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HiSorb sorptive extraction for determining salivary short chain fatty acids and hydroxy acids in heart failure patients

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ABSTRACT

Variations in salivary short-chain fatty acids and hydroxy acids (e.g., lactic acid, and 3-hydroxybutyric acid) levels have been suggested to reflect the dysbiosis of human gut microbiota, which represents an additional factor involved in the onset of heart failure (HF) disease. The physical–chemical properties of these metabolites combined with the complex composition of biological matrices mean that sample pre-treatment procedures are almost unavoidable. This work describes a reliable, simple, and organic solvent free protocol for determining short-chain fatty acids and hydroxy acids in stimulated saliva samples collected from heart failure, obese, and hypertensive patients. The procedure is based on in-situ pentafluorobenzyl bromide (PFB-Br) derivatization and HiSorb sorptive extraction coupled to thermal desorption and gas chromatography-tandem mass spectrometry. The HiSorb extraction device is completely compatible with aqueous matrices, thus saving on time and materials associated with organic solvent-extraction methods. A Central Composite Face-Centred experimental design was used for the optimization of the molar ratio between PFB-Br and target analytes, the derivatization temperature, and the reaction time which were 100, 60 °C, and 180 min, respectively. Detection limits in the range 0.1–100 μM were reached using a small amount of saliva (20 μL). The use of sodium acetate-1-^{13}C as an internal standard improved the intra- and inter-day precision of the method which ranged from 10 to 23%. The optimized protocol was successfully applied for what we believe is the first time to evaluate the salivary levels of short chain fatty acids and hydroxy acids in saliva samples of four groups of patients: i) patients admitted to hospital with acute HF symptoms, ii) patients with chronic HF symptoms, iii) patients without HF symptoms but with obesity, and iv) patients without HF symptoms but with hypertension. The first group of patients showed significantly higher levels of salivary acetic acid and lactic acid at hospital admission as well as the lowest values of hexanoic acid and heptanoic acid. Moreover, the significant high levels of acetic acid, propionic acid, and butyric acid observed in HF respect to the other patients suggest the potential link between oral bacteria and gut dysbiosis.

1. Introduction

Heart failure (HF) is a serious and a long-term condition affecting 1–2% of the population of industrialized countries, with a prevalence of > 10% in subjects aged over 70 years [1]. It is caused by an abnormal function of the heart's pumping action, resulting in a poor delivery of oxygen and nutrients to tissues and organs [2]. Pathological conditions such as heart attack, high blood pressure, diabetes, and stroke as well as life-style related conditions (e.g., smoking, and physical inactivity) cause or exacerbate HF [3].

Heart failure patients are a frail population who are prone to frequent hospitalizations (about 30% in one year [4]), thus creating a huge economic and management burden for healthcare systems [5]. The medical community is continuously debating the best management approach to improve both HF diagnosis and patient treatments [2]. Nowadays, the European Society of Cardiology recommends multidisciplinary management of HF based on the routine evaluation of various symptoms, e.g., dyspnoea and fatigue, as well as instrumental tests such as transthoracic Doppler 2D and chest X-ray [2]. Blood natriuretic peptides (i.e., BNP and/or NT-proBNP) are potential prognostic biomarkers of HF [2], although their use in monitoring the pharmacological therapy is still under evaluation mainly because of the contrasting

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results reported in the literature [6].

Human gut microbiota, especially when gut dysbiosis occurs, could be an additional factor involved in the onset of HF, mainly due to the predisposition of a low-grade inflammatory state in the host [7]. Gut dysbiosis can modify the physiological homeostasis of short-chain fatty acids (SCFAs), increasing intestinal permeability and leading to the subsequent migration of pro-inflammatory bacterial metabolites into the systemic circulation [7]. Oral microbiota has been proposed as an alternative and reliable way to mirror changes in the gut microbiota providing a non-invasive approach to monitor several systemic diseases in the host [8]. SCFAs and branched-chain fatty acids are the main metabolites produced by the anaerobic metabolism of gastrointestinal bacteria, but they can also be found in periodontal pockets [9]. They are aliphatic saturated organic acids with a chain length from one to seven carbon atoms [7]. Although they have beneficial effects when produced in the human gut, SCFAs can be considered virulence factors when produced locally in the oral cavity at high concentrations (units-tenths of mM [10]), causing the disruption of periodontal epithelial cells [9]. Lactic acid and 3-hydroxybutyric acid are other metabolites that are likely to be linked to the diversity of microbial metabolomics [11] as well as certain HF-health related conditions, such as fatty acid oxidation and the breakdown of carbohydrates under low levels of oxygen [12].

The analysis of the salivary metabolome is a promising approach to evaluate the dysbiosis of the oral microbiota [11]. Saliva is mainly composed of water (99%) and minor constituents (1%), such as proteins, organic, and inorganic substances. Compared to plasma, saliva can be easily collected, stored, and transported and does not require highly trained personnel, making it easy to handle [13].

Several techniques can be used to collect stimulated (or unstimulated) saliva with a large variety of collection devices [13]. From an analytical point of view, the determination of SCFAs and hydroxy acids in saliva can be challenging due to their relatively low concentrations (from tenths up to hundreds of μM for SCFAs and 3-hydroxybutyic acid [14]) as well as their hydrophilic nature and limited volatility [15]. Most LC- and GC-based protocols require time-consuming and labour-intensive analytical workflows aimed at modifying their polar functional group using derivatization reagents [16,17] and the subsequent extraction of the reaction's derivatives from the aqueous-based matrix [17,18]. In this regard, pentafluorobenzyl bromide (PFB-Br) is a versatile derivatization agent, widely used in GC–MS approaches due to its peculiar physicochemical properties [19] that allow to generate volatile and thermally stable derivatives characterised by well-recognized mass spectra. Stir bar sorptive extraction (SBSE) and its analogous (HiSorb probe) are a possible alternative to the traditional sample preparation approaches since they are completely compatible with aqueous matrices and thus do not require any extraction solvents [20]. This characteristic makes sorptive extraction techniques particularly interesting for salivary metabolomics analysis. Sorptive extraction is an equilibrium process in which compounds are partitioned between the aqueous sample and the polymeric phase according to their "octanol–water" partition coefficient ($K_{o/w}$) [21]. Compared to the common solid phase microextraction (SPME) technique, HiSorb has a larger volume of the PDMS sorbent (0.5 μL *vs.* 65 μL), thus improving the extraction capabilities of the device [21].

The aim of this work was to develop and validate an analytical workflow based on in-situ derivatization with PFB-Br and HiSorb-probe sorptive extraction coupled to TD-GC–MS/MS for the simultaneous determination of short-chain fatty acids (i.e., formic acid, acetic acid, propionic acid, butyric acid, valeric acid, hexanoic acid, and heptanoic acid), branched-chain fatty acids (i.e., isobutyric acid, isovaleric acid, and isohexanoic acid), 3-hydroxybutyric acid and lactic acid in saliva. The validated workflow was employed to monitor SCFAs and hydroxy acids in stimulated saliva samples collected from HF, obese, and hypertensive patients. The aim was to make a preliminary evaluation of whether these compounds could act as non-invasive indicators of gut dysbiosis as well as of the progression of HF disease.

2. Materials and methods

2.1. Chemicals and materials

Volatile free acid (VFA mixture) (certified reference material, 10 mM in water composed of formic acid, acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, hexanoic acid, isohexanoic acid, and heptanoic acid), labelled internal standard (sodium acetate-1-^{13}C, IS, purity of 99%), 3-hydroxybutyric acid (purity of 95%), and DL-lactic acid (purity of about 90%) were purchased from Merck Chemical (Milan, Italy). LC-MS grade water, methanol, and acetonitrile, at a purity of $\geq$ 99.9%, were purchased from Merck Chemical (Milan, Italy). The derivatization reagent (2,3,4,5,6-pentafluorobenzyl bromide, PFB-Br, purity $>$ 99%) was purchased from Supelco (Milan, Italy).

Synthetic air (hydrocarbon free, purity of 99.5%), helium 5.5 IP, and nitrogen 5.0 IP were purchased from Sol Group Spa (Monza, Italy). Each gas was further purified with a super clean filter purchased from Agilent Technologies (Santa Clara, USA) to remove water, oxygen, and hydrocarbon contaminants. Calibration Solution Loading Rig (CSLR), 10 mL headspace vials equipped with crimp-top sealing, inert HiSorb^{TM} probes (short length 55 mm), and commercial thermal desorption sorbent tubes, (stainless steel, O.D. 6.4 mm, I.D. 5 mm, 89 mm length) empty or packed with 250 mg of 60/80 mesh Tenax GR phase (70% Tenax TA, 2,6-diphenyl-p-phenylene-oxide and 30% graphite), were purchased from Markes International (Cardiff, UK). Each HiSorb metal probe supports a section of polydimethylsiloxane (PDMS) with a phase volume of 65 μL.

One milliliter amber glass vial equipped with a screwcap mininert valve was purchased from Sigma-Aldrich (Milan, Italy). One microliter syringe was purchased from Hamilton (Reno, USA).

