



# Intermittent fasting-induced biomolecular modifications in rat tissues detected by ATR-FTIR spectroscopy and machine learning algorithms

Taha Ceylani<sup>a</sup>, Hikmet Taner Teker<sup>b</sup>, Gizem Samgane<sup>c</sup>, Rafiq Gurbanov<sup>d,e,\*</sup>

<sup>a</sup> Department of Food Quality Control and Analysis, Muş Alparslan University, Muş, Turkey

<sup>b</sup> Department of Medical Biology, Ankara Medipol University, Ankara, Turkey

<sup>c</sup> Department of Biotechnology, Bilecik Şeyh Edebali University, Bilecik, Turkey

<sup>d</sup> Department of Bioengineering, Bilecik Şeyh Edebali University, 11230, Bilecik, Turkey

<sup>e</sup> Central Research Laboratory, Bilecik Şeyh Edebali University, 11230, Bilecik, Turkey

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## ABSTRACT

This study aimed to reveal the intermittent fasting-induced alterations in biomolecules of the liver, ileum, and colon tissues of rats using Support Vector Machine (SVM) and Linear Discriminant Analysis (LDA) algorithms developed on infrared spectrochemical data. LDA prediction accuracies were generally calculated in the range of 95–100%, while training and validation accuracies of SVM were in the range of 91–100% and 83–91%, respectively. The quantitative measurements of spectral bands at the CH (lipids), Amide (proteins), and PO<sub>2</sub> antisymmetric (nucleic acids) stretching regions were performed to monitor modulated metabolic processes. The concentration of biomolecules and phosphorylation rate of proteins were found higher in studied tissues. The altered conformations and low rates of carbonylation (oxidation) were also common in proteins. No significant change was recorded for the length of fatty acid acyl chains ( $A_{2922}/A_{2955}$  band area ratio) in the liver, whereas the shortening of acyl chains was calculated as 23% and 27% in ileum and colon tissues, respectively. Enhanced membrane dynamics ( $Bw_{2922}/Bw_{2955}$  bandwidth ratio) were depicted in the liver (35% increase), while a decline in dynamics was apparent in the ileum (36% decrease) and colon (31% decrease). The study revealed important alterations in major biomolecules of studied tissues.

## 1. Introduction

Intermittent fasting (IF) is one of the dietary approaches that involves the restriction of regular food intake. Preventive roles of IF against metabolic illnesses, aging, as well as cardiovascular and neurological diseases have recently been recognized by the scientific community. IF diet is suggested as a prophylactic and therapeutic intervention for various chronic diseases [1]. Many studies have been conducted focusing on fasting practices, revealing the beneficial effects of various fasting practices on both the metabolism and cognitive status of the person [2]. IF, lasting from 16 to 48 h, is seen as one of the most preferred fasting practices but there are also certain discussions on possible negative effects. However, with recent pieces of evidence IF might be both safe and highly effective for health [3,4]. One of the most striking effects of IF might be healthy weight loss, but recent studies also find out many other beneficial effects at the cellular level [5–7]. Because of a calorie restriction for a certain period, there is also a healthy

decrease in blood sugar levels. Accordingly, the blood insulin level diminishes, which may lead to the prevention of insulin resistance during metabolic diseases [8–10].

Fatty liver disease is a very common condition in modern societies, and it can be a very important risk factor for many diseases such as diabetes, liver cirrhosis, cardiovascular diseases, and cancer [11]. It is observed that the rate of fat in the liver begins to decline rapidly after metabolism enters the fat-burning mode during IF [12,13]. One of the most striking features of fasting may be its ability to suppress inflammation, which is one of the most important risk factors for many diseases, from cancers to heart disease [14]. Recent shreds of evidence have shown that fasting significantly reduces indicators of inflammation such as C-reactive protein and interleukin-6 in the circulatory system [15]. In addition, IF provides significant protection against cardiovascular diseases by suppressing blood sugar, unhealthy cholesterol, and inflammation [16,17]. Also, significant normalizations in blood pressure are observed in many patients who are included in the IF program due to

\* Corresponding author. Department of Bioengineering, Bilecik Şeyh Edebali University, 11230, Bilecik, Turkey.

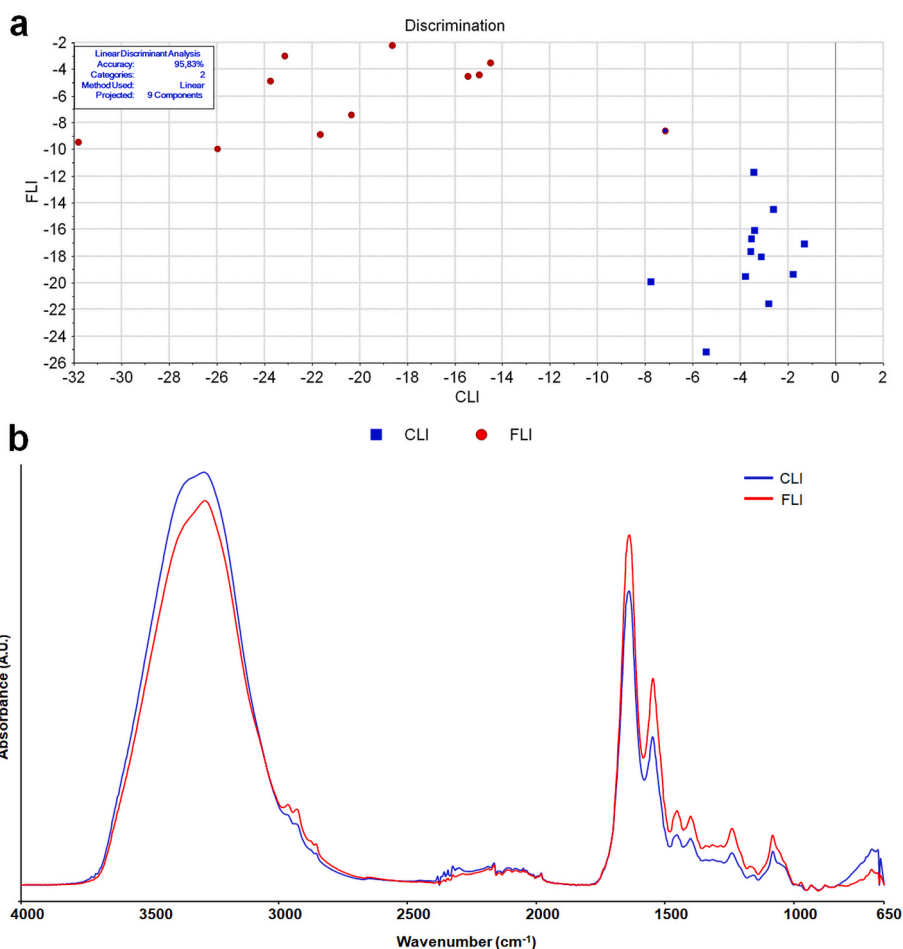
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**Fig. 1.** Intermittent fasting affects the whole biomolecular profile in the liver samples. a) LDA discrimination plot and b) baseline-corrected average spectra in full (4000–650  $\text{cm}^{-1}$ ) spectral region. CLI (control rats), FLI (rats on intermittent fasting).

obesity and diabetes [18–20].

