Experimental Metabonomic Model of Dietary Variation and Stress Interactions


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Stress in the form of moderate periods of maternal separation of newborn rats has been postulated to cause permanent changes in the central nervous system and diseases in later life. It is also considered that dietary supplementation with long chain polyunsaturated fatty acids (LC-PUFAs) can potentially ameliorate the effects of stress. The metabolic consequences of early life maternal separation stress were investigated in rats (2–14 days after birth), either alone or in combination with secondary acute water avoidance stress at 3–4 months of age. The effect of a LC-PUFA-enriched dietary intervention in stressed animals was also assessed. Systematic changes in metabolic biochemistry were evaluated using 1H nuclear magnetic resonance spectroscopy of blood plasma and multivariate pattern recognition techniques. The biochemical response to stress was characterized by decreased levels of total lipoproteins and increased levels of amino acids, glucose, lactate, creatine, and citrate. Secondary acute water avoidance stress also caused elevated levels of O-acetyl glycoproteins in blood plasma. LC-PUFAs dietary enrichment did not alter the metabolic response to stress, but did result in a modified lipoprotein profile. This work indicates that the different stressor types resulted in some common systemic metabolic responses that involve changes in energy and muscle metabolism, but that they are not reversible by dietary intervention.

Keywords: metabonomics • stress • blood plasma • lipoprotein • metabolites • long chain polyunsaturated fatty acid • LC-PUFAs dietary intervention • multivariate data analysis • 1H NMR spectroscopy • O-acetyl glycoprotein

Introduction

A growing awareness of the impact of diet and nutrition, combined with healthy lifestyle and efficient stress management, on the health and well-being of human populations has led to a rising number of experimental studies on the interactive effects of various dietary components and stresses. It has been reported that stress leads to elevation of hormones, such as the glucocorticoids and catecholamines. Stressors throughout life have been shown to play a major role in the vulnerability of rats to develop irritable bowel syndrome later in life and maladaptive responses to various conditions. Chronic neonatal stress in the form of moderate periods of maternal separation of newborn rats has been shown to play a major role in the vulnerability of rats to develop irritable bowel syndrome later in life and maladaptive responses to stressors throughout life.

Chronic psychological stress can be an initiating factor in intestinal inflammation. Although the hormone change induced by stressors has been studied, less attention has been paid to the systemic metabolic consequences of such interventions, although it has been hypothesized that certain dietary components have ameliorated effects. For example, it is well documented that an intake of polyunsaturated fatty acids (LC-PUFAs) are essential for good health.
addition of LC-PUFAs to the diet can possibly be helpful in preventing or delaying the effects some chronic diseases, such as, hypertension, cancer, diabetes, inflammatory and autoimmune disorders, atopic eczema, Alzheimer’s dementia, major depression, schizophrenia, and multiple sclerosis. Dietary enrichment of LC-PUFAs has been shown to be particularly effective in inflammatory bowel conditions. Understanding the overall metabolic effects of stress and dietary intervention with LC-PUFAs will undoubtedly enrich our current understanding of stress-induced diseases and provide insights into the beneficial effects of dietary intervention. However, such studies are difficult to conduct in human populations that encompass huge diversity of genetic backgrounds and environmental exposures, and therefore, animal models are often used in such studies.

Conventional biochemical approaches for assessing metabolic responses to stimuli are typically time-consuming and fragmentary (such as a series of targeted clinical assays). More recently, platforms capable of monitoring many parameters simultaneously, such as gene expression arrays, proteomic data, and metabolic profiles, have been favored for investigation of biochemical consequences of diet and diseases.