Salivette roll-shaped polyester swabs were purchased from Sarstedt (Nümbrecht, Germany). Pehanon narrow-range pH paper strips (5.2 $<$ pH $<$ 8.1, resolution of 0.3 pH units) were purchased from Macherey Nagel (Düren, Germany).

All liquid solutions and saliva samples were stored in sterile polypropylene containers from Eppendorf (Milan, Italy).

2.2. Equipment

A VELP Scientifica ZX4 Advanced Vortex Mixer (Usmate, Italy) and an Eppendorf Centrifuge 5804 R equipped with an A-4–44 swinging bucket rotor (Milan, Italy) were used for sample vortex-mixing and centrifugation, respectively. A Julabo SW22 thermostatic water bath (Milan, Italy) was used to control the temperature (resolution of 0.1 °C) of the derivatization reaction.

Thermal desorption (TD) unit was composed of a Markes International TD-100 multi-tube auto-sampler equipped with an automated recollection system (Cardiff, UK). Analyses were performed using an Agilent 7890B Gas Chromatograph coupled with an Agilent 7010 GC/MS Triple Quad Mass Detector (Santa Clara, USA). Analytes were separated using an Agilent DB-5 ms capillary column (60 m length, I.D. 0.25 mm and 1 μm film thickness) (Santa Clara, USA), which is composed of a (5%-phenyl)-methylpolysiloxane stationary phase.

2.3. Standard solutions and quality control samples

Stock solutions of DL-lactic acid (130 mM) and 3-hydroxybutyric acid (100 mM) were prepared gravimetrically by diluting 20 μL of each pure compound in 2 mL of LC-MS grade water and stored in sterile polypropylene containers at 4 °C for up to one week. Intermediate stock solutions were prepared daily by diluting (10-fold) stock solutions with LC-MS water and storing them at 4 °C.

A stock solution of sodium acetate-1-^{13}C (IS, 100 mM) was prepared gravimetrically by dissolving the appropriate amount of pure compound in 4 mL of LC-MS water and stored at 4 °C for up to one week. A working solution of IS was then prepared daily by diluting (100-fold) stock

solution with LC-MS water.

A stock solution of phosphate buffer solution (PBS) (1 M, pH 7) was prepared by dissolving appropriate amounts of NaH_2PO_4 and K_2HPO_4 in 500 mL of water and storing them at 4 °C in a glass flask for up to six months. This solution was diluted (2.5-fold) with water every week.

A stock solution of PFB-Br (2.8 M) was prepared gravimetrically by diluting 85 μL of pure compound (6.6 M) to 200 μL with acetonitrile and storing it in an amber vial at room temperature for up to one week.

Working solutions of the target compounds were prepared at desired concentration by diluting with water aliquots of VFA mixture and intermediate stock solutions of DL-lactic acid and 3-hydroxybutyric acid. These solutions were freshly prepared each working day.

Pooled saliva samples were obtained by mixing known aliquots of stimulated saliva samples collected from 20 nominally healthy control volunteers. This pooled sample was daily spiked with known amounts of target analytes to obtain quality control samples of the target analytes at seven quality control levels (5, 25, 50, 250, 500, 2500, and 5000 μM).

2.4. HiSorb sorptive extraction procedure

HiSorb probes were conditioned under a permanent N_2 flow rate (70 mL/min) at 280 °C for 2 h. Before use, each probe was conditioned again for 30 min using the same parameters. The conditioning procedure entailed a pre-purge step (20 mL/min of N_2 for 10 min) aimed at preventing PDMS oxidation at high temperatures [22]. A flow path temperature of 140 °C was used during the conditioning procedure.

An aliquot (20 μL) of saliva or standard solution was placed into a 10 mL headspace vial together with 20 μL of sodium acetate-1-^{13}C (1 mM), 900 μL of PBS (400 mM, pH 7), and 5 μL of PFB-Br (2.8 M). The solution was diluted up to 8 mL using LC-MS grade water. Each vial was then sealed with a crimped HiSorb septum cap and a metal-core PDMS HiSorb was inserted into the vial through the septum. The vial was kept in the thermostatic water bath for 3 h at 60 °C to enhance the derivatization procedure and to improve the extraction of the analyte-PFB-Br adducts. Then, the HiSorb probe was removed from the vial, rinsed with LC-MS grade water to clean up the matrix residual, and gently dried with tissue paper. Finally, the HiSorb probe was inserted into a conventional empty TD tube for TD-GC–MS/MS analysis.

2.5. Instrumental analysis

For HiSorb probes, primary desorption was performed in splitless mode at 250 °C for 15 min with a N_2 flow rate of 50 mL/min. Before the primary desorption, probes were pre-purged with 20 mL/min of N_2 for 10 min. The cold trap was desorbed (secondary desorption) in split mode (split ratio of 11) at 300 °C for 20 min. A flow path temperature of 140 °C was used during the TD analysis. Analytes were separated on the DB-5 ms column at a flow rate of 1 mL/min using the following oven temperature program: 150 °C for 1 min, 5 °C/min to 240 °C (no hold). A post run step of 6 min with an oven temperature of 250 °C and a carrier gas flow of 1 mL/min was included. A solvent delay was set in the first 6 min of the analysis. The electron ionization source was set at 70 eV and the triple quadrupole mass spectrometer operated in MS/MS mode (Multiple Reaction Monitoring, MRM). The temperatures of the transfer line, ion source and quadrupoles were set at 260, 250 and 150 °C, respectively. Helium was used as a quench gas at a flow of 4 mL/min, and N_2 as a collision gas at a flow of 1.5 mL/min. The TD-100 and GC–MS/MS systems were controlled by Maverick TD software (Markes International, UK) and MassHunter Workstation software (Agilent Technologies, USA), respectively.

2.6. Optimization of the derivatization reaction

The influence of temperature (x_1, °C), time (x_2, min) and PFB-Br to analyte molar ratio (x_3) on the yield of the reaction was evaluated using a Central Composite Face-Centred (CCF) experimental design. Three central points were also included in the model to provide additional information on the experimental error, and to check for possible non-linear relationships in the middle of the intervals. In this design, the star points (α) correspond to the centre of each face of the factorial space, so that α is equal to ± 1. In our experiments, the temperature was evaluated between 20 and 60 °C, whereas the time ranged from 20 up to 180 min, and the PFB-Br to analyte molar ratio between 20 and 100. Temperature and time values were chosen by considering the parameters employed by Pan *et al* [23]. The PFB-Br to analyte molar ratio range was chosen to ensure a great molar excess of the derivatization reagent over the analytes to account for the maximum reaction yield despite its unavoidable hydrolysis in aqueous samples, as suggested in previous work [19].

MODDE software (Umetrics, Sweden) was used to generate a table of 17 experiments, which were conducted randomly on the same day. For this purpose, a standard working solution containing VFA, lactic acid, and 3-hydroxybutyric acid was prepared at 500 μM in water and mixed with an aliquot (20 μL) of IS (1 mM). The resulting solution was derivatized according to the values provided for each factor in the CCF matrix.

2.7. Method validation

The analytical approach was validated following the guidelines of the International Union of Pure and Applied Chemistry (IUPAC) [24] and included an evaluation of the limits of detection (LOD) and quantification (LOQ), relative response factors, calibration curves, matrix effect, recovery, as well as intra-day and inter-day precision.

LOD and LOQ were calculated as the ratio between three and ten times the standard deviation of the low-level spiked blank or blank sample and the slope of the calibration curve, respectively. Analyses were carried out in triplicate.

Relative response factors were calculated as the product of the ratio between the area of each target analyte and the IS, and the ratio between the concentration of the IS and the concentration of the analyte.

Calibration curves (n = 3 at each concentration level) were obtained in the range: a) 5–5000 μM for isobutyric, isovaleric, and valeric acid; b) 25–5000 μM for butyric acid; c) 50–5000 μM for propionic, isohexanoic, 3-hydroxy-butyric, hexanoic and heptanoic acid, d) 500–5000 μM for acetic and lactic acid.

Recovery and precision of the analytical procedure were evaluated by analysing on the same day and on three consecutive days, a pooled saliva sample spiked with: a) 5, 25, 50, 250, 500, 2500, and 5000 μM of isobutyric, isovaleric, and valeric; b) 25, 50, 250, 500, 2500, and 5000 μM of butyric acid; c) 50, 250, 500, 2500, and 5000 μM of propionic, isohexanoic, 3-hydroxybutyric, hexanoic and heptanoic acid; d) 500, 2500, and 5000 μM of acetic and lactic acid and e) 2500 and 5000 μM of formic acid. Experiments were performed in triplicate at each concentration level. The recovery was calculated as the percentile ratio of the difference between the analyte concentration measured in the spiked and the non-spiked samples to the nominal spiked concentration. Intra- and inter-day precision were defined in terms of the relative standard deviation (RSD%) of measurements performed on the spiked samples on a single day and on three consecutive days, respectively.

The matrix effect was evaluated by comparing the slopes of the calibration curves obtained with both standard aqueous solutions and spiked saliva samples at a confidence level of 95%.