Recent studies revealed that the most crucial effect of IF may be its role in the initiation and progression of the autophagy cascade. With the autophagy process, aged proteins, organelles, and also damaged DNA is digested and degraded to be used as an energy source and replaced with newly generated ones [21]. The ability of IF to balance sugar and cholesterol metabolism and suppress inflammation is also reflected in the brain as well as in all other organs. Brain-derived neurotrophic factor (BDNF) begins to be secreted in the brain after fasting for a certain period. As it is known, BDNF has very crucial functions in the regeneration of brain cells [22–24]. Another important impact of IF is its possible role in cancer protection [25]. It has been shown that there is an increase in the level of certain so-called “anticancer proteins” in the blood during fasting exceeding 4 weeks. These substances slow down the reproduction of cancer cells and also increase the sensitivity of cancer cells to chemotherapy [26].

Fourier Transform Infrared (FTIR) Spectroscopy, which detects molecule vibrations and generates molecular spectral bands in the mid-infrared region, is a technique used to gather broad-spectrum data in a rapid, easy, and non-invasive manner in biological analyses [27–29]. The Attenuated Total Reflection (ATR) mode of FTIR spectroscopy is a useful tool for studying biological samples [30–32]. Chemometrics is a branch of chemistry that covers the computer-assisted analysis of chemical data, as well as statistics and mathematics. The appeal of these chemometric approaches is due to their ability to provide flexible and adaptable solutions for obtaining rapid, accurate, precise, and trustworthy findings in the analysis of complicated materials. Therefore, it encompasses a range of disciplines, including descriptive and inferential

statistics, signal processing, experimental design, modeling, calibration, optimization, pattern recognition, classification, artificial intelligence approaches, image processing, and information and system theory. Chemometric approaches (multivariate pattern recognition methods or machine learning approaches) are used for the mining and classification of data gathered by a variety of analytical procedures in fields such as analytical chemistry, biology, archaeology, as well as clinical and forensic medicine [33].

Studies on fasting practices show that the hunger period is as important as the satiety condition for human metabolism. It seems that human metabolism can show the ability to turn the hunger period in its favor. Many studies have mentioned the numerous effects of fasting on regeneration. The regeneration that starts at the cellular level as a result of autophagy is then reflected in all tissues and organs [21]. However, the initiation of autophagy requires a certain period of starvation. Therefore, the time interval of daily food restriction in the fasting program must be long enough to initiate the autophagy process. Although fasting for 16 h does not normally result in ketosis, it is sufficient to encourage many pathways associated with prolonged fasting such as autophagy [34]. Because of the crucial role of the liver and intestine in the regulation of metabolism, digestion, also host-microbiome interaction; this study aimed to investigate the impact of 18-h IF administered for 5 weeks on the liver, ileum, and colon tissues of rats. For this purpose, two different supervised machine learning techniques (Support Vector Machine/SVM and Linear Discriminant Analysis/LDA) were applied to big data obtained from FTIR spectral measurements of tissues. The quantitative measurements of FTIR spectral bands specific to lipids, proteins, and nucleic acids and their ratio indices were also calculated

**Table 1**

SVM classification for liver samples in full (4000–650  $\text{cm}^{-1}$ ) spectral region. CLI (control rats), FLI (rats on intermittent fasting). SVM type: Classification (nu-SVC). Method: Radial Basis Function. \* Highlights the wrong classification.

Accuracy (%)			
Training	91.66	Validation	87.50
Classification			
Samples	Class		
CLI1	1	1	CLI
CLI2	2	2	CLI
CLI3	3	3	CLI
CLI4	4	4	CLI
CLI5	5	5	CLI
CLI6	6	6	CLI
CLI7	7	7	CLI
CLI8	8	8	CLI
CLI9	9	9	CLI
CLI10	10	10	CLI
CLI11	11	11	CLI
CLI12	12	12	CLI
FLI1	13	13	FLI
FLI2	14	14	FLI
<b>FLI3</b>	<b>15</b>	<b>15</b>	<b>*CLI</b>
FLI4	16	16	FLI
FLI5	17	17	FLI
FLI6	18	18	FLI
FLI7	19	19	FLI
FLI8	20	20	FLI
FLI9	21	21	FLI
FLI10	22	22	FLI
FLI11	23	23	FLI
FLI12	24	24	FLI

for the monitoring of modulated metabolic processes at the biomolecular level. Both quantitative data analyses and machine learning approaches effectively enabled rapid and accurate detection of IF-induced biomolecular changes.

## 2. Materials and methods

### 2.1. Animal studies

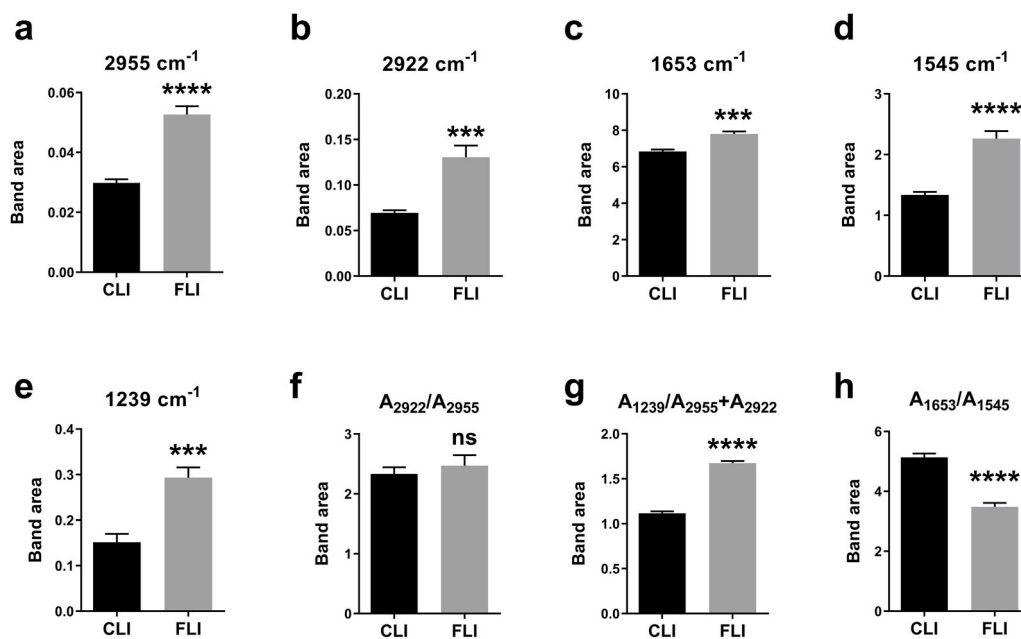
The male Wistar rats (12-month-old) were used as a model organism in the study. IF was applied to the rats ( $n = 6$ ) in the IF experimental group for 35 days. While the rats in the IF group were always able to drink water, their access to food was restricted for 18 h, and they were only allowed to feed for 6 h. The food access interval of the animals in the IF group was determined to be between 09:00 a.m. and 03:00 p.m. The control group ( $n = 6$ ) was allowed access to water and food for 24 h. The animals were fed a standard rodent diet on an *ad libitum* basis [35]. The body weight of the animals, the feed, and the consumed water were followed for 35 days. The blood glucose levels were also measured at the end of the application. One day after the end of the application, the animals in the control and IF groups were slightly stunned by treatment with ether and sacrificed. The extracted tissues were shocked on dry ice and left in the  $-80\text{ }^{\circ}\text{C}$  deep freezer until the time to be studied. All animals were housed under standard animal care conditions. The study was carried out with the approval of the Ethics Committee (approval number: 2021/05) from the Saki Yenilli Experimental Animal Production and Practice Laboratory.

