Proteomnic proof that a probiotic elevates glutathione level in human serum

Abstract: Lactobacillus fermentum ME-3 (LfME-3) has been proven to synthesize and secrete glutathione. A regular use of the foods fermented by it has shown a favourable influence on human lipid profiles and several antioxidant parameters. We administered the LfME-3-fermented kefir for 14 days to 43 human subjects and evaluated their serum with MALDI-TOF mass spectrometer at the beginning and end of the test period. We found an increase of the peak at m/z 308 (corresponding to glutathione) and a new peak at m/z 1467.

Keywords: probiotic Lactobacilli, glutathione, peptidomics

1 Introduction

Lactobacilli are normal inhabitants of human microbiota and some strains have been proven to have beneficial properties for the host. Viable lactic acid bacteria (LAB) of human origin help to restore normal intestinal microbial functions, alleviating disease symptoms in patients with gastrointestinal tract (GIT) infections, stimulating the immune system, expressing anti-atherogenic effects and having possible anti-carcinogenic influence [1-5]. Evidence indicates that many of these effects are strain-specific. Lactobacillus fermentum ME-3 (DSM14241, LfME-3) has a human origin, it is isolated from the human gastrointestinal tract (GIT) [6-8]. Previously it has been proven that LfME-3 possesses substantial antimicrobial and antioxidative-antiatherogenic activity, expresses manganese superoxide dismutase, eliminates hydroxyl radicals and contains a whole glutathione (GSH) system: synthesis, transport and redox cycling [4,8-10]. The latter is important as without GSH and the glutathione redox cycle, LAB cannot effectively protect themselves against reactive oxygen species (oxidative stress) as the antioxidative potency in lactobacilli has a significant positive correlation with cellular GSH levels [11,12]. At the same time, the ability to synthesize GSH is rare in LAB [13]. Glutathione (L-gamma-Glu-L-Cys-Gly or GSH) is a major cellular non-enzymatic antioxidant. It eliminates reactive oxygen species (ROS) such as lipid and hydrogen peroxides, hydroxyl radicals and peroxynitrite, mainly via cooperation with selenium-dependent glutathione peroxidase [14,15]. Confirmation of the presence of all the components of the glutathione system in a LAB strain gives very valuable information as it shows that this specific LAB strain has especially high oxygen and ROS tolerance under different stress conditions. Tolerance to stress in the digestive system as well as during the production of a functional food is an essential physiological trait for probiotics [16,17].

For some years now LfME-3 has been used as a probiotic in commercial milk products in the Baltic countries and Finland; the strain is patented in Russia, USA and European Union. The positive health effects are documented in [4,9,18,19].

For instance, the postprandial lipemic response is widely accepted as an independent risk factor for cardiovascular diseases. Any aggravation of the postprandial pro-oxidative situation may have negative consequences as postprandial oxidative stress has been suggested to be the unifying mechanism in the connection between cardiovascular diseases (CVD), metabolic syndrome, insulin resistance and Type 2 diabetes [20,21]. As metabolic syndrome and CVD, both associated with a pro-oxidant situation and often with a lowered serum HDL, are an increasing problem, it is noteworthy that consuming LFME-3 products regularly increases the HDL level and reduces the oxidized LDL level [5].

Increasing the levels of GSH in human subjects is
indicated in many situations characterized with increased oxidative stress. If the limiting factor of GSH synthesis is the deficiency of cysteine, the routine practice and partial solution is administration of N-acetyl-cysteine. In other cases (where the deficiency of cysteine is not the main issue) we do lack a good solution. Orally administered GSH is not well-absorbed [22]. A new approach is using Lactobacilli for that purpose [23,24].