Metabonomics involves the study of the multivariate metabolic response of complex multicellular organisms to physiological and/or pathological stressors and the consequent disruption of system regulation. It is now well-established that NMR spectroscopy, coupled with appropriate data reduction techniques, offers a powerful approach to generating and analyzing high information density metabolic data on biofluids and tissues. This approach is capable of simultaneously detecting a wide range of small molecule metabolites, thus providing a wide ranging “metabolic fingerprint” of the major extracellular components that are in exchange with the blood and other biofluids and tissues. A series of multivariate statistical analyses, including linear projection methods, such as principal component analysis (PCA), orthogonal signal correction-projection to latent structure-discriminant analysis (O-PLS-DA), Bayesian probabilistic methods, and neural networks can all be applied to complex spectral data to aid visualization and characterization of changes relating to biological perturbations. NMR-based metabonomics has become a well-established analytical tool with successful applications in diverse fields, such as the study of disease processes, drug toxicity, detection of genetic disorders, and the study of mammalian-parasite interactions. This metabolic profiling strategy has also been applied to the investigation of the more subtle metabolic signatures of dietary interventions such as soy consumption, chamomile ingestion and alcohol intake. More recently, the use of directly coupled of HPLC–MS profiling techniques allied with multivariate data analysis has also been applied to biofluids to study drug toxicity and disease process, providing a complementary approach to the NMR technique. In this study, we have applied a NMR-based metabonomic approach to evaluate the systemic metabolic consequences of rats in response to maternal separation stress in isolation or followed by a secondary stressor (water avoidance) later in life. In addition, the effects of intervention with a high LC-PUFA diet on stressed rats were assessed.

Experimental Methods

Animal Handling. The models for stress used herein, namely maternal separation and water avoidance, are well-authenti-

### Table 1. Chemical Composition of Diets (g/100 g)

<table>
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<tr>
<th>ingredients</th>
<th>control diet</th>
<th>LC-PUFA-enriched diet</th>
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<tr>
<td>K-caseinate (87.5% protein)</td>
<td>20.00</td>
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<tr>
<td>Corn starch</td>
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<td>Sucrose</td>
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<tr>
<td>AIN-93A Mineral mix</td>
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<tr>
<td>AIN-93A Vitamin mix</td>
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<tr>
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<td>Cholinehydrogenate DAB 10</td>
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<td>Cellulose (Bulk)</td>
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<td>Fat mix</td>
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### Table 2. Treatment and Experimental Design

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</table>

* denotes no. + denotes yes. LC-PUFA: long chain polyunsaturated fatty acid rich diet.
alone or in un stressful animals due to lack of facilities at the time and the desire to minimize animal usage. This could form the basis of further studies. For the animals fed with the LC-PUFAs enriched diet, the dietary intervention was initiated after the weaning period (21 days) and continued until sacrifice. Animals were sacrificed at 3–4 months by decapitation and samples of blood plasma were taken immediately by collection from the throat into Li-heparin tubes and centrifuged at 2000 × g for 10 min at 5 °C. Plasma was divided into two aliquots and frozen at −80 °C until NMR analysis was performed.

**1H NMR Spectroscopy of Plasma.** Samples were prepared by mixing 200 µL of blood plasma with 400 µL of saline containing 10% D2O as a field frequency lock. All 1H NMR spectra were recorded on a Bruker DRX 600 NMR spectrometer (Bruker, Germany), equipped with a Bruker 5 mm triple resonance probe with inverse detection, operating at 600.13 MHz for 1H. Three types of 1H NMR spectra were acquired for each sample. First, a standard one-dimensional water peak presaturation pulse sequence (90°–t1–90°–t2–90°–acq) was applied. The inter-pulse delay t1 was 3 ms, and the mixing time t2 was 100 ms. A weak irradiation field was applied at the water resonance frequency during both the mixing time and the recycle delay. Second, water-suppressed Carr–Purcell–Meibom–Gill (CPMG)50 spectra were acquired using the pulse sequence [RD–90°–(r–180°–r)n–ACQ]. This acts as a T2 relaxation filter to suppress signals from macromolecules and other molecules with constrained molecular motions. A total spin–spin relaxation delay (2πr) of 160 ms was used for all samples and water signal irradiation was applied during the relaxation delay. Finally, water-suppressed diffusion-edited 1H NMR spectra were acquired to remove peaks from low molecular weight components by using the bipolar-pair longitudinal-eddy-current (BPP-LED) pulse sequence [RD–90°–G1–r–180°–G2–r–90°–Δ–90°–G3–r–180°–G4–r–90°–T1–90°–ACQ].51 where G represents a pulsed field gradient. A sineshaped gradient with strength of 32 G/cm and duration of 2.5 ms was used, followed by a delay (Δ) of 400 μs to allow for the decay of eddy currents. A diffusion time (Δ) of 100 ms and a delay T1 of 5 ms was used. Water suppression was applied during the recycle delay.