### 2.2. Analysis of samples by Attenuated total reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy

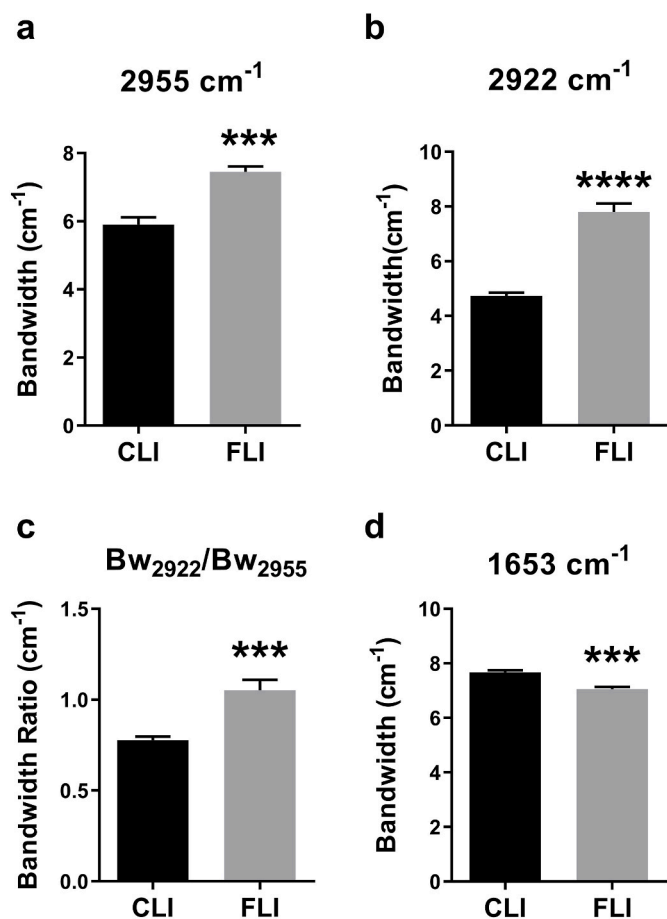
Liver, ileum, and colon tissues of all animals were compressed on the Zn/Se crystal of the ATR unit (PerkinElmer) without any pretreatment and each tissue was examined twice with an ATR-FTIR spectrometer (PerkinElmer) at a resolution of  $4\text{ cm}^{-1}$  and a scan number of 32. The spectra were obtained with the Spectrum One (PerkinElmer) software in the wavelength range of  $4000\text{--}650\text{ cm}^{-1}$  [27].

### 2.3. Prediction studies with different machine learning approach based on big spectral data

Linear Discriminant Analysis (LDA), a machine learning approach, was applied to differentiate the experimental groups from each other.



**Fig. 2.** The changes in the FTIR spectral band areas for liver samples. The area values of a)  $\text{CH}_3$  antisymmetric ( $2955\text{ cm}^{-1}$ ), b)  $\text{CH}_2$  antisymmetric ( $2922\text{ cm}^{-1}$ ), c) Amide I ( $1653\text{ cm}^{-1}$ ), d) Amide II ( $1545\text{ cm}^{-1}$ ), e)  $\text{PO}_2$  antisymmetric ( $1239\text{ cm}^{-1}$ ) bands. The indices for f) acyl chain length of fatty acids ( $A_{2922}/A_{2955}$ ), g) protein phosphorylation ( $A_{1239}/A_{2955}+A_{2922}$ ), and h) protein conformation ( $A_{1653}/A_{1545}$ ). CLI (control rats), FLI (rats on intermittent fasting), A (Absorbance).



**Fig. 3.** The changes in the FTIR spectral bandwidths for liver samples. The bandwidth values of a) CH<sub>3</sub> antisymmetric (2955 cm<sup>-1</sup>), b) CH<sub>2</sub> antisymmetric (2922 cm<sup>-1</sup>), c) Bw<sub>2922</sub>/Bw<sub>2955</sub> ratio index, and d) Amide I (1653 cm<sup>-1</sup>). CLI (control rats), FLI (rats on intermittent fasting), Bw (Bandwidth).

Spectral data were used in pattern recognition analysis. To make the analyzes as independent as possible from the FTIR spectrometers, each sample spectrum was preprocessed on The Unscrambler® X 10.3 (CAMO Software AS, Norway) software with a baseline offset transformation in the 4000–650 cm<sup>-1</sup> region. Spectra processed in this way were first subjected to unsupervised Principal Component Analysis (PCA) [36–38]. Spectra were passed from standard deviation normalization (mean centering normalization) and full-cross random validation. Subsequently, the spectra were examined in lipid (3000–2700 cm<sup>-1</sup>), protein (1700–1500 cm<sup>-1</sup>), nucleic acid (1200–650 cm<sup>-1</sup>), and full (4000–650 cm<sup>-1</sup>) regions by Singular Value Decomposition (SVD) algorithm.

LDA is a supervised classifier in which n-dimensional feature samples are linearly transformed into an m-dimensional space. PCA only uses sample spectra to determine the transformation, while LDA also uses class information in training samples leading to better classification. PCA data were used as LDA model inputs with The Unscrambler® X 10.3 (CAMO Software AS, Norway) multivariate analysis (MVA) software. The category variable column was included in a data matrix and then all spectra of different sample categories were used to generate a training set. The linear method using the projections of the 9 PCA components was used for the prediction. Prior probabilities were calculated from the training set. The results are presented as a discrimination plot, as well as

prediction and confusion matrices [39,40].

Support Vector Machine (SVM) is another very popular machine learning approach. SVM classification method was accomplished via The Unscrambler® X 10.3 (CAMO Software AS, Norway) multivariate analysis (MVA) software. All spectra were pre-processed as explained above and subsequently different sample categories were used to generate a training set. Classification (nu-SVC) was chosen as SVM type using a linear method as Kernel type. Nu value was set to 0.5, weights as all 1.00. The 9 segments of cross-validation were used in the calculation of training and cross-validation accuracies. Finally, the generated training dataset was applied to all sample datasets to obtain an SVM classification model.

#### 2.4. Quantification studies of FTIR spectral bands

Spectral data analysis was performed using OPUS 5.5 (Bruker) software. The average spectra of 2 replicates from each sample were baseline corrected using the Rubberband correction method with 128 baseline points prior to the band quantification analyses. In detailed band analyses, the bands with the highest absorbance values in different spectral regions of the spectra were selected and the beginning and ending frequencies of the bands were determined with precision. The areas of bands specific to various biomolecules were analyzed by taking the integral areas of the determined frequency ranges with the OPUS 5.5 (Bruker) software. In addition, a virtual line was drawn vertically from the midpoint of the band baseline to the peak of the band, and the length of the line was measured with the help of a virtual ruler. Then, by marking the point where 0.75 times the length of the line coincides with the line, a horizontal line was drawn along the band from this point and bandwidth values were obtained.

