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a methodological journal

JIOMICS

Journal of Integrated OMICS

Focus and Scope

Journal of Integrated OMICS, JIOMICS, provides a forum for the publication of original research papers, preliminary communications, technical notes and critical reviews in all branches of pure and applied "-omics", such as genomics, proteomics, lipidomics, metabolomics or metallomics. The manuscripts must address methodological development. Contributions are evaluated based on established guidelines, including the fundamental nature of the study, scientific novelty, and substantial improvement or advantage over existing technology or method. Original research papers on fundamental studies, and novel sensor and instrumentation development, are especially encouraged. It is expected that improvements will also be demonstrated within the context of (or with regard to) a specific biological question; ability to promote the analysis of molecular mechanisms is of particular interest. Novel or improved applications in areas such as clinical, medicinal and biological chemistry, environmental analysis, pharmacology and materials science and engineering are welcome.

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Myosin light chain and calcium regulating protein differences in chronic musculoskeletal neck and shoulder pain

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ABSTRACT

Proteomic screening analysis has detected myosin light chain (MLC) as a protein implied to be involved in chronic musculoskeletal neck and shoulder pain. Several analyses of MLC proteins have stated a difference in phosphorylation being the determining factor for protein activation hence altered contrability of the muscle in i.e. senescence. In continuation of a previous publication, this study is an attempt to analyze the different MLC isoforms by mass spectrometry and immune-analyses in myalgic and healthy trapezius muscle. In the present study no differences in phosphorylation level between the corresponding individual proteins were detected using LC-MSMS and immunoblotting; instead we assigned different isoforms of regulatory MLCs. To further elucidate the contrability: calcium (Ca^{2+}) regulatory proteins, sarco (endo)plasmic reticulum Ca^{2+} ATPase 1 (SERCA-1) and calsequestrine (CSQ) were analyzed by western blot. The analysis revealed a significantly increased abundance of SERCA-1 protein in the myalgic muscle and a significantly increased abundance of CSQ in healthy muscle. Myalgic muscle contraction patterns have in previous studies shown to differ from healthy muscle which may be connected to the Ca^{2+} availability in the muscle. Here we present the proteomic characterization of differences in Ca^{2+} regulating proteins and particularly regulatory MLCs in trapezius muscle of women with chronic musculoskeletal neck and shoulder pain.

Keywords: calcium (Ca^{2+}), mass spectrometry, phosphorylation, muscle pain, myosin light chain, trapezius.

Abbreviations

MLC: myosin light chain; **MHC:** myosin heavy chain; **SERCA 1:** sarco(endo)plasmic reticulum Ca^{2+} ATPase 1; **CSQ:** calsequestrin; **two dimensional – differential gel analysis:** 2D-DIGE; **liquid chromatography mass spectrometry mass spectrometry:** LCMSMS.

1. Introduction

The introduction of proteomics and metabolomics to the field of work related musculoskeletal disorders [1, 2] have suggested a difference in a number of proteins and metabolites relating to chronic musculoskeletal neck and shoulder pain (or chronic trapezius myalgia). These screening studies have presented proteins correlating to an altered metabolism, different contractibility and inflammatory and acute stress responses to differ in abundance between myalgic and healthy muscle [3].

Regulatory myosin light chain 2 (MLC2) was one protein appearing more abundant in chronic trapezius myalgia, according to a proteomic systematic explorative screening of the present biopsy material [2]. MLC2 has been a protein of interest in previous studies concerning sarcopenia (ageing muscle) [4-6] and muscle inactivity, such as during bed rest [4, 7, 8]. Phosphorylation of MLC2 has previously shown to affect the calcium sensitivity of skeletal muscle contraction [9], affecting the muscles ability to contract [10]. Fast regulatory MLC2 traditionally correlate with fast contracting type 2 fibers in skeletal muscle [11]. During low load work it

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has been shown that the muscle contraction in myalgic muscle is activated by a higher contribution of type 2 motor units compared to healthy muscle [12]. These several electromyographic studies indicate altered activation patterns of the trapezius muscle in subjects with chronic neck-shoulder pain conditions [13-15]. There is also discussed whether painful muscles are disused and clinically deconditioned [16]. One or both of these circumstances may be associated with altered contractility. A better understanding of molecular events related to contractility aspects can facilitate the development of targeted treatment interventions in subjects with chronic myalgia. Introducing proteins of Ca^{2+} regulation into this area of research may explain the excitation-coupling properties of the myalgic muscle. The result from the proteomic study suggesting the Ca^{2+} regulatory MLC2 fast to be more abundant in myalgic muscle [2], are one protein that may explain the differences in muscle contractility. The muscular symptoms associated with chronic trapezius myalgia are stiffness, tension and a decreased maximal force torque rate compared to healthy muscle [17, 18]; and also the inability to relax the muscle during the naturally occurring pauses during dynamic work [19, 20].