**Spectral Processing and Analysis.** For all 1D 1H NMR spectra, free induction decays were multiplied by an exponential function corresponding to a 0.3 Hz line-broadening prior to Fourier transformation and were automatically phased and baseline-corrected using an in-house developed MATLAB script (The MathWork inc.) (T. Ebbels, Imperial College London). The spectra were referenced to the anomic proton α-glucose resonance at δ 5.223. The spectra over the range δ 0.6–8.0 were digitized into 14 800 points using a MATLAB script developed in-house (O. Cloarec, Imperial College London). The region δ 4.65–5.15 was removed to avoid the residual spectral effects of imperfect water suppression. Normalization of the spectra to a constant sum was carried out on these data prior to pattern recognition analyses. Principal Components Analysis (PCA) was performed using mean centering with the Simca-P 10.0 software (Umetrics, Umeå, Sweden). Data were then visualized in the form of the PC scores plots and loadings plots. Each coordinate on the scores plot represents an individual sample and each coordinate on the loadings plots represents one NMR spectral region. Thus the loadings plots provide information on spectral regions responsible for the position of coordinates or clusters of samples in the corresponding scores plots.

**Figure 1.** 600 MHz 1H spin–echo NMR spectra of blood plasma from a control rat. A: A standard one-dimensional water presaturation 1H NMR spectrum; B: spin–echo 1H NMR spectrum; C: diffusion-edited 1H NMR spectrum. Keys: 1: β-glucose; 2: α-glucose; 3: lactate; 4: alanine; 5: creatine; 6: citrate; 7: glutamine; 8: O-acetyl-glycoproteins; 9: N-acetyl-glycoproteins; 10: valine; 11: leucine/isoleucine; 12: lipoprotein CH3(CH2)n; 13: lipoprotein CH3(CH2)n; 14: lipoprotein CH2CH2CO; 15: lipoprotein CH2C=C; 16: lipoprotein CH3CH2CO; 17: lipoprotein CH3CH2CH=C; 18: choline in phosphatidylcholine; 19: lipoprotein CH=CH2; 20: 3-α-hydroxybutyrate; 21: triglyceride; Insert: an expansion of the resonances from the methyl groups of lipoproteins indicating HDL, LDL, and VLDL.

**Results**

**1H NMR Spectroscopy of Plasma.** Examples of a typical standard one-dimensional 1H NMR spectrum, a spin–echo NMR spectrum (CPMG) and a diffusion-edited NMR spectrum of a plasma sample obtained from a 3–4 month old control rat are shown in Figure 1. The spectrum acquired with standard water presaturation alone, without any spectral editing (Figure 1A) includes signals from both small molecules and macromolecules and is generally dominated by lipoprotein composition. Resonance assignments were made according to the literature. In addition to the lipoprotein peaks, the spectra
contained numerous resonances from small molecule metabolites, including glucose, valine, leucine, isoleucine, lactate, acetate, creatine, citrate, 3-\textbf{D}-hydroxybutyrate, plus acetyl signals from \textit{N}-acetyl-glycoprotein and \textit{O}-acetyl-glycoprotein moieties with high motional freedom.\textsuperscript{53,54} The effect of the spin–echo sequence (Figure 1B) was to attenuate the spectral contributions from macromolecules, which have short spin–spin relaxation times. By using a total spin–spin relaxation delay (2\(\pi\)) of 160 ms, the relative intensities of those resonances from the large molecules such as lipoproteins, albumin and immunoglobulins were attenuated relative to those from small molecules.\textsuperscript{29,60} In contrast to the spin–echo sequence, the diffusion-edited pulse sequence effectively edits out low molecular weight components with high translational diffusion rates (Figure 1C), and the insert shows the region where methyl groups from fatty acyl chains in lipoproteins appear.