The stability of PFB-adducts in the PDMS of the HiSorb probe during a typical sequence of chromatographic analyses (i.e., storage at room temperature in the thermal desorption autosampler for 24 h) was evaluated. For this purpose, six aqueous standard solutions were prepared at 500 μM and analysed as described in Section 2.5. Three HiSorb probes were immediately analysed (t_0), whereas the remaining three probes were inserted into conventional empty TD tubes which were closed with diffusion-locking caps (Markes International, UK) and then kept in the TD unit. These latter tubes were analysed after 24 h (t_0 + 24 h).

Short-term stability of the target SCFAs in the sample was evaluated at both room temperature and 4 °C for up to six hours, simulating a typical working day. A pooled saliva sample was thus spiked with 500 μM of target analytes and then split into nine aliquots of 50 μL each. Three aliquots were immediately analysed and used as reference values. A first set of three samples were stored at room temperature (25 ± 1 °C), and another set was stored at 4 °C. Each of aliquots in the second set was analysed after six hours of storage.

2.8. Application to real samples: Preliminary monitoring of heart failure, hypertensive, and obese patients

The HiSorb-TD-GC–MS/MS approach was employed to determine the target analytes in stimulated saliva samples collected from four groups of patients enrolled at the Fondazione Toscana Gabriele Monasterio (Pisa, Italy): i) patients admitted to hospital with acute HF symptoms (AHF, n = 11), ii) patients with chronic HF symptoms (CHF, n = 13), iii) patients without HF symptoms but with obesity (OB, n = 14), and iv) patients without HF symptoms but with hypertension (HTN, n = 16).

For hospitalized patients with acute HF symptoms, samples were collected at the time of hospital admission (AHF_A) and at discharge (AHF_D), whereas for the other three groups (i.e., CHF, OB, and HTN), the saliva sampling was carried out during the outpatient check-up. The study was approved by the local Ethics Committee (CEAVNO-Tuscany Region, protocol number 54764). Stimulated saliva was collected by placing the synthetic Salivette swab into the mouth and moving it around the mouth for 1 min. Each swab was weighed before (S_b) and after (S_a) the sampling procedure to determine the amount of saliva collected (A_s) as the difference between S_a and S_b. The salivary flow rate (grams per minute) was calculated as the ratio between A_s (g) and collection time (minute), assuming the density of saliva of 1 g/mL [25]. Samples were then recovered by centrifuging the swabs at 3000 rpm for 5 min at room temperature (25 ± 1 °C). After that, two independent operators evaluated the salivary pH using narrow range pH paper strips. Saliva samples were then stored at −80 °C until their analysis. Table 1 reports the clinical data of the four groups of patients enrolled within this study. Physiological and clinical parameters such as full blood count (g/dL), blood levels of NT-proBNP (pg/mL), creatinine (mg/dL), urea (mg/dL), sodium (mmol/L), potassium (mmol/L), and iron (mmol/L) were monitored.

Table 1
Characteristics of enrolled patients. Data are shown as mean ± standard deviation or median [interquartile range, calculated as 75th − 25th percentiles]. AHF_A: acute HF at hospital admission; AHF_D: acute HF at hospital discharge; CHF: chronic HF patients; HTN: hypertensive patients; OB: obese patients. NT-proBNP: N-terminal prohormone B-type natriuretic peptide.

Characteristics	AHF_A(n = 11)	AHF_D(n = 11)	CHF(n = 13)	HTN(n = 14)	OB(n = 16)
Age (years)	78 [10]	–	72 [3]	71 [16]	62 [15]
Full Blood Count (g/dL)	13 [2]	12 [3]	14 [2]	14 [1]	14[1]
Blood NT-proBNP (pg/mL)	5702 [9151]	2222 [3886]	755 [2035]	64 [69]	44 [113]
Creatinine (mg/dL)	1.5 [0.7]	1.4 [0.7]	1.0 [0.4]	0.9 [0.4]	0.9 [0.2]
Urea (mg/dL)	65 [42]	65 [44]	50 [8]	40 [11]	41 [17]
Sodium (mM)	141 ± 3	142 ± 2	141 ± 1	140 ± 2	139 ± 2
Potassium (mM)	3.8 ± 0.3	3.8 ± 0.3	4.4 ± 0.4	4.1 ± 0.4	4.4 ± 0.4
Iron (mM)	52 ± 21	41 ± 10	89 ± 29	85 ± 18	77 ± 21
Salivary flow rate (mL/min)	0.5 [0.6]	0.7 [0.4]	0.8 [0.4]	0.5 [0.4]	0.5 [0.5]
Salivary pH	6.8 ± 0.1	6.9 ± 0.1	6.9 ± 0.1	6.8 ± 0.1	6.8 ± 0.1

2.9. Statistical analysis

Shapiro-Wilk test was used to test the normality. Continuous variables with a normal and a skewed distribution were reported as mean and standard deviation as well as median with lower (25th percentile) and upper (75th percentile) quartiles, respectively. T-test and Mann-Whitney test was employed to evaluate potential differences between groups. The relationships between demographic and clinical variables were examined by Pearson's correlation. A two-tailed p-value of < 0.05 was considered statistically significant. Data were analysed using GraphPad Prism (v. 8.0) from GraphPad Software Inc. (La Jolla, USA) and R software (RStudio, USA).

3. Results and discussion

3.1. Optimization of the GC–MS/MS conditions

Sorbent tubes, prepared as described in the Supplementary section, were analysed in full-scan mode (*m/z* 35–500) to evaluate the retention times of the target analytes and to select suitable precursor ions for the tandem mass spectrometry experiments. Mass spectra of PFB-Br derivatives showed an intense fragment ion at *m/z* 181, corresponding to pentafluorobenzyl moiety $[C_6F_5\text{-}CH_2\bullet]^+$, and at *m/z* = M – 181. Mass spectra also highlighted a fragment ion at *m/z* 161, corresponding to a loss of hydrogen fluoride from the pentafluorobenzyl fragment. In the case of carboxylic acid derivatives, the most abundant ions were found at *m/z* = M – 197 and at *m/z* = M – 225, resulting from a loss of $C_6F_5\text{-}CH_2\text{-}O$ and $C_6F_5\text{-}CH_2\text{-}O\text{-}CO$, respectively [26]. Except for PFB-3-hydroxybutyrate adduct, all SCFAs-PFB derivatives showed the molecular fragment ion (M^+) with abundances ranging between 1 and 20%, confirming the results reported elsewhere [27]. The full-scan spectra of the PFB-3-hydroxybutyrate adduct showed a characteristic fragment ion at *m/z* = M – 15 linked to the loss of a methyl group. All these specific fragment ions were used to create an MRM method, which entailed monitoring selected transitions (precursor ion → product ion) within defined time windows.

Table 2 reports the retention time, molecular weight, MRM transition with the corresponding collision energy, and qualifier (q)-to-quantifier (Q) ratio of the analyte-PFB adducts. The dwell time of each transition was selected to obtain at least 20–25 data points, thus improving the instrumental sensitivity.

Fig. 1 shows the selected reaction chromatogram (quantifier transition) of a working standard solution at 500 μM of each target analyte, a pooled saliva sample, and a pooled saliva sample spiked with 500 μM of each analyte.

3.2. Central composite face-centred experimental design for the optimization of the derivatization reaction

A central composite face-centred experimental design was used to establish the optimal conditions (temperature, time, and PFB-Br to analyte molar ratio) for the derivatization reaction. This design involves the use of a two-level factorial design combined with axial points, factorial points, and centre runs.

Fig. 2 shows an example of a typical overview plot of the CCF experimental design for propionic acid.

The plot of replicates (Fig. 2a) enables the raw data to be studied and to evaluate the peak area (y-axis) of each experiment. The blue square points (experiments 15–17) represent the replicate values at the centre point of the experimental domain (40 °C, 100 min, and PFB-Br to analyte molar ratio of 60) and should be less than the overall variation of responses (see light blue column in the fit plot summary).

The summary of fit plot (Fig. 2b) reports the basic model diagnostic parameters, i.e., coefficient of determination R^2 (green bar), predictive power Q^2 (blue bar), model validity (yellow bar) and reproducibility (light blue bar). R^2 indicates the ability of the model to reproduce the

Table 2
Retention time, molecular weight, multiple reaction monitoring (MRM) transitions (precursor ion → product ion) with the corresponding collision energy (CE, eV), and qualifier (q)-to-quantifier (Q) ratio of the target analytes.

