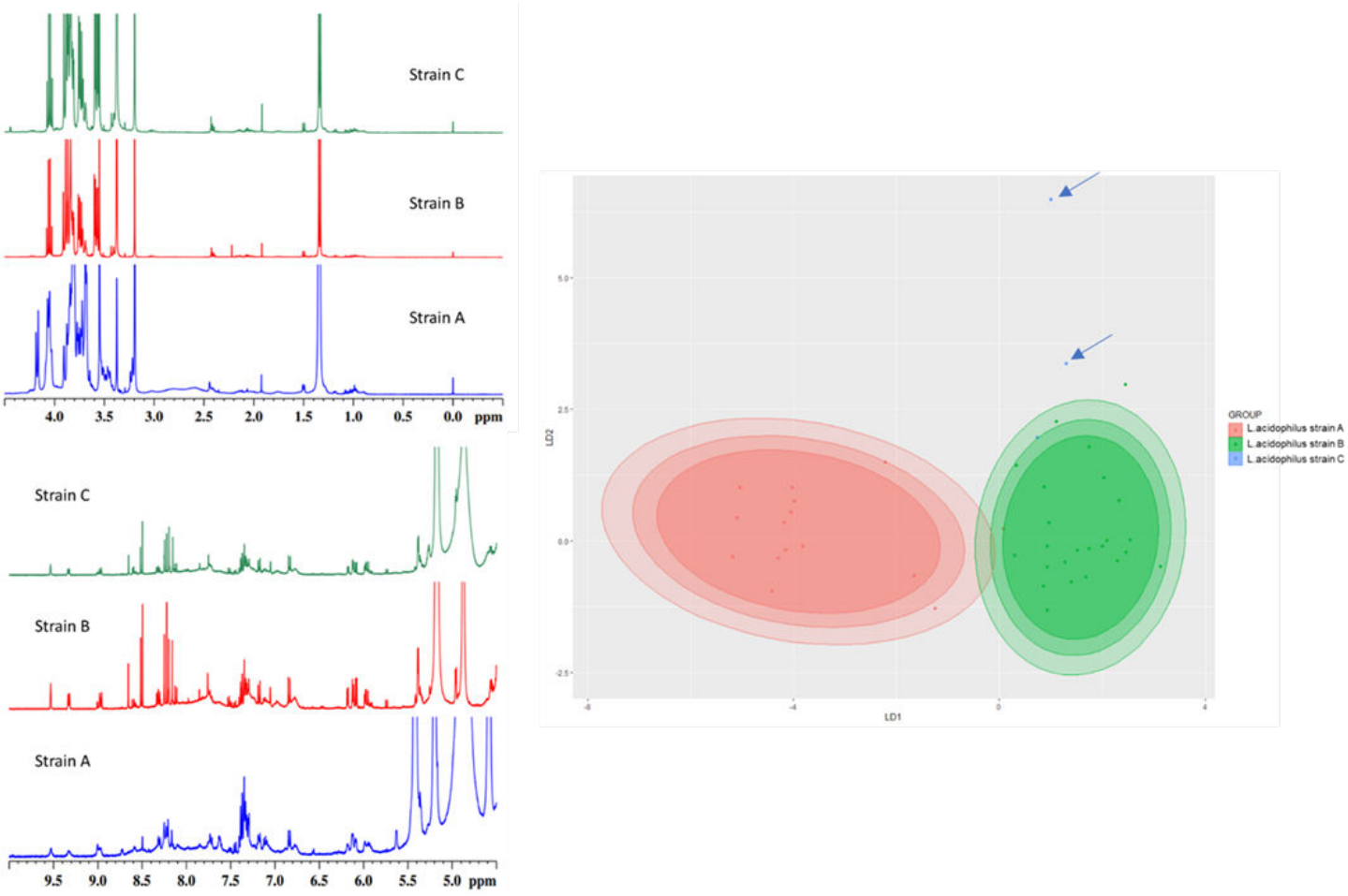

Figure 1. Representative ^{1}H NMR spectra of samples with three different *Lactobacillus acidophilus* strains (left), and the Linear Discriminant Analysis plot showing the clustering of data points representing the three strains. The ordination plot for *L. acidophilus* strain C (data points marked with blue arrows) is not shown due to low sample volume.