Peptidomics with mass spectrometry has shown a promising potential in detecting peptide/protein spectrum changes in biological samples in a wide variety of situations from cancer research to rheumatology [25,26]. So we made an attempt to detect the peptidomic changes in human sera induced by a regular use of the probiotic LfME-3. Although we had detected a change in serum glutathione content in earlier experiments with biochemical methods it was a bit discouraging to read the results of Feng et al. [27] and the MALDI spectrometry difficulties in glutathione measurements. Lactobacilli are proved to have a wide variety of exocytosed peptides and proteins [28] so it cannot be excluded that some small peptides are absorbed and detectable in human sera or that some peptides are synthesized in response to the cross-talk of Lactobacilli with host cells in the gut and secreted into blood.

2 Methods

The subjects were the 48 volunteers described in [5] as the study group, briefly 34 women and 14 men with mean BMI $27 \pm 2$ kg/m$^2$ and mean age $55 \pm 3$ years with no current or chronic illness or medication. All participants signed their written informed consent and had the option of withdrawing from the study at any time. The Ethics Review Committee (ERC) on Human Research of the University of Tartu approved the study protocol. This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association. The participants consumed 200 ml of kefir containing LfME-3 (viable counts $2 \times 10^8$ CFU/g kefir) for 14 days. The postprandial serum sample (obtained 2.5 hours after a standard meal described in [5]) was taken from the participants before and after the intervention. The serum was collected into vacutainer test tubes without gel, allowed to clot for 30 min and centrifuged at 1800 g for 10 minutes at room temperature. The serum was pipetted off and stored in aliquots at -80°C until analysed. From that investigation group we obtained paired sera of 43 persons of the study group (results of previous investigations have shown no change of glutathione system members in the control group). The serum from either point 0 or endpoint was not available for 4 women and 1 man.

The specimen preparation procedure is based on the method of [29]. For further purification the protocol of Purification kit MB-HIC with C18 beads was applied [30]. And finally the phase of concentrating the material was added between two steps of purification inspired by [31]. Mass-spectrometry grade acetonitrile, water, formic acid and α-cyano-4-hydroxycinnamic acid matrix were all purchased from Fluka/Sigma-Aldrich, Germany. Here is the final protocol briefly.

In order to concentrate the possible LfME-3 induced small (signalling) molecules, 100 μl of acetonitrile were added to 400 μl of serum. After mixing the solutions were centrifuged at $14000 \times g$ at $20°C$ for 45 minutes. 300 μl of the upper fraction was transferred to cutoff filter (Microcon® centrifugal filter devices with nominal molecular weight limit 10,000 from Millipore, Billerica, USA) and centrifuged at $14000 \times g$ for 60 min at $20°C$. The filtrate was kept at -80°C for 30 minutes and then lyophilized overnight. The next day the lyophilisate was suspended with 10 μl water and treated by the magnetic purifying beads according to the manufacturer (100 MB-HIC 18 purification kit from Bruker Daltonics, Germany) protocol: 10 μl of buffer solution and 5 μl of magnetic beads added to 10 μl sample, mixed, in 60 seconds separated on magnetic field (Promega MagneSphere® Technology Magnetic Separation Stand for $2 \times 0.5$ ml). Beads were washed twice with 100 μl of washing solution and finally resuspended in 5 μl 50% acetonitrile in water (MilliQ). Elution was repeated once again and the obtained liquid phases were pooled. From the 10 μl solution 1 μl was mixed with 1 μl a-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 50 % acetonitrile/water) and transferred onto a MALDI sample plate.

The spectra were obtained on Voyager PerSeptive DE-PRO MALDI-TOF (PerSeptive Biosystems, USA) in linear mode with a delayed extraction mode (extraction delay time 100 ns) at acceleration voltage of 20000 V. The acquisition mass range was set to m/z 250-3000 and 50 shots were taken to form a spectrum. The laser intensity was set at 1961 arbitrary units and low mass gate to m/z 200. Before these parameters were chosen, several preliminary trials were conducted with different voltage and laser intensity levels to determine the conditions giving the optimal signal to noise ratio. Fragmentation analyses were performed on Shimadzu Prominence high-performance liquid chromatography (Shimadzu Inc. Japan) and QTrap 3200 (Applied Biosystems, USA) tandem mass spectrometry. 20 μl sample was separated in C18 column (Luna C18, 5 × 150 mm, Phenomenex, USA) and...
detected for fragments of given ions at declustering and collision potentials 40 V. Electrospray ionization was at 300°C and 4500 V.