RT (min)	Compound	MW	MRM CE (eV)	Transitions	q/Q ratio (%)
7.72	PFB-formate	226	6	(Q) 226 → 178	–
				(q) 226 → 181	48
8.76	PFB-1-^{13}C-acetate	241	9	(Q) 241 → 44	–
				(q) 241 → 197	47
8.76	PFB-acetate	240	4	(Q) 240 → 178	–
				(q) 240 → 181	23
10.32	PFB-propionate	254	2	(Q) 254 → 181	–
				(q) 254 → 178	57
11.01	PFB-isobutyrate	268	10	(Q) 268 → 43	–
				(q) 268 → 181	81
12.03	PFB-butyrate	268	1	(Q) 268 → 203	–
				(q) 268 → 60	82
12.72	PFB-lactate	270	20	(Q) 270 → 45	–
				(q) 270 → 56	28
13.06	PFB-isovalerate	282	9	(Q) 282 → 176	–
				(q) 282 → 181	59
14.07	PFB-valerate	282	11	(Q) 282 → 176	–
				(q) 282 → 181	81
15.42	PFB-isohexanoate	296	8	(Q) 296 → 97	–
				(q) 296 → 115	90
15.61	PFB-3-hydroxybutyrate	284	20	(Q) 269 → 181	–
				(q) 269 → 87	1
16.22	PFB-hexanoate	296	7	(Q) 296 → 181	–
				(q) 296 → 176	62
18.36	PFB-heptanoate	310	8	(Q) 310 → 83	–
				(q) 310 → 181	36

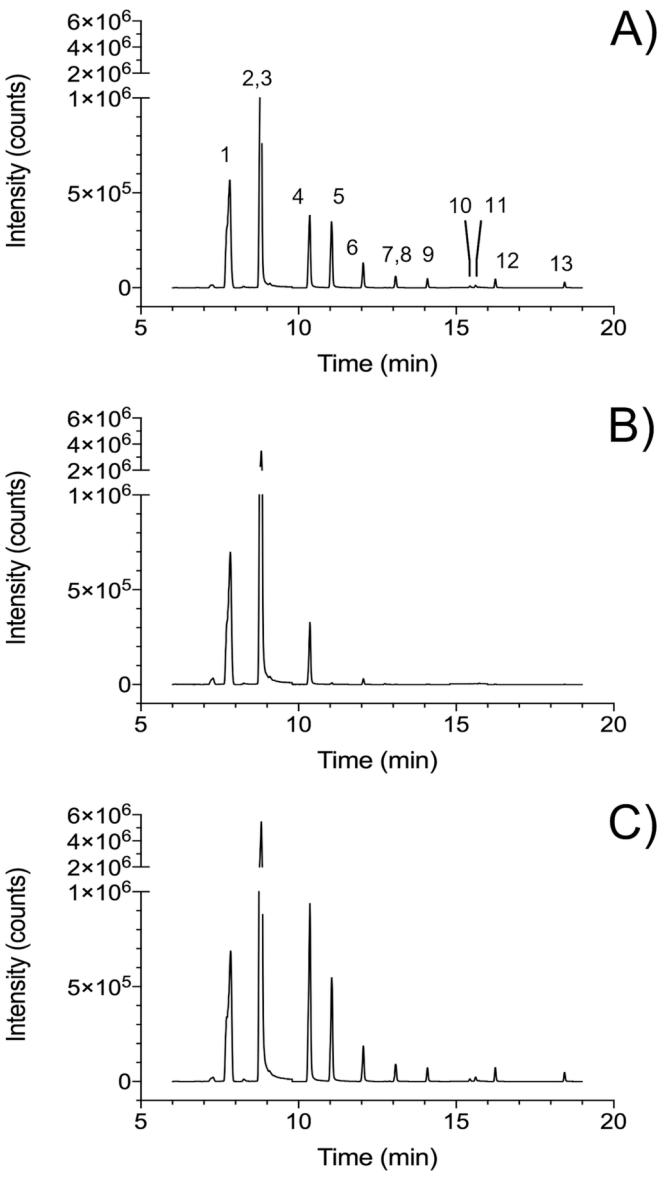

Fig. 1. Selected reaction monitoring chromatogram (quantifier transition) a working standard solution at 500 μM of each target analyte A), a pooled saliva sample, B) and a pooled saliva sample spiked with 500 μM of each analyte C). Elution order: 1) PFB-formate; 2) PFB-1-^{13}C acetate; 3) PFB-acetate; 4) PFB-propionate; 5) PFB-isobutyrate; 6) PFB-butyrate; 7) PFB-lactate; 8) PFB-isovalerate; 9) PFB-valerate; 10) PFB-isohexanoate; 11) PFB-3-hydroxybutyrate; 12) PFB-hexanoate; 13) PFB-heptanoate.

training set data (experiments 1–14), whereas Q^2 provides information on the predictivity power of the model.

A significant model is characterized by R^2 and Q^2 being higher than 0.5 and 0.1, respectively. The presence of outliers and/or the existence of statistically significant deviations from the model used to fit the raw data is highlighted by model validity values (yellow bar) lower than 0.25. The light blue bar indicates the experimental variability of the replicates performed at the centre point. The model is validated if the reproducibility value is higher than 0.5.

In our case, all these indexes were satisfied, thus highlighting the validity of our experimental model. This is further confirmed by the good linear relationship between predicted and observed values (model prediction, Fig. 2d). Table S1 reports all the indexes obtained with the central composite face-centred experimental design.

The plot of the coefficients (Fig. 2c) shows the effect of variables on the peak area of each target analyte. In this plot, variables with a positive sign led to an increase in the peak area of the analyte, whereas the opposite was true for negative values. Error bars represent the uncertainty associated with each variable, which are non-significant when they overlap the x-axis of the plot.

Except for lactic acid, temperature (x_1) and derivatization time (x_2) were the most significant coefficients (positive sign), whereas the PFB-Br to analyte molar ratio (x_3) was less important (Figure S2). The positive effect of the derivatization temperature could be due to the increased efficiency of the mass transfer of analytes from aqueous solution to PDMS [28]. Similarly, the effect of derivatization time is probably due to the combination of two factors: i) the negative effect of the protic solvents (e.g. water) on the derivatization reaction since the carboxylate ion is not able to react properly with the electrophilic substrate (PFB-Br) [29], and ii) the longer times required for the analyte extraction due to the larger capacity of the PDMS sorbent (65 μL) compared to the SPME fibre (0.5 μL) [21].

The negligible effect of the PFB-Br to analyte molar ratio (x_3) on the derivatization reaction could be explained by considering that the high amount of PFB-Br in the water solution (2.8, 8.4 and 14 mmol) may easily cover the PDMS surface, as already reported elsewhere for SPME

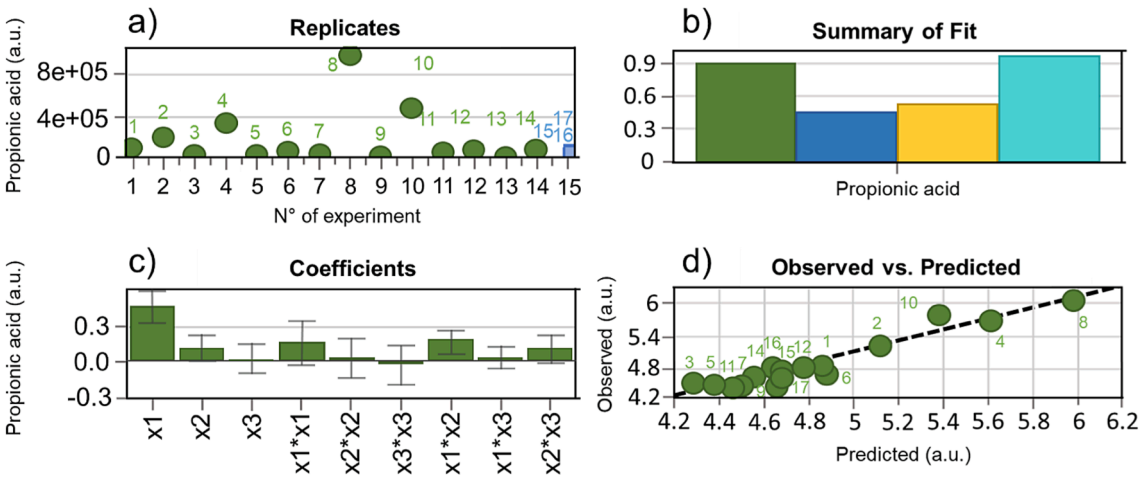

Fig. 2. Overview plot of propionic acid: plot of replicates (a), summary of fit (R^2 (green), Q^2 (blue), model validity (yellow), and reproducibility (light blue)) (b), plot of coefficients (c), and model predictions (observed *vs.* predicted), (d). Confidence interval bars at 95% of the coefficients are also shown. In the plot of coefficients x1 corresponds to derivatization temperature (° C), x2 to derivatization time (min), and x3 to PFB-Br to analyte molar ratio. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[23]. In this scenario, the reaction between the target analytes and PFB-Br takes place in the PDMS phase rather than in the water solution, producing a sort of on-sorbent derivatization [23,30,31]. For lactic acid a positive effect of both temperature (x_1) and PFB-Br to analyte molar ratio (x_3) on the peak area was obtained, whereas the derivatization time (x_2) showed a limited impact. The chemical structure of α-hydroxy acids is characterized by an internal hydrogen bond between the hydrogen of the hydroxyl group and one of the oxygen atoms of the carboxylic group [32]. This implies the generation of a pseudo-5-atom ring, making the carboxylate group of the lactic acid less available for the reaction with PFB-Br. High values of temperature and PFB-Br to analyte molar ratio are thus required to enhance the derivatization reaction, justifying the results of the CCF experimental design.