Differences in overall composition between plasma samples obtained from the control and treated groups were observed (data not shown). For example, reduced levels of lipids were observed in the plasma of rats from all groups in comparison with control and elevated levels of creatine, 3-\textbf{D}-hydroxybutyrylate, acetate, acetoacetate, and oxaloacetate were observed in rats exposed to water avoidance stress. Since visual analysis of the \(1^H\) NMR spectral profiles is a subjective process and inter-animal variation can easily distort interpretation of these data, multivariate data analysis of NMR spectra was performed in order to form a general overview of metabolite patterns of the effects of stresses and dietary intervention.

**Metabolic Effects of Early Maternal Separation.** PCA of the mean-centered normalized spin–echo NMR dataset indicated that maternally separated and control rats could be differentiated. However, since the both PC1 and PC3 contributed to the class separation, interpretation of the metabolites accountable for the separation from the corresponding loading plot is difficult. These data were further analyzed using the O-PLS-DA method that was developed in-house and has been described previously.\textsuperscript{33} This method incorporates back-transformed loadings of a unit variance scale model and variable weights and allows simplified interpretations of loadings in the same format as an NMR spectrum.\textsuperscript{32} Here, two orthogonal components and one PLS components were calculated using unit variance scaling and the resulting model explained 98\% of variance in the dataset (\(R^2\)) with a cross-validation of 0.42 (\(Q^2\)). The O-PLS-DA coefficient plot that illustrated metabolites responsible for the separation between plasma samples from the control rats and from rats that had been separated from their mother during days 2–14 after birth is shown in Figure 2. Here, the observed phase (positive or negative) of the resonance signals is determined by the relative concentration variation of metabolites in the stress treatment group with respect to the control group as calculated from the covariance matrix, thus giving the appearance of an NMR difference spectrum. The colors projected onto the spectrum are associated with the correlation of NMR data with the class of treatment, and give an indication of the significance of those metabolites in characterizing the metabolic effects of stress; with red corresponding to highly correlated (\(r > 0.6\)) and blue (\(r < 0.2\)) indicating no correlation with sample class. The coefficient suggests that the rats in the maternal separation group (B) displayed decreased levels of lipoproteins and triglycerides and elevated levels of amino acids, glucose, lactate, creatine and citrate in comparison with control rats (Table 3).

PC analysis of diffusion-edited NMR plasma spectra obtained a clear separation between the control and maternal separated rats in the second PC component (Figure 3). Although NMR resonances of lipoproteins including HDL, LDL, and VLDL are heavily overlapped, direct correlation between chemical shift and lipoprotein particle size, and density can provide some indication of changes in HDL, LDL, and VLDL concentration\textsuperscript{10} (Figure 3). Rat pups exposed to maternal separation in early life revealed decreases in relative concentration of choline and HDL together with an increase in relative concentration of VLDL in the plasma at 3–4 months in comparison with control animals.

**Effects of Water Avoidance and Double Stress.** PCA was carried out on the mean-centered normalized \(1^H\) spin–echo NMR spectra from plasma from the control rats (blue boxes), rats exposed to water avoidance stress only (red triangles) and rats that had undergone maternal separation followed by the secondary stressor, water avoidance (green diamonds) (Figure 4). Separation between both of the treatment groups and the control group was observed. Rats that had been subjected to double stressors (green diamonds) were more metabolically similar to the controls than those which were subjected to water avoidance stress only (red triangles). The metabolites accountable for the separation between both of the stressed groups and control were similar to the results obtained from the maternal separation treated group and included elevated plasma levels of 3-\textbf{D}-hydroxybutyrate, amino acids, glucose, lactate, citrate, creatine, \textit{O}-acetyl-glycoproteins and reduced levels of lipoprotein lipids. Inspections of NMR spectra con-
firmed this observation (Table 3). Diffusion-edited NMR spectra were also exploited to investigate modification of lipoproteins by the water avoidance stress and the double stress using PCA. The plasma obtained from rats subjected to water avoidance (red triangles) and double stressors (green diamonds) were differentiated from the controls (blue boxes) in the first PC component (Figure 5). Again rats that endured double stressor (green diamonds) were closer to the controls than those from the water avoidance stress alone (red triangles) and this is similar to the PCA pattern obtained from the spin-echo NMR spectra. The loadings plot suggested that plasma obtained from stressed animals contained relatively high concentrations of choline, acetyl-glycoproteins, HDL and relatively low concentrations of VLDL, triglyceride, and unsaturated lipids in comparison with control rats.