#### 2.5. Statistics

Statistical evaluations and graph plots of the results were made using GraphPad Prism 6.01 (GraphPad, USA). The data were analyzed using an unpaired *t*-test, and the significance levels were stated as  $P \leq 0.05$  \*,  $P \leq 0.01$  \*\*,  $P \leq 0.001$  \*\*\*, and  $P \leq 0.0001$  \*\*\*\*. Results are presented as mean  $\pm$  SEM (standard error of the mean).

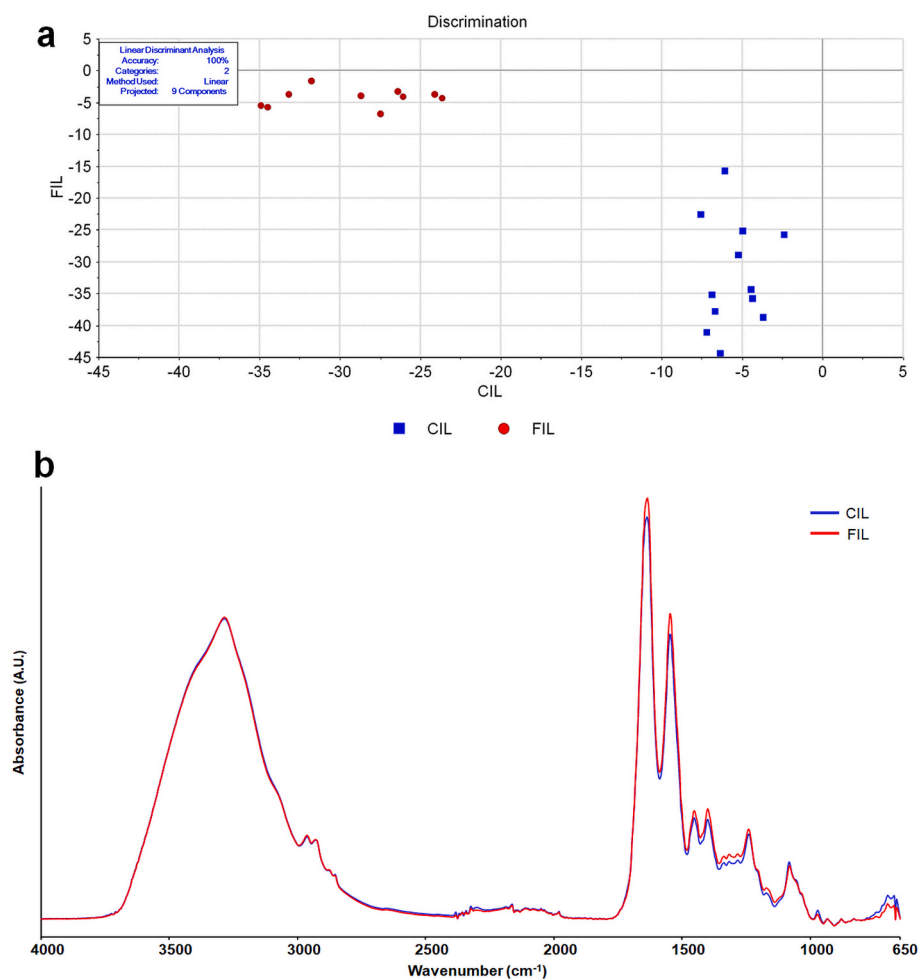
### 3. Results

#### 3.1. Intermittent fasting affects the water and feed consumption of animals

The body weights of the rats in the IF group did not change significantly ( $P = 0.795$ ). However, there was a significant increase in the body weights of the rats in the control group ( $P \leq 0.001$ ). The difference between the control and IF groups was also significant ( $P \leq 0.0001$ ) (Fig. S1a). There was also a significant difference in food ( $P \leq 0.0001$ ) and water consumption ( $P \leq 0.0001$ ). In the days after the application started, the rats in the IF group tended to eat and drink more as time went on (Figs. S1b–c). At the end of the application, the blood glucose levels of rats were measured. While the glucose values recorded in the control group were seen as more stable, there was no significant change between the two groups ( $P = 0.25$ ) (Fig. S1d).

#### 3.2. Intermittent fasting affects lipid, nucleic acid, and protein profiles in the liver

When the spectra of samples taken from the liver tissue at the end of the application were examined by the LDA method, there was a significant differentiation in terms of the whole biomolecular profile between



**Fig. 4.** Intermittent fasting affects the whole biomolecular profile in the ileum samples. a) LDA discrimination plot and b) baseline-corrected average spectra in full (4000–650  $\text{cm}^{-1}$ ) spectral region. CIL (control rats), FIL (rats on intermittent fasting).

the control and IF groups, with a high accuracy rate (95.83% for whole biomolecular profile) (Fig. 1a, Tables S1–S2). As seen in the LDA discrimination plot, the data for the control and fasting groups are clustered in completely different places (Fig. 1a). This indicates the absolute effect of IF on liver tissue. We observed the same effect when we examined the lipid profiles of the liver (100% for lipids) (Fig. S2, Tables S3–S4). As shown in Fig. S2, the data obtained from the control and IF groups in terms of lipids are located in completely different places. This IF-induced differentiation was similarly realized in nucleic acid and protein profiles of the liver with the highest accuracies (100% for nucleic acids, 100% for proteins) (Figs. S3–S4, Tables S5–S8). When Fig. S3 and Fig. S4 are examined, it is seen that the data obtained for both nucleic acid and protein profiles are located in completely different clusters, and IF has a significant effect on these parameters. A comparable classification was obtained with the SVM method with 91.66% training and 87.50% cross-validation accuracies, for the whole biomolecular profile of liver tissues (Table 1).

Average spectra (full infrared region/4000–650  $\text{cm}^{-1}$ ) for liver samples of control and IF groups demonstrate visible changes in many spectrochemical bands, each associated with specific functional groups of biomolecules (Fig. 1b). According to Beer-Lambert law, the intensity and/or the area of the infrared absorption bands rising from a particular

functional group of the relevant molecule is directly proportional to the concentration of that molecule. Therefore, we used areas under the bands to obtain relative concentration information about biomolecules [41]. To monitor the possible origin of IF-associated differentiation in the liver, the changes in FTIR spectral bands related to different functional groups in biomolecules were calculated. Accordingly, the spectral parameters such as band areas, band area ratios, and bandwidths were analyzed for the elucidation of molecular modifications in lipids, proteins, and nucleic acids. The analyses mainly covered the bands at 2955  $\text{cm}^{-1}$  ( $\text{CH}_3$  antisymmetric stretching: lipids and proteins), 2922  $\text{cm}^{-1}$  ( $\text{CH}_2$  antisymmetric stretching: lipids), 1653  $\text{cm}^{-1}$  (Amide I:  $\alpha$ -helical structure of proteins), 1545  $\text{cm}^{-1}$  (Amide II:  $\beta$ -sheet structure of proteins), and 1239  $\text{cm}^{-1}$  ( $\text{PO}_2$  antisymmetric stretching: nucleic acids) positions. The band areas were significantly increased (76% in 2955  $\text{cm}^{-1}$ , 88% in 2922  $\text{cm}^{-1}$ , 14% in 1653  $\text{cm}^{-1}$ , 69% in 1545  $\text{cm}^{-1}$ , and 93% in 1239  $\text{cm}^{-1}$ ) for liver tissues of IF group (Fig. 2a–e).