The *Optimizer* tool of the MODDE software enabled us to evaluate the best experimental conditions that maximize the response of each analyte. The best desirability function enables results from each analyte to be combined and to obtain the overall maximum for the derivatization reaction using 60 °C, 180 min and a PFB-Br to analyte molar ratio of 100. Note that even if the procedure required a relatively long derivatization time, the possibility to fully automatize the entire procedure with dedicated autosampler may increase the instrumental throughput by derivatizing six sample at the same time. The possibility to daily analyse about 15 samples makes our HiSorb-TD-GC–MS/MS approach competitive with other methods for SCFAs analysis [16,18,33–35].

3.3. Method validation

3.3.1. Limits of detection, relative response factors, and method precision

Table 3 reports the calibration ranges, mean relative response factors, limits of detection as well as intra- and inter-day recovery and precision for the investigated analytes.

The use of sodium acetate-1-^{13}C as internal standard improved the overall precision of the HiSorb-TD-GC–MS/MS approach, leading to a significant decrease (ranged between 3- and 5-fold) of the target analytes detection limits. For example, for valeric acid, the use of IS correction reduced the variability by a factor of 3 and the detection limit from 3 μM down to 1 μM. Limits of detection were in the range of 0.1–100 μM which is slightly higher than those reported elsewhere [17,36–38]. Note that our LODs were reached using a small amount of saliva (20 μL) in a relatively solvent-free approach, whereas most of the already published protocols entail the use of hundreds of microliters of sample or the employment of organic solvents for analytes extraction. It is worth to mention that none of these works determined SCFAs in saliva samples by means of GC–MS technique.

The analysis of an empty sorbent tube at the beginning of each TD-GC–MS/MS sequence highlighted the presence of PFB-formate and PFB-acetate at approximately 1000 and 500 μM, respectively. This was probably due to a residual amount of PFB-Br in the cold trap that induces the derivatization of formic acid and acetic acid present in our lab as air contaminants [39]. Cleaning the cold trap at 300 °C with approximately

Table 3
Mean relative response factors, limits of detection (LODs), intra- and inter-day recovery and precision values of SCFAs and hydroxy acids.

Compound	Mean relative response factor (RSD*)	LOD (μM)	Intra-day recovery (%)	Intra-day precision (RSD)	Inter-day recovery (%)	Inter-day precision (RSD)
Formic acid	1.50 (56%)	1250	123	30%	120	35%
Acetic acid	6.32 (25%)	100	94	1%	95	23%
Propionic acid	4.89 (23%)	10	93	3%	98	17%
Isobutyric acid	2.41 (12%)	0.2	95	6%	91	11%
Butyric acid	0.51 (13%)	6	95	2%	90	18%
Lactic acid	0.002 (20%)	100	85	10%	83	21%
Isovaleric acid	0.30 (8%)	0.1	91	5%	94	10%
Valeric acid	0.21 (9%)	1	88	5%	93	13%
Isohexanoic acid	0.02 (19%)	10	87	8%	92	8%
3-hydroxy-butyric acid	0.07 (16%)	10	87	6%	91	12%
Hexanoic acid	0.17 (6%)	10	87	5%	80	12%
Heptanoic acid	0.17 (8%)	10	86	5%	78	13%

*RSD = Relative Standard Deviation.

2 L of carrier gas before the TD-GC–MS/MS analysis (e.g., using the heat trap mode of the TD-100) reduced the PFB-formate and PFB-acetate signals at about 400 and 30 μM, respectively).

Except for formic acid, whose recovery was about 120%, the analyte recovery ranged from 78 to 98%. A slight decreasing trend in recovery values over the number of carbon atoms present in the structure was observed probably due to a potential interaction between the free compound and the PDMS phase. The recovery value of lactic acid close to 80% was probably due to the formation of an internal hydrogen bond making the carboxylate group less reactive to PFB-Br. The high method variability obtained for formic acid was probably due to the contamination of our air lab as mentioned before. Thus, we decided to not determine it in saliva samples.

A matrix effect was ruled out by comparing the slope of calibration curves obtained from spiked saliva and standard solutions at a confidence level of 95%. No significant difference between slope values was found for any of the analytes (Fig. 1). All these figures of merit confirmed the ability of our protocol to determine SCFAs and hydroxy acids in saliva. Unlike most protocols, our approach did not require any organic extraction solvents and enabled the derivatization and extraction of the target analytes to be performed in a single step.

3.3.2. *Stability study*

Fig. 3 shows the stability of SCFAs and hydroxy acids in saliva at two storage temperatures (room temperature and 4 °C) up to 6 h and the stability of PFB-adducts in the PDMS during a typical TD-GC–MS/MS sequence (storage in the TD autosampler at room temperature for up to 24 h).

Only lactic acid showed an increase in the signal of about 40% when saliva was stored at room temperature for up to 6 h. This effect could be related to the presence of lactate dehydrogenase in saliva, which is an enzyme that catalyses the interconversion of pyruvic acid in lactic acid [40,41]. Storing samples at 4 °C improved the stability of lactic acid in saliva thereby reducing the signal increase to 20%. No significant variations in the PFB-SCFA adducts were observed when HiSorb probes were stored in the TD autosampler at room temperature for 24 h.

3.4. *Application to real samples: Preliminary monitoring of heart failure, hypertensive, and obese patients*

Fig. 4 shows the box-plot of the salivary levels of acetic acid, propionic acid, butyric acid, lactic acid, isobutyric acid, isovaleric acid, valeric acid, hexanoic acid, heptanoic acid, and blood NT-proBNP measured in all samples collected from the four groups of patients, i.e., acute HF, chronic HF, hypertensive, and obese patients. Salivary levels of isohexanoic acid and 3-hydroxybutyric acid were below the LODs in all the samples analysed. Table S2 reports the concentrations of all the target analytes in the investigated groups.

Patients with acute HF symptoms showed a significant ($p < 0.05$) decrease in salivary levels of acetic acid (Fig. 4A) and lactic acid (Fig. 4E), as well as an increase in hexanoic acid (Fig. 4H) and heptanoic acid (Fig. 4I) in saliva during hospitalization. The acute phase of the disease is characterized by a poor organ perfusion which becomes inadequate to meet the metabolic demands of tissues in the organism [2]. The body activates several compensatory mechanisms aimed at restoring tissue perfusion, as an example, natriuretic peptides are released from the myocardium to increase water and electrolyte excretion [42]. The high levels of acetic acid at hospital admission may be due to the hypoxia occurring during the acute phase of HF since low levels of oxygen in tissue induce a shift from aerobic to anaerobic metabolism. This leads to a higher production of acetic acid as the most abundant metabolite, followed by propionic acid, and butyric acid in the oral cavity [43]. Furthermore, the release of metabolites such as acetic acid and lactic acid may decrease the salivary pH contributing to the degradation of gingival tissues and leading to the development of a low-grade inflammatory state, which may exacerbate the onset of

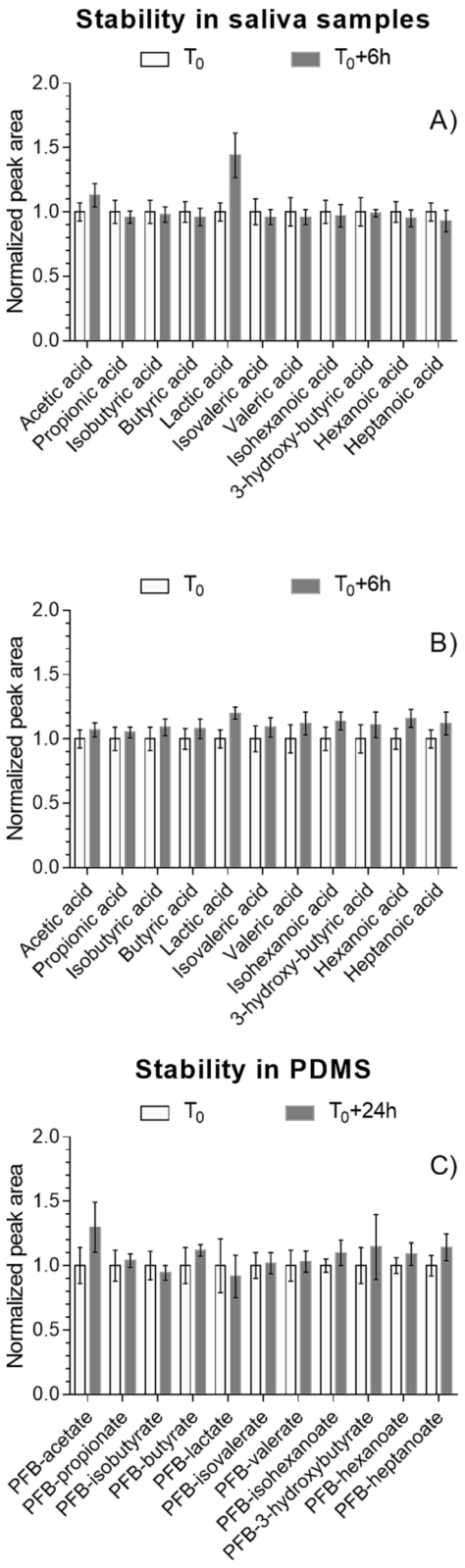

Fig. 3. Stability of i) SCFAs and hydroxy acids in saliva stored at room temperature (A) and 4 °C (B) up to 6 h (t0 + 6 h), and of ii) PFB-SCFA adducts adsorbed into PDMS sorbent phase stored in the thermal desorption unit for up to 24 h (t0 + 24 h) (C). Data were normalized with respect to the PFB-1-13C acetate peak area and to the area of the peak corresponding to the first observation time (t0). Error bars correspond to the standard deviation of three replicates (n = 3).