Effects of Dietary Intervention. PCA of 1H spin-echo NMR spectra of plasmas from control rats, rats treated with water avoidance and fed with normal diets and rats treated with water avoidance and fed with LC-PUFAs enriched diets was carried out. Samples from the rats exposed to water avoidance and fed with LC-PUFAs enriched diets were clustered with those from rats fed with normal diets in the scores plot derived from spin-echo NMR spectra. However, PCA of diffusion-edited NMR spectra suggested a separation existed in the plasma profile of rats treated with water avoidance (red triangles) and water avoidance but fed with LC-PUFAs enriched diets (indigo star) (Figure 6). In this model, one animal from the control group was removed due to the presence of high levels of lipids which introduced a high degree of leverage on the model. The water avoidance treated rats with and without dietary supplementation of LC-PUFAs were separated from the rats subjected to water avoidance and fed with normal diet in the second PC of the diffusion-edited spectra (Figure 6). The corresponding loading plot indicated that rats exposed to the water avoidance stress consistently contained relative higher levels of HDL compared to both the control rats and those rats that had been exposed to water avoidance and fed with LC-PUFAs diets.

Discussion

The primary aim of the present study was to investigate the effects of single and combined stresses including an early life stress in which rat pups were separated from their mothers daily for 12 days with duration of 180 min after birth and an acute water avoidance stress at 3–4 months. Both stresses caused depletion in the levels of total lipoproteins and elevated levels of amino acids, glucose, lactate, creatine, citrate in plasma, when compared to samples obtained from the control rats. Additional elevated intensity of O-acetyl glycoprotein signal was observed in plasma of rats subjected to water avoidance stress.53,54 It has been well documented that stress causes increases in the secretion of hormones such as glucocorticoids and catecholamines.3,5 Glucocorticoids stimulate gluconeogenesis, mobilize amino acids from protein, inhibit glucose uptake in muscle and adipose tissue, and stimulate lipolysis,9,56–60 resulting in elevated blood glucose and amino acids levels and depleted lipids. The fatty acids released by lipolysis are used for production of energy in muscle, and the released glycerol provides an alternative substrate for gluconeogenesis. The results presented here are, therefore, consistent with earlier studies investigating the metabolic response to stress. Lactate can act as a precursor of glucose in liver and elevated lactate could be used to provide glucose synthesis in the stressed...
The O-acetylated carbohydrate-bound protein resonance is absent from NMR spectra of human blood plasma but is found in rat blood plasma and could be considered as an alternative “acute-phase” glycoproteins in model animals of human inflammatory conditions. The concentrations of human plasma “acute phase” N-acetyl glycoproteins are known to be markedly elevated in a range of abnormal clinical conditions, e.g., inflammatory disease, cancer, certain liver diseases, and also during surgical trauma. These “acute phase” acetyl glycoproteins are predominantly synthesized in liver parenchymal cells in response to cytokines. Recently, work has demonstrated that psychological stress stimulates the production of pro-inflammatory cytokines which was in parallel with elevated levels of catecholamines and cortisol, for example in an academic examination situation. In the current study, we found that O-acetyl glycoproteins were present with higher signal intensity in the spectra of plasma from rats exposed to the water avoidance stress than those in the control group. Elevated of O-acetyl glycoproteins in blood plasma of stressed animals is consistent with previous investigations of the metabolic response to stress.

It is also interesting to note that rats exposed to water avoidance alone were separated further from the control rats than those exposed to both maternal separation and water avoidance (Figure 4 & 5). This could infer that rats had been previously stressed may have become desensitized to stress and would accommodate secondary stress better than those that had not been exposed to this first early life stressor. However, in contrast, Caldji et al. and Liu et al. suggested that adult offspring with low level of interactions with their mothers exhibited increased behavioral fearfulness in response to novelty and increased levels of adrenocorticotropic hormone and corticosterone response to acute stress.