MALDI-TOF mass spectra were corrected for background and calibrated with the help of matrix dimer signal at m/z 379.093. By visual inspection it was confirmed that main signals from all spectra over all m/z range overlapped satisfactorily and instrumental and calibration shifts were marginal. Each spectrum was binned with bin length 1 amu leading to data reduction to 2750 data points per spectrum. Data analysis was done with R 2.12.2 (R Foundation for statistical computing, Vienna, Austria).

3 Results

Molecular cut-off filters and C18 magnetic beads were used to extract peptides and other possible low molecular compounds from 43 healthy individuals before and after a 2 week period of consumption of a LfME-3 containing diet. First multivariate data analysis methods were used to allow reduction of large data matrices into fewer components or factors and visualize the variations among data. In a principal component analysis the treatment effect could not be associated with any obtained principal component, meaning that other sources of variation (e.g. genetic/metabolic background, dietary differences other than LfME-3 etc.) are more important in defining blood serum peptide profiles than LfME-3 diet. Partial least squares discriminant analysis (PLSDA) is an analogous multivariate data analysis method, but searches specifically for data patterns that distinguish two user defined groups. The two groups in our study were defined via the LfME-3 diet. Although not complete, a partial separation of sera from pre- and post LfME-3 diet into two groups can be seen in Fig. 1. Hence LfME-3 consumption causes a specific, although minor, alteration in serum peptidomic profile. Analysis of loading factors for the same PLSDA model revealed that the strongest contributors were compounds with mass to charge ratio (m/z) 308 and 307. A number of other compounds with molecular mass 365, 323, 466, 310, 945, 730, 825 contributed to the model to a lesser extent.

In multivariate statistics it was not possible fully to take into account that the samples were paired due to each subject having one sample before and one after the diet intervention. The average change in intensity of mass spectrometry signals by the diet and the significance of these changes were calculated for every m/z value (Fig. 2). The diet caused 43-47% increase in the mean signal of m/z values 304-308 and 1465-1466. The change

Figure 1. Graph presentation of partial least squares discriminant analysis. It indicates a separation of peptidomic profile of blood sera before (empty triangles) and after (full circles) the 2 weeks of diet containing L.f. ME-3. X-variates 1 and 2 are the two first latent factors generated by the PLS-DA model.
in m/z values 307 and 308 was statistically significant with p < 0.001 and 306, 1465, 1466 were significant at the level of p < 0.05. In large scale “-omics” data analysis the significance levels need to be corrected by an increased false positive rate and multiple comparison. It has been shown in actual proteomic or metabolomic studies the significance levels are slightly higher than predicted by Bonferroni correction [32]. For our dataset a threshold 0.0001 should be appropriate for a corrected significance level. But the fact that some m/z values appear in isotopic clusters (close m/z values locating close) further confirms that these signals are not standing out due to an increased chance of false positives in multiple comparison. Signal decreases due to probiotic diet were either small in effect or insignificant (Fig. 2). M/z values with either the greatest (> 41%) or the most significant (p < 0.001) changes in mean values were plotted as a heatmap to visualize the responses per individual. For each chosen m/z the average signal intensity among all individuals (including both pre- and post-diet samples) was calculated and each sample represented as having a higher or lower signal. Based on the response pattern among the study cohort the m/z values could be clustered into 5 groups. Group A includes signals with m/z 304-306, which all had a large, but not highly significant change after LfME-3 diet. These signals have very high variation between subjects. Group B (signals at m/z 945, 1612, 1273) was different from group A as the low p-values stemmed from a minimal but persistent shift from relatively stable signals. Group C had only m/z 781. Decrease in this signal was low (ca. 15%), but it seems to occur in most of the subjects. Group D had 3 m/z values (m/z 427, 308 and 307), which all increase after LfME-3 consumption. For m/z 308 28 (65%) subjects had an increase, 6 (14%) subjects had a decrease and for 9 (21%) subjects the level remained constant. M/z 307-308 (and also m/z 306) are probably isotopes of one compound. It is interesting that for these there is a clear difference that the right half (after probiotic diet) has more blue and the left side (before diet) more red colour. Not every individual has responded the same way, but in general there is an universal shift towards an increase in this compound. Members of group E (m/z 1465, 1466, 1467) had mediocre p-values and, as seen on Fig. 3, there are two kinds of study subjects with respect to this change. 20 subjects have all three signals increased, while 23 have a decrease or no change. It is remarkable that the (positive) responders have this signal above median also before LfME-3 diet challenge. It can be seen that m/z 1465, 1466 and 1467 (rows 11-13) share a strongly correlated pattern suggesting that they are isotopic signals for the same molecule. M/z 1465-1467 group has a subgroup of 10 subjects, who have an elevated signal before probiotic diet and have it even more pronouncedly up after diet. The response of other subjects is less clear and is giving ground to a hypothesis that the observed change in this marker is not a universal response to LfME-3, but something which has its origin elsewhere, but, if present, is amplified by the probiotic diet.