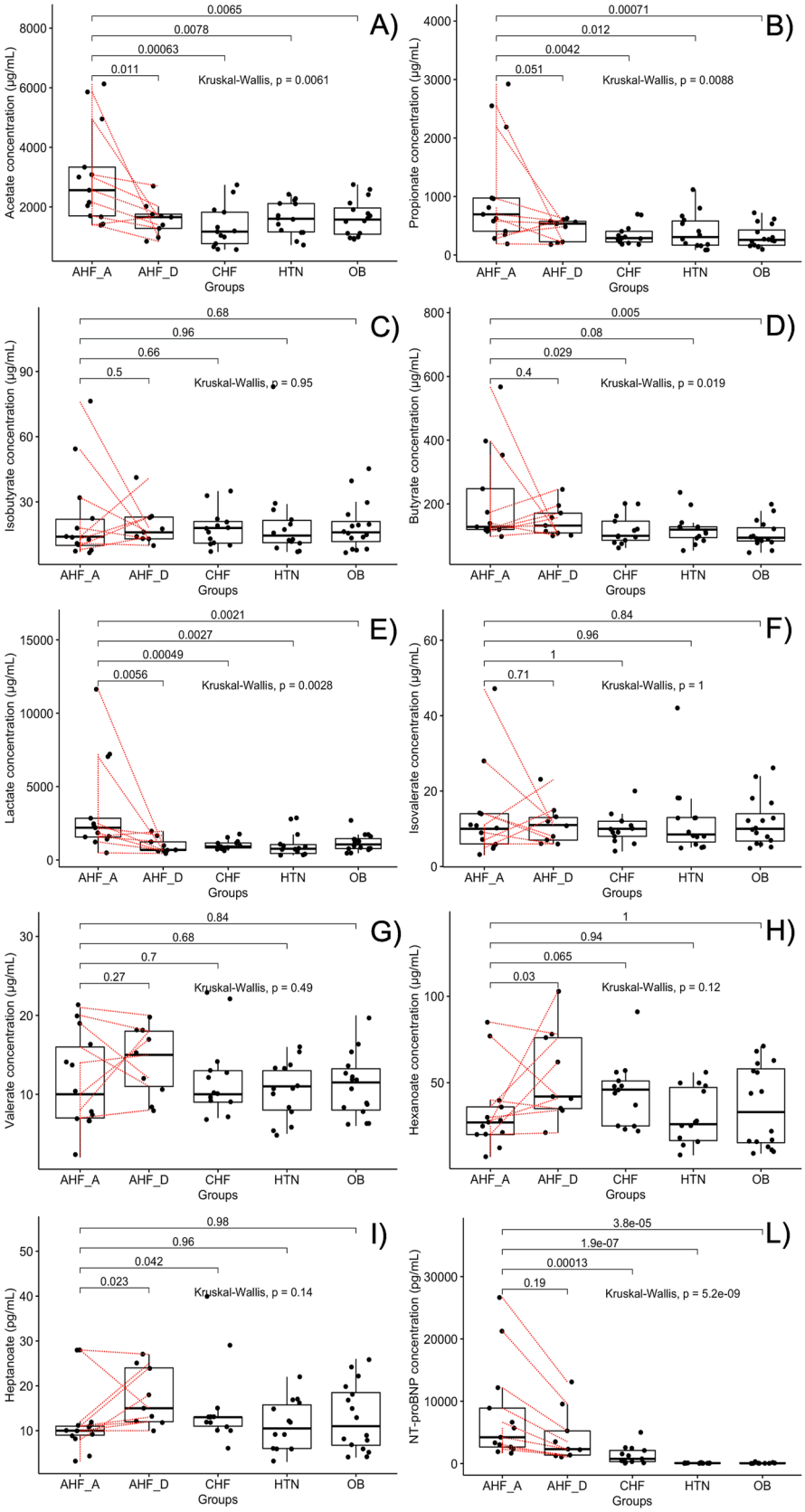

Fig. 4. Box-plot of acetic acid (A), propionic acid (B), isobutyric acid (C), butyric acid (D), lactic acid (E), isovaleric acid (F), valeric acid (G), hexanoic acid (H), heptanoic acid (I) and blood NT-proBNP (L) for acute HF at hospital admission (AHF_A) and discharge (AHF_D), chronic HF (CHF), hypertensive (HTN), and obese (OB) patients. The box plot shows the minimum, 25th percentile, median, 75th percentile, and the maximum value. Paired samples are connected each other with red lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cardiovascular diseases [44,45]. Likewise, reduced tissue perfusion enhances anaerobic glycolysis, thus modifying the lactic acid production in the skeletal muscle and the clearance (i.e., liver metabolism and kidney removal) [46]. Such variations increase the plasmatic and salivary levels of lactic acid, confirming the data reported elsewhere [47]. Even if most of samples showed hexanoic acid and heptanoic acid values close to their LOQs (10 μM) reducing the accuracy of their determination, we observed an increase of hexanoic acid and heptanoic acid values at hospital discharge probably due to their consumption in heart cells to produce citric acid cycle intermediates lacking in the acute phase of heart failure [48].

In addition, our results highlighted that the salivary concentrations of acetic acid, propionic acid, butyric acid, and lactic acid, measured at hospital admission (AHF_A), were significantly different compared to the chronic HF (CHF), hypertensive (HTN), and obese (OB) groups. Carley *et al.* discussed the potential role of SCFAs as fuel oxidative metabolism in the failing heart [49]. They reported that SCFAs are a preferred energy source over ketones for the failing heart. The accumulation of acidic metabolites, such as propionic acid, in body fluids (acidaemia) has also been linked to the exacerbation of cardiac dysfunction and diseases [50]. Moreover, high levels of acetic acid, propionic acid, and butyric acid may be related to the altered oral bacteria, which may travel from the mouth to the gut through the salivary membrane. Such condition induces local imbalances in gut microbial communities, resulting in gut dysbiosis [51]. Recently, gut dysbiosis induced by oral bacteria has been linked with increased gut permeability that triggering the chronic inflammation in HF patients [52]. Interestingly, hypertension and obesity are the two main comorbidities affecting HF patients, in fact 65% of HF patients have hypertension, and 23% have obesity [3]. Our findings suggest that the variations in acetic acid, propionic acid, butyric acid, and lactic acid were mainly related to HF rather than other comorbidities, thus confirming the ability of our analytical approach to monitor HF patients.

4. Conclusions

We have described a reliable analytical approach for the determination of SCFAs and hydroxy acids in saliva by in-situ PFB-Br derivatization and HiSorb sorptive extraction coupled to TD-GC–MS/MS analysis. The good analytical performance of the proposed protocol guaranteed the reliable determination of the target analytes.

Our protocol was successfully employed to monitor acute and chronic HF, hypertensive, and obese patients. Compared to discharge, HF patients showed significantly higher salivary acetic acid and lactic acid levels, as well as lower hexanoic acid and heptanoic acid concentration values at hospital admission. Our results also highlighted that the salivary concentrations of acetic acid, propionic acid, butyric acid, and lactic acid, at hospital admission, were significantly different compared to the other groups. These preliminary findings suggest the role of these compounds as potential salivary indicators of the progression of HF disease and could pave the way for future applications in this clinical field.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2023.123826.

References

[1] H.J. Aparicio, E.J. Benjamin, C.W. Callaway, A.P. Carson, S. Cheng, M.S.V. Elkind, K.R. Evenson, J.F. Ferguson, K.L. Knutson, C.D. Lee, T.T. Lewis, M.S. Loop, P. L. Lutsey, J. Mackey, D.B. Matchar, Heart disease and stroke statistics-2021 update a report from the american heart association (2021), https://doi.org/10.1161/CIR.0000000000000950.