The acyl chain length of fatty acids can be calculated by band area ratio  $A_{2922}/A_{2955}$ . However, no significant changes were recorded in the liver of rats subjected to IF (Fig. 2f). In the literature, the band area ratio  $A_{1239}/A_{2955}+A_{2922}$  is usually referred to as the protein phosphorylation index. This index was found to be increased by 50% in the liver of rats subjected to IF, indicating high concentrations of phosphorylated

**Table 2**

SVM classification for ileum samples in full (4000–650  $\text{cm}^{-1}$ ) spectral region. CIL (control rats), FIL (rats on intermittent fasting). SVM type: Classification (nu-SVC). Method: Linear. \* Highlights the wrong classification.

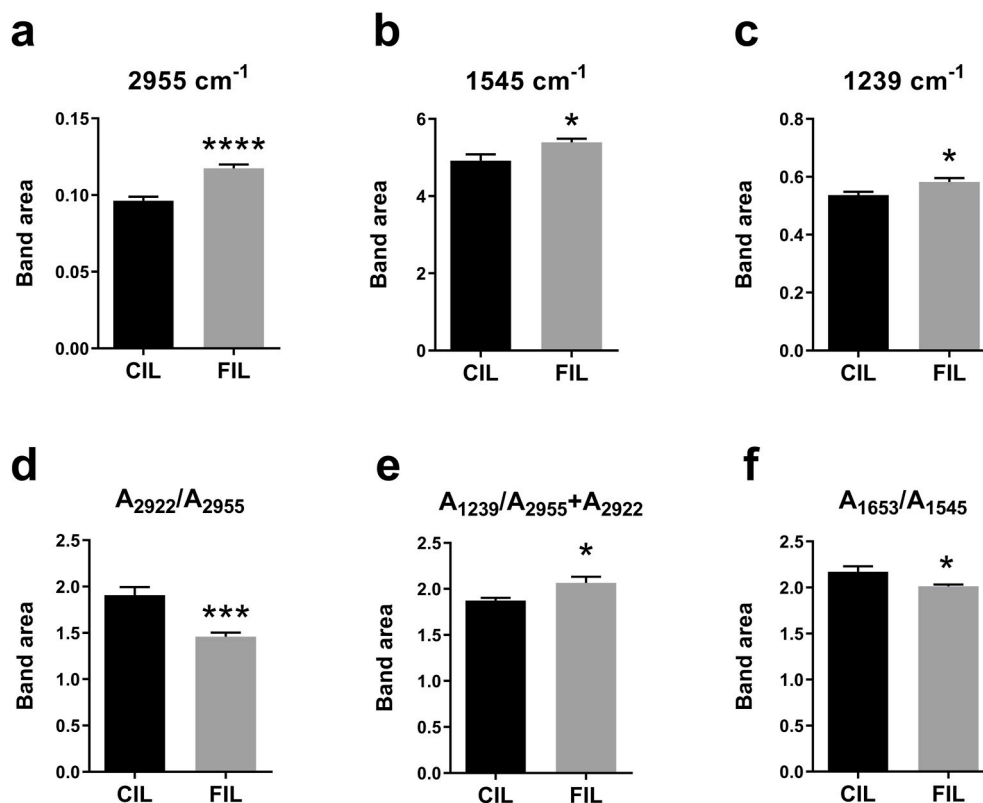
Accuracy (%)			
Training	100	Validation	83.33
Classification			
Samples		Class	
CIL1	1	1	CIL
CIL2	2	2	CIL
CIL3	3	3	CIL
CIL4	4	4	CIL
CIL5	5	5	CIL
CIL6	6	6	CIL
CIL7	7	7	CIL
CIL8	8	8	CIL
CIL9	9	9	CIL
<b>CIL10</b>	<b>10</b>	<b>10</b>	<b>*FIL</b>
CIL11	11	11	CIL
CIL12	12	12	CIL
FIL1	13	13	FIL
FIL2	14	14	FIL
FIL3	15	15	FIL
FIL4	16	16	FIL
FIL5	17	17	FIL
FIL6	18	18	FIL
FIL7	19	19	FIL
FIL8	20	20	FIL
FIL9	21	21	FIL
FIL10	22	22	FIL
FIL11	23	23	FIL
FIL12	24	24	FIL

proteins (Fig. 2g). The reduction (67%) in area ratio  $A_{1653}/A_{1545}$ , an indicator of protein conformation, was also seen in the liver of the IF group, pointing out conformational alterations in liver proteins (Fig. 2h).

The bandwidth of  $\text{CH}_2$  antisymmetric stretching bands gives information about membrane dynamics since it is related to the motion rates of the lipid molecule [42]. The increase in the bandwidths of these bands implies an increase in membrane dynamics. It was also reported that the disruption of membrane organization is directly associated with the decrease in membrane fluidity [43]. Therefore, the changes in membrane dynamics were assessed through the calculation of bandwidths of 2955  $\text{cm}^{-1}$  and 2922  $\text{cm}^{-1}$  bands, and the most reliable  $Bw_{2922}/Bw_{2955}$  ratio index. It was estimated that IF elevates the membrane dynamics (35% in  $Bw_{2922}/Bw_{2955}$  bandwidth ratio) in liver tissues (Fig. 3a–c). Broadening of Amide I band i.e., an increase in bandwidth of band at 1653  $\text{cm}^{-1}$  is an indicator of protein carbonylation. The formation of additional carbonyls on some amino acid residues results from protein oxidation [42]. IF lowered the protein carbonylation (oxidation) index in liver tissues by 9% (Fig. 3d).

### 3.3. Intermittent fasting affects lipid, nucleic acid, and protein profiles in the ileum

The effect of IF was seen when the spectra of samples obtained from the intestinal tissue's ileum region were examined using the LDA method (Fig. 4a, Tables S9–S10). The differentiation of groups in terms of the whole biomolecular profile was calculated with a higher degree of accuracy than that of liver tissue, and the data of control and IF groups are clustered in completely different places (100% for the whole biomolecular profile). This difference in accuracy rate was also reflected in the distribution, and the effect of IF on the ileum occurred with greater clarity. When the data distribution was examined in terms of lipid,



**Fig. 5.** The changes in the FTIR spectral band areas for ileum samples. The area values of a)  $\text{CH}_3$  antisymmetric (2955  $\text{cm}^{-1}$ ), b) Amide II (1545  $\text{cm}^{-1}$ ), c)  $\text{PO}_2$  antisymmetric (1239  $\text{cm}^{-1}$ ) bands. The indices for d) acyl chain length of fatty acids ( $A_{2922}/A_{2955}$ ), e) protein phosphorylation ( $A_{1239}/A_{2955}+A_{2922}$ ), and f) protein conformation ( $A_{1653}/A_{1545}$ ), CIL (control rats), FIL (rats on intermittent fasting), A (Absorbance).