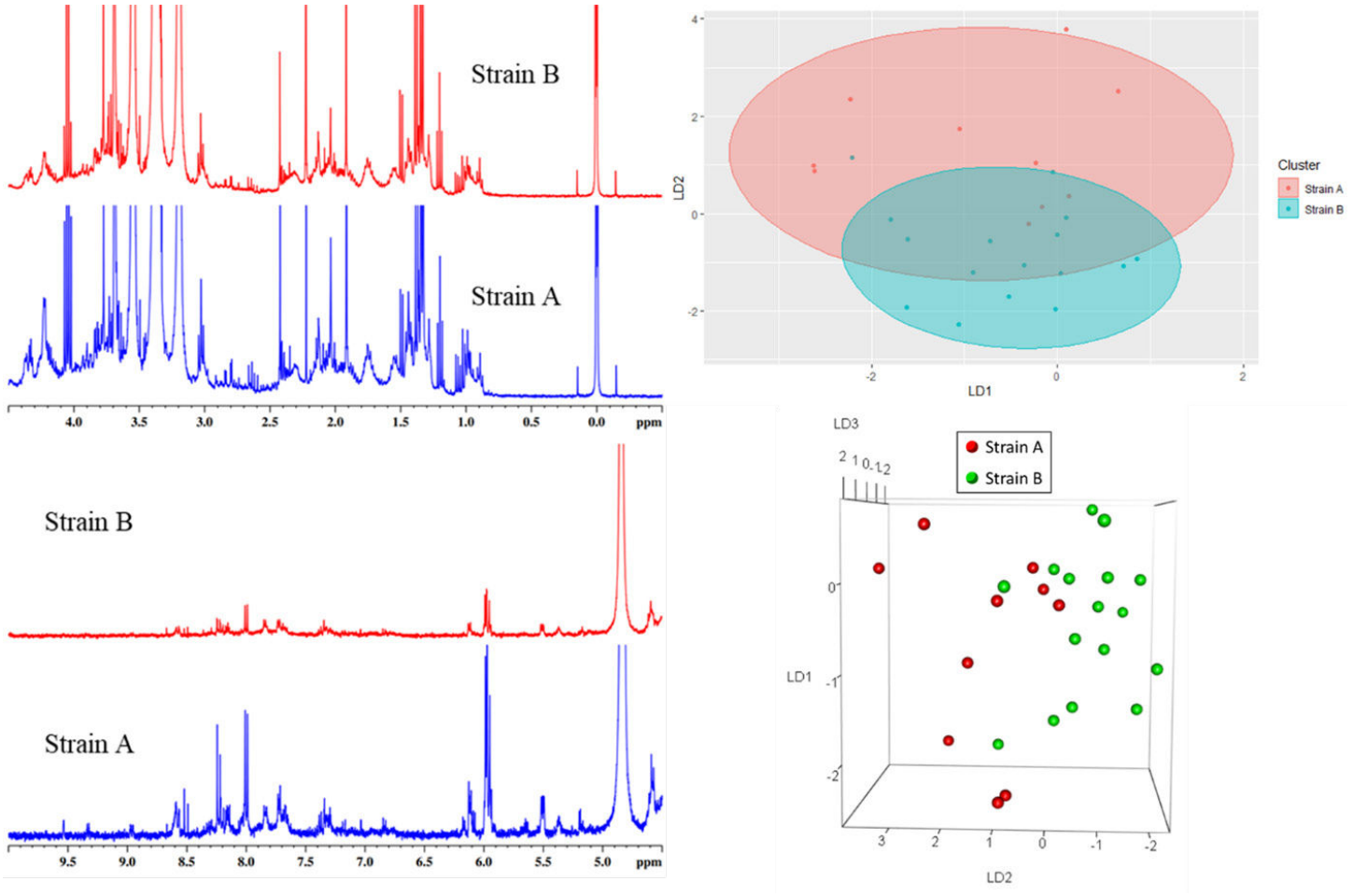

Figure 2. Representative ^{1}H NMR spectra of two cultured *Lactobacillus acidophilus* strains (left), and the Linear Discriminant Analysis plot in 2-D and 3-D formats showing the clustering of data points representing two of the strains (right).