[2] T.A. McDonagh, M. Metra, M. Adamo, R.S. Gardner, A. Baumbach, M. Böhm, H. Burri, J. Butler, J. Čelutkienė, O. Chioncel, J.G.F. Cleland, A.J.S. Coats, M. G. Crespo-Leiro, D. Farmakis, M. Gilard, S. Heymans, A.W. Hoes, T. Jaarsma, E. A. Jankowska, M. Lainscak, C.S.P. Lam, A.R. Lyon, J.J.V. McMurray, A. Mebazaa, R. Mindham, C. Muneretto, M. Francesco Piepoli, S. Price, G.M.C. Rosano, F. Ruschitzka, A. Kathrine Skibelund, R.A. de Boer, P. Christian Schulze, M. Abdelhamid, V. Aboyans, S. Adamopoulos, S.D. Anker, E. Arbelo, R. Asteggiano, J. Bauersachs, A. Bayes-Genis, M.A. Borger, W. Budts, M. Cikes, K. Damman, V. Delgado, P. Dendale, P. Dilaveris, H. Drexel, J. Ezekowitz, V. Falk, L. Fauchier, G. Filippatos, A. Fraser, N. Frey, C.P. Gale, F. Gustafsson, J. Harris, B. Iung, S. Janssens, M. Jessup, A. Konradi, D. Kotecha, E. Lambrinou, P. Lancellotti, U. Landmesser, C. Leclercq, B.S. Lewis, F. Leyva, A. Linhart, M.-L. Løchen, L. H. Lund, D. Mancini, J. Masip, D. Milicic, C. Mueller, H. Nef, J.-C. Nielsen, L. Neubeck, M. Noutsias, S.E. Petersen, A. Sonia Petronio, P. Ponikowski, E. Prescott, A. Rakisheva, D.J. Richter, E. Schlyakhto, P. Seferovic, M. Senni, M. Sitges, M. Sousa-Uva, C.G. Tocchetti, R.M. Touyz, C. Tschoepe, J. Waltenberger, M. Adamo, A. Baumbach, M. Böhm, H. Burri, J. Čelutkienė, O. Chioncel, J.G. F. Cleland, A.J.S. Coats, M.G. Crespo-Leiro, D. Farmakis, R.S. Gardner, M. Gilard, S. Heymans, A.W. Hoes, T. Jaarsma, E.A. Jankowska, M. Lainscak, C.S.P. Lam, A. R. Lyon, J.J.V. McMurray, A. Mebazaa, R. Mindham, C. Muneretto, M.F. Piepoli, S. Price, G.M.C. Rosano, F. Ruschitzka, A.K. Skibelund, ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure, Eur. Heart J. 42 (2021) (2021) 3599–3726, https://doi.org/10.1093/eurheartj/ehab368.

[3] C.A. Lawson, F. Zaccardi, I. Squire, H. Okhai, M. Davies, W. Huang, M. Mamas, C.S. P. Lam, K. Khunti, U.T. Kadam, Risk factors for heart failure: 20-year population-based trends by sex, socioeconomic status, and ethnicity, Circ. Hear. Fail. (2020), https://doi.org/10.1161/CIRCHEARTFAILURE.119.006472.

[4] M.G. Crespo-Leiro, S.D. Anker, A.P. Maggioni, A.J. Coats, G. Filippatos, F. Ruschitzka, R. Ferrari, M.F. Piepoli, J.F.D. Jimenez, M. Metra, C. Fonseca, J. Hradec, O. Amir, D. Logeart, U. Dahlström, B. Merkely, J. Drozdz, E. Goncalvesova, M. Hassanein, O. Chioncel, M. Lainscak, P.M. Seferovic, D. Tousoulis, A. Kavoliuniene, F. Fruhwald, E. Fazlibegovic, A. Temizhan, P. Gatzov, A. Erglis, C. Laroche, A. Mebazaa, European society of cardiology heart failure follow-up outcomes and differences across regions, ESC Hear. Fail. 18 (2016) 613–625, https://doi.org/10.1002/ejhf.566.

[5] M. Kilgore, H.K. Patel, A. Kielhorn, J.F. Maya, P. Sharma, Economic burden of hospitalizations of Medicare beneficiaries with heart failure, Risk Manag. Healthc, Policy 10 (2017) 63–70, https://doi.org/10.2147/RMHP.S130341.

[6] A.K. Saenger, O. Rodriguez-Fraga, R. Ler, J. Ordonez-Llanos, A.S. Jaffe, J.P. Goetze, F.S. Apple, Specificity of B-type natriuretic peptide assays: Cross-reactivity with different BNP, NT-proBNP, and proBNP peptides, Clin. Chem. 63 (2017) 351–358, https://doi.org/10.1373/clinchem.2016.263749.

[7] S. Madan, M.R. Mehra, Gut dysbiosis and heart failure: navigating the universe within, Eur. J. Heart Fail. 22 (2020) 629–637, https://doi.org/10.1002/ejhf.1792.

[8] D.T. Graves, J.D. Corrêa, T.A. Silva, The oral microbiota is modified by systemic diseases, J. Dent. Res. 98 (2019) 148–156, https://doi.org/10.1177/0022034518805739.

[9] G.L. Magrin, F.J. Strauss, C.A.M. Benfatti, L.C. Maia, R. Gruber, Effects of short-chain fatty acids on human oral epithelial cells and the potential impact on periodontal disease: A systematic review of in vitro studies, Int. J. Mol. Sci. 21 (2020) 1–19, https://doi.org/10.3390/ijms21144895.

[10] S. Takigawa, N. Sugano, R. Nishihara, R. Koshi, M. Murai, N. Yoshinuma, K. Ochiai, K. Ito, The effect of butyric acid on adhesion molecule expression by human gingival epithelial cells, J. Periodontal Res. 43 (2008) 386–390, https://doi.org/10.1111/j.1600-0765.2007.01048.x.

[11] A. Gardner, H.G. Parkes, P.W. So, G.H. Carpenter, Determining bacterial and host contributions to the human salivary metabolome, J. Oral Microbiol. 11 (2019), https://doi.org/10.1080/20002297.2019.1617014.

[12] B. Phypers, J.M.T. Pierce, Lactate physiology in health and disease, Contin. Educ. Anaesthesia, Crit. Care, Pain (2006), https://doi.org/10.1093/bjaceaccp/mkl018.