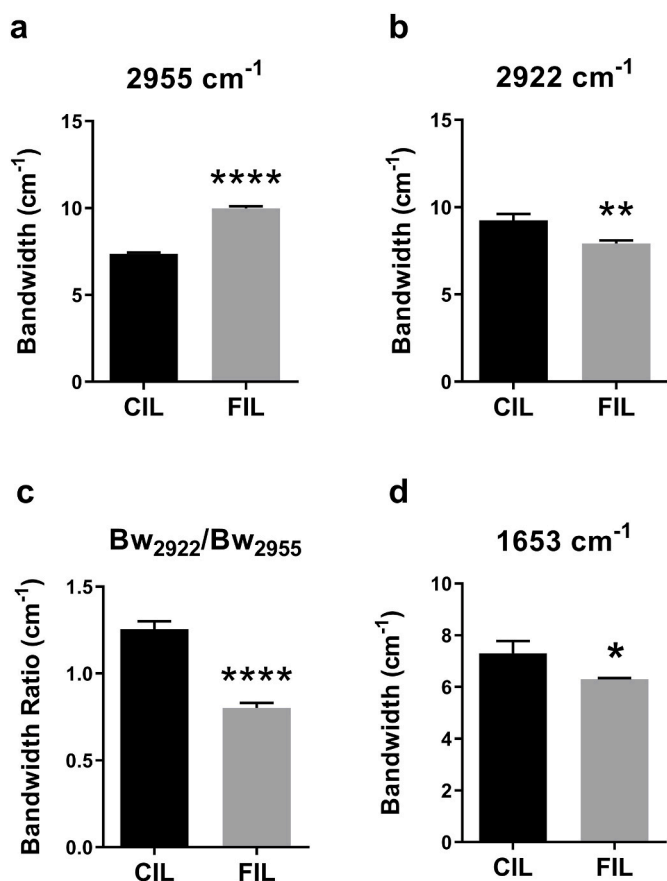


Fig. 6. The changes in the FTIR spectral bandwidths for ileum samples. The bandwidth values of a) CH<sub>3</sub> antisymmetric (2955 cm<sup>-1</sup>), b) CH<sub>2</sub> antisymmetric (2922 cm<sup>-1</sup>), c) Bw<sub>2922</sub>/Bw<sub>2955</sub> ratio index, and d) Amide I (1653 cm<sup>-1</sup>). CIL (control rats), FIL (rats on intermittent fasting), Bw (Bandwidth).

nucleic acid, and protein profiles with the same highest accuracies (100% for all these biomolecules), the deepest differentiation occurred for lipids, then for nucleic acids and proteins, respectively (Figs. S5–S7, Tables S11–S16). Similar to liver samples, there was a sharper differentiation for lipids, while a similar differentiation for nucleic acids and proteins was depicted in ileum samples. In the case of the SVM method conducted for the whole biomolecular profile of ileum tissues, the accuracies for training and cross-validation were revealed as 100% and 83.33%, respectively (Table 2).

Infrared spectra of ileum samples show the main differences between control and IF groups (Fig. 4b). However, the given full infrared region (4000–650 cm<sup>-1</sup>) does not visualize all the subtle changes. According to quantitative band analyses; IF led to significant increments in marker bands specific to lipids (22% in CH<sub>3</sub> antisymmetric at 2955 cm<sup>-1</sup>), proteins (10% in Amide II at 1545 cm<sup>-1</sup>), and nucleic acids (8% in PO<sub>2</sub> antisymmetric at 1239 cm<sup>-1</sup>) (Fig. 5a–c). In addition, IF caused a 23% decline in A<sub>2922</sub>/A<sub>2955</sub> band area ratio index demonstrating shorter acyl chains in fatty acids (Fig. 5d). Significant modifications were also measured in the band area indices for protein phosphorylation (10% increase in A<sub>1239</sub>/A<sub>2955</sub>+A<sub>2922</sub>) and protein conformation (7% decrease in A<sub>1653</sub>/A<sub>1545</sub>) (Fig. 5e–f).

In contrast to the increase in liver tissue, the Bw<sub>2922</sub>/Bw<sub>2955</sub> bandwidth ratio index for membrane dynamics was diminished (36%) in the

ileum (Fig. 6a–c). As in the liver, the protein carbonylation (oxidation) index which is the bandwidth of 1653 cm<sup>-1</sup> was found to be decreased by 14% in the ileum (Fig. 6d). Although many biomolecular changes in the ileum and liver of rats are similar, it is obvious that the membrane fluidity parameters (membrane dynamics values) change in the opposite direction.

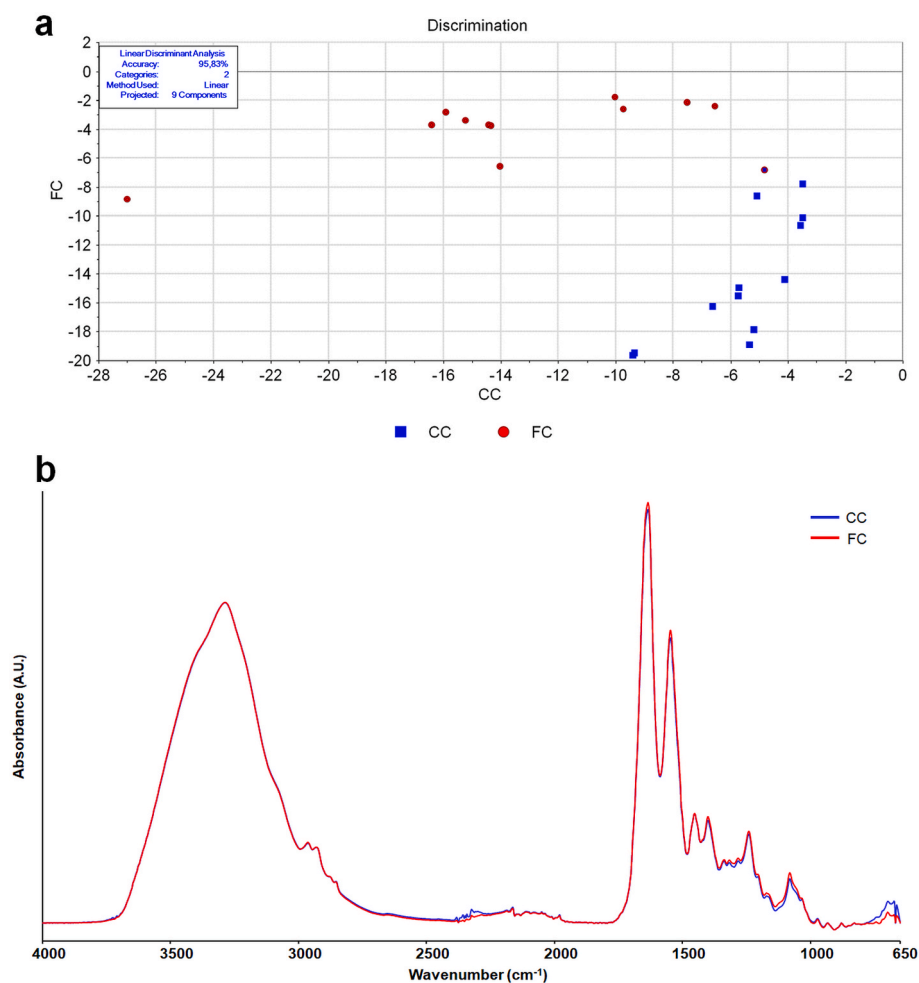
#### 3.4. Intermittent fasting affects lipid, nucleic acid, and protein profiles in the colon