Conclusion

The NMR spectroscopy-based statistical model successfully differentiates the products of different *Lactobacillus acidophilus* strains produced by different manufacturers and offers good insights into sample consistency, therefore demonstrating that the technique is well-suited for routine differentiation and consistency testing of probiotic products. The developed statistical model will be strengthened by analyzing more samples of the studied strains as the model relies on the volume of the spectral database. In addition, other bacterial strains will be included in this model to increase robustness for successful routine testing. The reported analytical approach for strain identification is quite promising and must be explored further to understand its full potential and suitability for routine strain-identification testing.

References

[1] A.H. Emwas, R. Roy, R.T. McKay, L. Tenori, E. Saccenti, G.A. Nagana Gowda, D. Raftery, F. Alahmari, L. Jaremko, M. Jaremko, D.S. Wishart, Nmr spectroscopy for metabolomics research, Metabolites. 9 (2019).

[2] S. Kostidis, R.D. Addie, H. Morreau, O.A. Mayboroda, M. Giera, Quantitative NMR analysis of intra- and extra cellular metabolism of mammalian cells: A tutorial, Anal Chim Acta. 980 (2017) 1–24.

[3] T. Gebregiworgis, R. Powers, Application of NMR Metabolomics to Search for Human Disease Biomarkers, Com. Chem. High Throughput Screen. 15 (2012) 595–610.

[4] X. Wang, P.D.B. Harrington, S.F. Baugh, Comparative study of NMR spectral profiling for the characterization and authentication of cannabis, J AOAC Int. 100 (2017) 1356–1364.

[5] J.M. Hicks, A. Muhammad, J. Ferrier, A. Saleem, A. Cuerrier, J.T. Arnason, K.L. Colson, Quantification of chloro genic acid and hyperoside directly from crude blueberry (Vaccinium angustifolium) leaf extract by NMR spectroscopy analysis: Single-laboratory validation, J AOAC Int. 95 (2012) 1406–1411.

[6] J. Trifković, F. Andrić, P. Ristivojević, E. Guzelmeric, E. Yesilada, Analytical methods in tracing honey authenticity, J AOAC Int. 100 (2017) 827–839.

[7] G. Valentino, V. Graziani, B. D'Abrosca, S. Pacifico, A. Fiorentino, M. Scognamiglio, NMR-based plant metabo lomics in nutraceutical research: An overview, Molecules. 25 (2020).

[8] A. Krishnamurthy, T. Arunachalam, Curcuma species identification and adulterant detection using nuclear magnetic resonance spectroscopy - A case study for botanicals, BT2022-01, Purity-IQ, Guelph, ON, Canada, 2022.

[9] A. Krishnamurthy, R. Shaykhutdinov, Mushroom species identification and sample consistencies using NMR metabolomics, BT2022-03, Purity-IQ, Guelph, ON, Canada, 2022.

[10] A. Krishnamurthy, T. Arunachalam, Differentiating elderberry products using NMR metabolomics, BT2022-02, Purity-IQ, Guelph, ON, Canada, 2022.

[11] P. Verma, Probiotics Market by Ingredient (Bacteria, and Yeast), Function (Regular, Preventative Healthcare, and Therapeutic), Application (Food & Beverages, Dietary Supplements, and Animal Feed), and End Use (Human Probiotics, and Animal Probiotics)-Global Opportunity Analysis and Industry Forecast, 2014-2022, 2016. https://www.alliedmarketresearch.com/probiotics-market (accessed August 18, 2022).

[12] R. George Kerry, J.K. Patra, S. Gouda, Y. Park, H.S. Shin, G. Das, Benefaction of probiotics for human health: A review, J Food Drug Anal. 26 (2018) 927–939.

[13] L.H. Shi, K. Balakrishnan, K. Thiagarajah, N.I. Mohd Ismail, O.S. Yin, Beneficial properties of probiotics, Trop Life Sci Res. 27 (2016) 73–90.

[14] M. Kechagia, D. Basoulis, S. Konstantopoulou, D. Dimitriadi, K. Gyftopoulou, N. Skarmoutsou, E.M. Fakiri, Health Benefits of Probiotics: A Review, ISRN Nutr. 2013 (2013) 1–7.