[13] F.G. Bellagambi, T. Lomonaco, P. Salvo, F. Vivaldi, M. Hangouët, S. Ghimenti, D. Biagini, F. Di Francesco, R. Fuoco, A. Errachid, Saliva sampling: Methods and devices. An overview, TrAC -, Trends Anal. Chem. 124 (2020), https://doi.org/10.1016/j.trac.2019.115781.
[14] X. Yu, A.-M. Shahir, J. Sha, Z. Feng, B. Eapen, S. Nithianantham, B. Das, J. Karn, A. Weinberg, N.F. Bissada, F. Ye, Short-chain fatty acids from periodontal pathogens suppress histone deacetylases, EZH2, and SUV39H1 to promote kaposi's sarcoma-associated herpesvirus replication, J. Virol. 88 (2014) 4466–4479, https://doi.org/10.1128/jvi.03326-13.
[15] F. Orata, Derivatization Reactions and Reagents for Gas Chromatography Analysis, in: 2007.
[16] W.S. Song, H.G. Park, S.M. Kim, S.H. Jo, B.G. Kim, A.B. Theberge, Y.G. Kim, Chemical derivatization-based LC–MS/MS method for quantitation of gut microbial short-chain fatty acids, J. Ind. Eng. Chem. 83 (2020) 297–302, https://doi.org/10.1016/j.jiec.2019.12.001.
[17] F. Xia, L. Guo, P. Cui, Q. Xu, J. Huang, H. Zhou, W. Shen, A sensitive and accurate GC–MS method for analyzing microbial metabolites short chain fatty acids and their hydroxylated derivatives in newborn fecal samples, J. Pharm. Biomed. Anal. 223 (2023), 115148, https://doi.org/10.1016/j.jpba.2022.115148.
[18] S. Zhang, H. Wang, M.J. Zhu, A sensitive GC/MS detection method for analyzing microbial metabolites short chain fatty acids in fecal and serum samples, Talanta 196 (2019) 249–254, https://doi.org/10.1016/j.talanta.2018.12.049.
[19] D. Tsikas, Pentafluorobenzyl bromide — A versatile derivatization agent in chromatography and mass spectrometry: I. analysis of inorganic anions and organophosphates, J. Chromatogr. B 1043 (2017) 187–201, https://doi.org/10.1016/j.jchromb.2016.08.015.
[20] Markes International Ltd, HiSorb sorptive extraction: brochure, (n.d.).
[21] Markes International Ltd, Introducing HiSorb Sorptive Extraction, (2016).
[22] N. Grassie, I.G. Macfarlane, The thermal degradation of polysiloxanes-I. Poly (dimethylsiloxane), Eur. Polym. J. 14 (1978) 875–884, https://doi.org/10.1016/0014-3057(78)90084-8.
[23] L. Pan, J. Pawliszyn, Derivatization/Solid-Phase Microextraction: New approach to polar analytes, Anal. Chem. 69 (1997) 196–205, https://doi.org/10.1021/ac9606362.
[24] M. Thompson, S.L.R. Ellison, R. Wood, Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report), Pure Appl. Chem. 74 (2002) 835–855, https://doi.org/10.1351/pac200274050835.
[25] E. Kubala, P. Strzelecka, M. Grzegocka, D. Lietz-Kijak, H. Gronwald, P. Skomro, E. Kijak, A review of selected studies that determine the physical and chemical properties of saliva in the field of dental treatment, Biomed Res. Int. 2018 (2018), https://doi.org/10.1155/2018/6572381.
[26] C.J. Chien, M.J. Charles, K.G. Sexton, H.E. Jeffries, Analysis of airborne carboxylic acids and phenols as their pentafluorobenzyl derivatives: Gas chromatography/ion trap mass spectrometry with a novel chemical ionization reagent, PFBOH, Environ. Sci. Tech. 32 (1998) 299–309, https://doi.org/10.1021/es970526s.
[27] J.H. Brill, B.A. Narayanan, J.P. McCormick, Selective determination of pentafluorobenzyl ester derivatives of carboxylic acids by GC using microwave plasma and mass selective detection, Appl. Spectrosc. (1991), https://doi.org/10.1366/0003702914335193.
[28] F.J. Camino-Sánchez, R. Rodríguez-Gómez, A. Zafra-Gómez, A. Santos-Fandila, J. L. Vílchez, Stir bar sorptive extraction: Recent applications, limitations and future trends, Talanta 130 (2014) 388–399, https://doi.org/10.1016/j.talanta.2014.07.022.
[29] M. Primec, D. Mičetić-Turk, T. Langerholc, Analysis of short-chain fatty acids in human feces: A scoping review, Anal. Biochem. 526 (2017) 9–21, https://doi.org/10.1016/j.ab.2017.03.007.
[30] N. Ochiai, K. Sasamoto, S. Daishima, A.C. Heiden, A. Hoffmann, Determination of stale-flavor carbonyl compounds in beer by stir bar sorptive extraction with in-situ derivatization and thermal desorption-gas chromatography-mass spectrometry, J. Chromatogr. A 986 (2003) 101–110, https://doi.org/10.1016/S0021-9673(02)01870-8.
[31] T. Lomonaco, A. Romani, S. Ghimenti, D. Biagini, F.G. Bellagambi, M. Onor, P. Salvo, R. Fuoco, F. Di Francesco, Determination of carbonyl compounds in exhaled breath by on-sorbent derivatization coupled with thermal desorption and gas chromatography-tandem mass spectrometry, J. Breath Res. 12 (2018), https://doi.org/10.1088/1752-7163/aad202.
[32] P.I. Nagy, Competing intramolecular vs. Intermolecular hydrogen bonds in solution, Int. J. Mol. Sci. (2014), https://doi.org/10.3390/ijms151119562.
[33] J.H. Na Hyun Park, Min-Sun Kim, Wonwoong Lee, Myoung Eun Lee, In situ extraction and derivatization method for rapid analysis of short-chain fatty acids in rat fecal sample by gas chromatography tandem mass spectrometry, (2017). https://doi.org/10.1039/C7AY00168A.
[34] X. Zheng, Y. Qiu, W. Zhong, S. Baxter, M. Su, Q. Li, G. Xie, B.M. Ore, S. Qiao, M. D. Spencer, S.H. Zeisel, Z. Zhou, A. Zhao, W. Jia, A targeted metabolomic protocol for short-chain fatty acids and branched-chain amino acids, Metabolomics 9 (2013) 818–827, https://doi.org/10.1007/s11306-013-0500-6.
[35] F. Bianchi, M.D. Asta, D. Del Rio, A. Mangia, M. Musci, F. Scazzina, Development of a headspace solid-phase microextraction gas chromatography – mass spectrometric method for the determination of short-chain fatty acids from intestinal fermentation, Food Chem. 129 (2011) 200–205, https://doi.org/10.1016/j.foodchem.2011.04.022.
[36] V. Ramos-Garcia, I. Ten-Doménech, A. Moreno-Giménez, L. Campos-Berga, A. Parra-Llorca, Á. Solaz-García, I. Lara-Cantón, A. Pinilla-Gonzalez, M. Gormaz, M. Vento, J. Kuligowski, G. Quintás, GC-MS analysis of short chain fatty acids and branched chain amino acids in urine and faeces samples from newborns and lactating mothers, Clin. Chim. Acta 532 (2022) 172–180, https://doi.org/10.1016/j.cca.2022.05.005.
[37] J.H. Zhu, Q. Mao, S.Y. Wang, H. Liu, S.S. Zhou, W. Zhang, M. Kong, H. Zhu, S.L. Li, Optimization and validation of direct gas chromatography-mass spectrometry method for simultaneous quantification of ten short-chain fatty acids in rat feces, J. Chromatogr. A 1669 (2022), 462958, https://doi.org/10.1016/j.chroma.2022.462958.
[38] J.K. Rohde, M.M. Fuh, I. Evangelakos, M.J. Pauly, N. Schaltenberg, F. Siracusa, N. Gagliani, K. Tödter, J. Heeren, A. Worthmann, A gas chromatography mass spectrometry-based method for the quantification of short chain fatty acids, Metabolites 12 (2022), https://doi.org/10.3390/metabo12020170.
[39] S. Madronich, R.B. Chatfield, J.G. Calvert, G.K. Moortgat, B. Veyret, R. Lesclaux, A photochemical origin of acetic acid in the troposphere, Geophys. Res. Lett. 17 (1990) 2361–2364, https://doi.org/10.1029/GL017i013p02361.
[40] K. Lokesh, J. Kannabiran, M.D. Rao, Salivary lactate dehydrogenase (LDH)- A novel technique in oral cancer detection and diagnosis, J. Clin. Diag Res. 10 (2016) ZC34–ZC37, https://doi.org/10.7860/JCDR/2016/16243.7223.
[41] T. Lomonaco, S. Ghimenti, D. Biagini, E. Bramanti, M. Onor, F.G. Bellagambi, R. Fuoco, F. Di Francesco, The effect of sampling procedures on the urate and lactate concentration in oral fluid, Microchem. J. 136 (2018) 255–262, https://doi.org/10.1016/j.microc.2017.02.032.
[42] Z. Abassi, T. Karram, S. Ellaham, J. Winaver, A. Hoffman, Implications of the natriuretic peptide system in the pathogenesis of heart failure: Diagnostic and therapeutic importance, Pharmacol. Ther. 102 (2004) 223–241, https://doi.org/10.1016/j.pharmthera.2004.04.004.
[43] F. Liu, T. Liang, Z. Zhang, L. Liu, J. Li, W. Dong, H. Zhang, S. Bai, L. Ma, L. Kang, Effects of altitude on human oral microbes, AMB Express 11 (2021), https://doi.org/10.1186/s13568-021-01200-0.
[44] E. Neculae, E.M. Gosav, E. Valasciuc, N. Dima, M. Floria, D.M. Tanase, The oral microbiota in valvular heart disease: current knowledge and future directions, Life. 13 (2023) 1–20, https://doi.org/10.3390/life13010182.
[45] Y. Li, M. Zhu, Y. Liu, B. Luo, J. Cui, L. Huang, K. Chen, Y. Liu, The oral microbiota and cardiometabolic health: A comprehensive review and emerging insights, Front. Immunol. 13 (2022) 1–15, https://doi.org/10.3389/fimmu.2022.1010368.
[46] C. Lazzeri, S. Valente, M. Chiostri, G.F. Gensini, Clinical significance of lactate in acute cardiac patients, World J. Cardiol. 7 (2015) 483, https://doi.org/10.4330/wjc.v7.i8.483.
[47] S. Ghimenti, T. Lomonaco, F.G. Bellagambi, D. Biagini, P. Salvo, M.G. Trivella, M. C. Scali, V. Barletta, M. Marzilli, F. Di Francesco, A. Errachid, R. Fuoco, Salivary lactate and 8-isoprostaglandin F2α as potential non-invasive biomarkers for monitoring heart failure: a pilot study, Sci. Rep. 10 (2020) 1–11, https://doi.org/10.1038/s41598-020-64112-2.
[48] I.C. Okere, T.A. McElfresh, D.Z. Brunengraber, W. Martini, J.P. Sterk, H. Huang, M. P. Chandler, H. Brunengraber, W.C. Stanley, Differential effects of heptanoate and hexanoate on myocardial citric acid cycle intermediates following ischemia-reperfusion, J. Appl. Physiol. 100 (2006) 76–82, https://doi.org/10.1152/japplphysiol.00255.2005.
[49] A.N. Carley, S.K. Maurya, M. Fasano, Y. Wang, C.H. Selzman, S.G. Drakos, E. D. Lewandowski, Short-chain fatty acids outpace ketone oxidation in the failing heart, Circulation 143 (2021) 1797–1808, https://doi.org/10.1161/CIRCULATIONAHA.120.052671.
[50] K.C. Park, S. Krywawych, E. Richard, L.R. Desviat, P. Swietach, Cardiac complications of propionic and other inherited organic acidemias, Front. Cardi. Med. 7 (2020) 1–20, https://doi.org/10.3389/fcvm.2020.617451.
[51] R. Kobayashi, Y. Ogawa, T. Hashizume-Takizawa, T. Kurita-Ochiai, Oral bacteria affect the gut microbiome and intestinal immunity, Pathog. Dis. 78 (2020) 1–9, https://doi.org/10.1093/FEMSPD/FTAA024.
[52] T. Kitai, W.H.W. Tang, Gut microbiota in cardiovascular disease and heart failure, Clin. Sci. 132 (2018) 85–91, https://doi.org/10.1042/cs20171090.