The effect of IF was also significant on colon tissues, as demonstrated by LDA. The effect of IF on the whole biomolecular profile of the colon was prominent with high accuracy (95.83%) and adequate separation rates (Fig. 7a, Tables S17–S18). When the impact of IF was evaluated for lipid and nucleic acid profiles of the colon; the differentiation occurred with the highest accuracy rates (100%) were similar to the lipid and nucleic acid profiles of the liver and ileum (Figs. S8–9, Tables S19–S22). In terms of the protein profile, the differentiation was detected with a lower accuracy rate (75%) (Fig. S10, Tables S23–S24). When all the tissues are evaluated; the effect of IF was most evident on lipid profiles, while its effect on protein profiles followed a more interesting and unique course. The SVM method revealed 95.83% training and 91.66% cross-validation accuracies for the whole biomolecular profile of the colon tissues (Table 3).

The full region infrared spectra and spectral band parameters for colon biomolecules are given in Figs. 7b and Figs. 8–9, respectively. Similar to other tissues, the increased concentrations of lipids (29% in CH<sub>3</sub> antisymmetric at 2955 cm<sup>-1</sup>), proteins (13% in Amide II at 1545 cm<sup>-1</sup>), and nucleic acids (19% in PO<sub>2</sub> antisymmetric at 1239 cm<sup>-1</sup>) were calculated in colon tissues (Fig. 8a–c). The shorter acyl chain length of fatty acids was also measured for the colon (27% diminish in A<sub>2922</sub>/A<sub>2955</sub> band area ratio index), as in the ileum (Fig. 8d). The changes in other important indices such as protein phosphorylation (34% increase in A<sub>1239</sub>/A<sub>2955</sub>+A<sub>2922</sub> ratio) and protein conformation (8% decrease in A<sub>1653</sub>/A<sub>1545</sub> ratio) were also depicted (Fig. 8e–f). While the membrane dynamics parameter (Bw<sub>2922</sub>/Bw<sub>2955</sub> bandwidth ratio index) of the colon decreases by 31% similar to the ileum (36%), it shows an opposite change with the liver (35%) (Fig. 9a–c). The 14% diminish in protein carbonylation (oxidation) index in colon samples was comparable with other tissues (Fig. 9d).

## 4. Discussion

Biological stress like IF improves metabolic health and extends lifespan by activating stress resistance pathways, triggering weight loss, and reducing blood pressure, LDL, cholesterol, and triglyceride levels. It also systematically reduces fasting insulin levels, insulin resistance, and inflammation, further decreasing oxidative stress at cellular levels [44]. Not only animal models, but also dozens of human trials are demonstrated reductions in body weight and metabolic disease risk parameters. The beneficial effect is observed at a cellular level initiating from gene transcription to protein translation and post-transcriptional modifications [44–46]. The liver is a key player in providing metabolic homeostasis for all other organs. IF induced serious increments in lipid, protein, and nucleic acid biomolecules of the liver, indicating crucial changes in hepatocellular metabolism. As it is known, autophagy begins at the cellular level when the subjects do not consume calories for at least 16 h. Autophagy enables the lysosomes to digest the old proteins, organelles, and damaged DNA in certain periods, and make new molecules while using digested ones. Prolonged fasting elevated the endogenous synthesis of molecules with antioxidant properties that function to



**Fig. 7.** Intermittent fasting affects the whole biomolecular profile in the colon samples. a) LDA discrimination plot and b) baseline-corrected average spectra in full (4000–650  $\text{cm}^{-1}$ ) spectral region. CC (control rats), FC (rats on intermittent fasting).

reduce free radicals and thus improve metabolism [47]. Several human studies also revealed positive effects of IF on concentrations of various lipid molecules circulating in blood [48]. A recent meta-analysis and systematic review study illustrated the potential of the IF diet in reducing lipid oxidative stress parameters and increasing total antioxidant capacities in humans [49]. Numerous animal and human studies have indicated an oxidative stress-reducing role of IF interventions in various metabolic disorders [50]. An elaborate metabolic response to fasting is orchestrated by the liver and is largely dependent on transcriptional regulation. Many transcription factors are activated in response to glucagon and glucocorticoid hormones and regulate various genes involved in gluconeogenesis, fatty acid oxidation, ketogenesis, and amino acid metabolism [51]. Targeted metabolomics analysis of liver metabolites in mice subjected to IF revealed a >1.5-fold increase in 12 molecules (PEP, citrate, succinate, NAD, NADH, NADPH, ATP, etc.) involved in glycolysis, tricarboxylic acid cycle, and oxidative phosphorylation which are central biochemical pathways in mammalian energy metabolism [52]. The intestinal epithelium is a rapidly renewing barrier of the body and the first-line sensor for dietary nutrients. The constant intestinal epithelial adaptation is required in response to stresses such as fasting stimuli alongside the changing nutrient stimuli. It

is recognized that fasting has a crucial role in intestinal health and disease through endogenous metabolites as well as hormones and growth factors [53].

According to the findings of this study; the IF diet initiates serious and mostly beneficial molecular modifications in the liver, ileum, and colon tissues of rats. Moreover, some unique biological consequences of IF were revealed depending on tissue type. Both supervised machine learning methods (LDA and SVM) were effective in the prediction/classification of datasets with high accuracies. The prediction accuracy range of LDA was generally calculated to be 95–100%, whereas this accuracy was 75% for colon samples in the protein (1700–1500  $\text{cm}^{-1}$ ) spectral region. The training and validation accuracies of SVM were found in the range of 91–100% and 83–91%, respectively. The effect of IF was broad on specific biomolecular parameters. Particularly, the concentrations of lipids, proteins, and nucleic acids, as well as the phosphorylation rate of proteins were found higher in studied tissues of rats subjected to IF. The altered conformations and low rates of carbonylation (oxidation) were also common in proteins. No significant change was recorded for the length of fatty acid acyl chains ( $A_{2922}/A_{2955}$  band area ratio index) in liver tissues, whereas the shortening of acyl chains was calculated as 23% and 27% in ileum and colon tissues,

**Table 3**

SVM classification for colon samples in full (4000-650  $\text{cm}^{-1}$ ) spectral region. CC (control rats), FC (rats on intermittent fasting). SVM type: Classification (nu-SVC). Method: Linear.