In the current study, PC analysis of diffusion-edited spectra was able to provide extra information regarding the effects of the stresses and dietary intervention on changes of lipoprotein compositions. This technique has previously been applied to study liver tissues from hydrazine-dosed rats and aid the resonance assignments of biofluids. Although both stressors had the effect of reducing overall levels of lipoproteins, the modification of lipoproteins varied in relation to the different stresses induced. The relative concentrations of phosphatidylcholine in lipoproteins and HDL were reduced in plasma from rats exposed to maternal separation alone whereas they were increased in plasma from those exposed to water avoidance. The underlying reason is unclear. However, the water avoidance stress was carried out by placing rats on a platform in a container filled with water, which inevitably introduced some effects from physical stress as well as psychological effects, and which may provide an explanation for the discrepancy observed from the two stressors. Furthermore, decreased concentration of HDL in relation to stress have also been observed previously and appeared to be associated with the elevated level of stress hormones, which is consistent with the effects of maternal separation in the current study.

The second objective of the current study was to address the hypothesis that a LC-PUFAs enriched dietary intervention can reverse the effects of stress-induced metabolic changes. Despite a vast amount of research that has inferred beneficial aspects for the balanced intake of polyunsaturated fatty acids (LC-PUFAs), such as in the prevention and the control of chronic diseases, hypertension, cancer, diabetes, inflammatory, and auto-immune disorders, atopic eczema, Alzheimer de-

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**Figure 5.** PC1 vs PC2 scores plots (A) and corresponding loadings (B) plots from the 1H diffusion-edited NMR spectra of plasma obtained from control rats (blue box) and rats were subjected to both maternal separation and water avoidance stresses (green diamond) and water avoidance alone (red triangle). Insert: expansion region of the loading plot comparing the diffusion-edited spectrum (red line) and the loadings (black line). Two PC components were calculated with 95% of variances being explained. See Figure 1 for metabolite identification key.

**Figure 6.** PC1 vs PC2 scores plots calculated from the 1H diffusion-edited NMR spectra of plasma obtained from control rats (blue box), rats exposed to water avoidance alone (red triangle) and rats were subjected to water avoidance and fed with LC-PUFAs enriched diets (indigo star). A total of two components were calculated with 92.9% of the total variances being explained.
mentia, depression, schizophrenia, multiple sclerosis, etc.\textsuperscript{13,17,18} The LC-PUFAs enriched dietary intervention exploited in the current study was unable to reverse the metabolic effects stimulated by stresses. Nevertheless, the dietary intervention had a measurable metabolic effect on rats exposed to stress as suggested in Figure 6 that rats fed with LC-PUFAs diets followed by exposure to the water avoidance stress presented relatively higher levels of VLDL and lower levels of HDL than those exposed to water avoidance alone and without the LC-PUFAs diet. Beneficial effects of LC-PUFAs diet are not therefore apparent from this current study.

In conclusion, metabolic effects were observed for rats undergoing maternal separation stress during early life and a secondary acute stressor, water avoidance, from blood plasma obtained from rats using an NMR-based metabolomic strategy. Both stressors had similar effects in which stressed rats showed depleted levels of lipoprotein lipids and elevated levels of ketone bodies, amino acids, glucose, lactate, creatine, and citrate in plasma compared to the control rats. In addition, increased levels of O-acetyl glycoproteins were observed in water avoidance stressed rats. Furthermore, both stresses had the effect of modification of the lipoprotein compositions. These effects can be explained by elevated levels of stress hormones. The LC-PUFAs dietary intervention was unable to reverse the metabolic changes induced by stress, but altered them to some extent. This study has also highlighted the advantages of using a combination of NMR spectra editing methods with pattern recognition techniques.

**Abbreviations.** CPMG, Carr–Purcell–Meiboom–Gill; LC-PUFAs, polysaturated fatty acids; NMR, nuclear magnetic resonance; PCA, principal component analysis; O-PLS-DA, orthogonal-projection on latent structure-discriminant analysis; COSY, \( ^1\text{H}–^1\text{H} \) correlation spectroscopy; TOCSY, \( ^1\text{H}–^1\text{H} \) total correlation spectroscopy; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; HDL, high-density lipoprotein.

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**References**