Next it was aimed to identify the most interesting markers with m/z 307-308 and 1465-1467. The former could fit with glutathione and double charged glutathione dimer, which is present in blood serum at more than micromolar concentration and would be an expected response for LfME-3-containing diet. Liquid chromatography – electrospray tandem mass spectrometry was used for fragmentation analysis. Fragmentation spectra of m/z 307-308 were compared with fragmentation spectra of glutathione standard. Signal from samples was very low as also seen in MALDI-TOF spectra. Two most intense GSH fragments 130 and 84
amu, were seen as fragments for m/z 308 from samples, but lack of other matches means it is not possible to fully confirm the presence of GSH in this signal. A more hydrophobic substance with higher retention time was found to degrade from 308 to 290, 262, 235, 141, 107 and 95.

According to previous studies [25,33] 1465.6 Da fibrinopeptide A (FPA) may be visible in MALDI-TOF based blood serum peptidomics. No fragmentation spectra with high enough quality for peptide full length sequencing could be obtained, but major fragment peaks corresponded very well to y14, y8, y5, y3 and y2 ions of theoretical FPA fragmentation.

4 Discussion

Detection of molecules with very low concentration in serum is a challenge even to a method like MALDI-TOF mass spectrometry, as the quantities of material used are extremely small [34,35]. There are basically two possible ways to overcome this disadvantage of an otherwise excellent method. One is an improvement in the technical parameters of the apparatuses, the other is increasing the concentration of the material prior to analysis [36]. As concentrating the material also means an elevation of possible noise signals we chose to purify the material with cut-off filters and C18 magnetic beads.

Although a method for glutathione quantification from substantially smaller amounts of blood has been developed [27], this approach includes derivatization and cannot be used in a complex analysis where different peaks are investigated simultaneously. The authors of this method state that they failed in measuring glutathione with MALDI-TOF mass spectrometry although it should be theoretically possible. We used a different matrix and the sample-preparation was drastically different – and we used sera as specimen, rather than whole blood. The positive aspects of using MALDI for glutathione analysis described in [27] still apply. In a review of glutathione measurements including peptidomic methods [36] only one group has used human serum as a specimen. Kato-Oamoto added compounds to GSH in methanol solution and analyzed them by MALDI -ToF, getting the m/z 308.1 – but it was not done with serum [37].