Accuracy (%)			
Training	95.83	Validation	91.66
Classification			
Samples	Class		
CC1	1	1	CC
CC2	2	2	CC
CC3	3	3	CC
CC4	4	4	CC
CC5	5	5	CC
CC6	6	6	CC
CC7	7	7	CC
CC8	8	8	CC
CC9	9	9	CC
CC10	10	10	CC
CC11	11	11	CC
CC12	12	12	CC
FC1	13	13	FC
FC2	14	14	FC
FC3	15	15	FC
FC4	16	16	FC
FC5	17	17	FC
FC6	18	18	FC
FC7	19	19	FC
FC8	20	20	FC
FC9	21	21	FC
FC10	22	22	FC
FC11	23	23	FC
FC12	24	24	FC

respectively. Another alteration was calculated for membrane fluidity in other words membrane dynamics considering the  $B_{W2922}/B_{W2955}$  bandwidth ratio index. Enhanced membrane dynamics were depicted in the liver (35% increase), while a decline in dynamics was apparent in the ileum (36% decrease) and colon (31% decrease) tissues. The study demonstrated biomolecular findings on IF-induced modulations in hepatic and intestinal tissues of rats, which may also be helpful for fasting-related human studies and clinical trials.

#### Declaration of interest statement

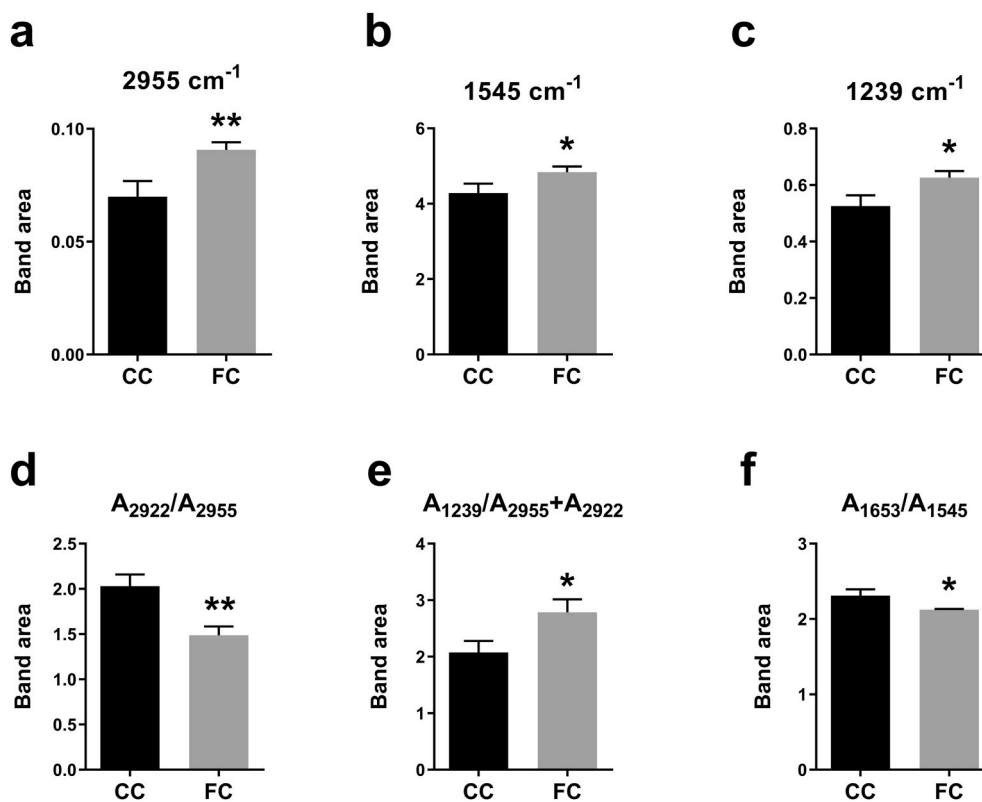
No potential conflict of interest was reported by the authors.

#### CRediT authorship contribution statement

**Taha Ceylani:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Hikmet Taner Teker:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Gizem Samgane:** Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Roles/Writing. **Rafiq Gurbanov:** Investigation, Methodology, Resources, Software, Supervision, Validation, Writing – review & editing, Visualization, Roles/Writing.

#### Data availability

Data will be made available on request.



**Fig. 8.** The changes in the FTIR spectral band areas for colon samples. The area values of a)  $\text{CH}_3$  antisymmetric ( $2955 \text{ cm}^{-1}$ ), b) Amide II ( $1545 \text{ cm}^{-1}$ ), c)  $\text{PO}_2$  antisymmetric ( $1239 \text{ cm}^{-1}$ ) bands. The indices for d) acyl chain length of fatty acids ( $A_{2922}/A_{2955}$ ), e) protein phosphorylation ( $A_{1239}/A_{2955}+A_{2922}$ ), and f) protein conformation ( $A_{1653}/A_{1545}$ ), CC (control rats), FC (rats on intermittent fasting). A (Absorbance).

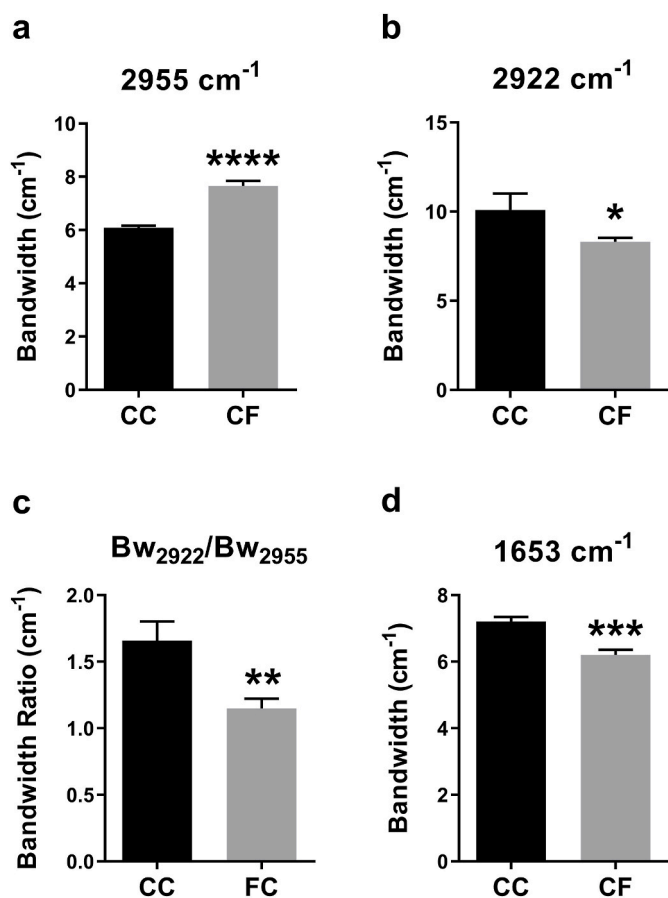


Fig. 9. The changes in the FTIR spectral bandwidths for colon samples. The bandwidth values of a) CH<sub>3</sub> antisymmetric (2955 cm<sup>-1</sup>), b) CH<sub>2</sub> antisymmetric (2922 cm<sup>-1</sup>), c) Bw<sub>2922</sub>/Bw<sub>2955</sub> ratio index, and d) Amide I (1653 cm<sup>-1</sup>). CC (control rats), FC (rats on intermittent fasting), Bw (Bandwidth).

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ab.2022.114825>.

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