A food containing an antioxidant LAB strain that has been proven to contain glutathione and has the ability to synthesize and handle glutathione [10] has shown clinically relevant positive effects in humans [4,18]. Now we are able to prove with MALDI-TOF mass spectrometry analysis that the concentration of glutathione in the

Figure 3. The heatmap presentation of the signals with the most significant and/or greatest changes. Red colour stands for lower than average, blue for higher than average and white for average values , the intensity of the colour represents the difference from the average and is capped at two-fold higher/lower than average. The 43 leftmost columns are the samples before diet intervention, 43 right columns are the samples from the same subjects after 2 week of LIME-3 kefir diet. Rows are clustered on the basis on the similarity of response patterns (dendrogram on the left). The letters on the denrogram indicate groups of signals with highly similar pattern of responders. The m/z values and p-value for pre- and post-diet differences are indicated on the right side.
blood of the product users increases substantially, which correlates well with earlier results [19]. It is therefore possible to conclude the existence of an inter-species signalling with (probably) peptidic compounds that results in an increase of human glutathione pool. We also found an increase in a bigger, presumably peptidic compound that is either produced in the intestine or whose production is induced by gut microflora. The structure of the molecule detected is not finally elucidated, but present evidence supports the possibility that we saw an increase in fibrinopeptide A (FPA) described in [25]. On the other hand the findings of Gianazza et al. [39] speak against the peak belonging to FPA as they identified the signal at m/z 1532 to originate from FPA. It could also be the molecule that distinguished healthy people from cancer patients in [40] but the possibility that it has not been described previously cannot be excluded either since a MASCOT search (http://www.matrixscience.com/search_form_select.html) did not elicit any highly probable candidates from SwissProt [41].

If it is indeed an increase in FPA that we recorded, its physiological or pathogenetical significance has yet to be clarified. Melis et al. [42] did not find any differences in FPA levels during different phases of the menstrual cycle (the biggest changes in our study were seen in women so it would be a cause to discuss, as synthetic oestrogens change FPA levels). At the same time Banfi and Del Fabbro [43] estimated that of hemostasis test parameters FPA is one with the highest within-person variabilities.

Apart from the benefit of the technical recording of GSH with MALDI-TOF, the actual physiological significance of improving a human GSH status is of greater importance. Several approaches have been tried to improve the human body’s glutathione status starting with oral supplementation. Although it proved to be ineffective years ago [22], researchers continue to test the version [44], probably encouraged by the ability of apical membrane cells to uptake GSH [45]. Lipoceutical and transdermal vehicles as well as nanoparticles are also in testing phase [46,47]. There is at least one Lactobacillus strain under investigation with local (gastrointestinal) beneficial effects presumably due to its release of GSH [48]. The synthesis of GSH by Lactobacilli is not a common property [13]. One approach is developing a microbe [24,49] that can induce GSH synthesis in humans, but biotechnological products have still some unsolved issues. Thus having a human-origin non-GMO Lactobacillus with that ability is a good option.

**Abbreviations used**

FPA – fibrinopeptide A

GIT – gastrointestinal tract

GSH – glutathione

CVD – cardiovascular disease

LIME-3 – Lactobacillus fermentum ME-3

LAB – lactic acid bacteria

MALDI-TOF – matrix-assisted laser desorption/ionization – time of flight

OxS – oxidative stress

PCA – principal component analysis

PLS - partial least squares regression

**Conflict of interest:** Authors declare nothing to disclose.

**References**


**Authors declare nothing to disclose.**
Lactobacillus casei and Lactobacillus acidophilus, in the TNBS model of rat colitis, J. Appl. Microbiol., 2007, 103, 836–844


Di Girolamo F., Alessandroni J., Somm, P., Guadaqni F., Pre-analytical operating procedures for serum Low Molecular Weight protein profiling, Proteomics, 2010, 73, 667-677


Melis G.B., Fruzzetti F., Paolotti, A.M., Carmassi F., Fioretti P., Fibrinopeptide A plasma levels during low-estrogen oral contraceptive treatment, Contraception, 1984, 30, 575-583