In muscle contraction Ca^{2+} is regulating the contractility of the muscle and the regulatory myosin light chains, situated on the myosin head regulates the calcium sensitivity of the myosin molecule [21]. Sarco(endo)plasmic reticulum Ca^{2+}ATPase 1 (SERCA-1) transfers Ca^{2+} from the cytosol of the cell to the lumen of the sarcoplasmatic reticulum. SERCA-1 is predominately abundant in type 2 fibers [22]. Calsequestrine (CSQ) is the most abundant Ca^{2+} binding protein of the sarcoplasmatic reiculum in skeletal muscle [23]. CSQ releases Ca^{2+} upon muscle contraction and are available in two different isoforms, CSQ1 predominantly in type 2 muscle fibers and CSQ2 predominantly in type 1 [23]. CSQ buffer Ca^{2+} in the sarcoplasmatic reticulum thus assisting SERCA to not having to pump Ca^{2+} against a high concentration gradient [24]. Accumulation of Ca^{2+} in the muscle has been shown in rats exposed to prolonged chronic low frequency muscle stimulation and is hence proposed to be a mechanism behind chronic muscle pain [25, 26]. The exact mechanisms behind this accumulation is yet not known, but serves as one of many hypothesis [27] behind the underlying pathophysiological mechanisms of chronic trapezius myalgia.

This study aims to characterize on proteomic level changes in Ca^{2+} regulating proteins in trapezius muscle of women with chronic trapezius myalgia and, in particular, to verify the possible difference in abundance of the phosphorylation of regulatory myosin light chain protein in healthy and myalgic trapezius muscle.

2. Material and Methods

The subjects, clinical examination, biopsy collection and Two-Dimensional Difference Gel Electrophoresis (2-D DIGE) image analysis are presented in a previously published paper presenting a proteomic screening analysis of myalgic and healthy muscle [2]. In short the 2D-DIGE method was applied on frozen muscle samples that were suspended in lysis buffer and homogenized followed by protein quantification. Labelling of protein samples was made with CyDye minimal dyes, Cy2, Cy3 and Cy5, in accordance to manufacturers' protocol. The internal standard method was used [28]. The samples were applied onto 24 cm 3-11 Non-Linear (NL) Immobilised pH gradient (IPG) strips and rehydrated at room temperature in the dark for 16 hours. The first dimension was run using Amersham Ettan™ IPGphor unit. Prior to the second dimension the gel strips was equilibrated and alkylated. The second dimension was conducted by using the Ettan DALT six apparatus. Gels were then immediately scanned with a Typhoon™ 9410 scanner. The scanned image was further processed using ImageQuant™ V5.2 and DeCyder™ V6.5. The 2D-DIGE protocol used has previously been evaluated [29]. A more detailed description of the analysis is available in Hadrevi et al 2013 [2].

Additional analysis and identification of the myosin light chain protein spots appearing on 2D-gels that according to a number of publications previously presented [4, 6, 7] are to be separated due to their individual level of phosphorylation. The main objective of our study is to identify the differences in phosphorylation between the different protein spots separated on a 2D-gel that are of interest in the previous screening analysis [2], by utilizing phosphorylation analysis and high resolution mass spectroscopy, in accordance to the methods presented below:

2.1. Phosphorylation analysis

A preparative gel (600 µg protein) stained with Sypro Ruby was used for analysis by mass spectrometry. 10 protein spots corresponding to the different isoforms of myosin light chains were excised and in-gel digestion was performed according to previously described protocols [30]. The samples were analyzed by mass spectrometry. 200 µg proteins were separated by 2D-PAGE. The ten protein spots corresponding to the different isoforms of myosin light chains were transferred from the gel by electroblotting to a PVDF membrane, blocked and incubated overnight with phosphor-threonine or phospho-tyrosine antibody (Cell Signaling Technology, Danvers, MA). Immunoreactive protein spots were detected using ECL prime detection system (GE Healthcare).

2.2. High-Resolution Mass Spectrometry

The obtained peptide mixtures were analyzed by LC-MSMS. Separation was done using nano-flow HPLC system (EASY-nLC II, Thermo Fisher Scientific) and data were acquired using LTQ Orbitrap Velos Pro hybrid mass spectrometer (Thermo Fisher Scientific). Peptides were separated during 90 min by reverse phase chromatography on a 20 mm×100 μm C18 pre column followed by a 100 mm×75 μm C18 column (particle size 5 μm, NanoSeparations, Netherlands) at a flow rate 300 nL/min. A gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was distributed as follows: starting with 3% B; linear gradient 3%–35% B in 0–60 min; 35%–90% B in 60–90 min. Automated online analyses were performed with LTQ Orbitrap Velos Pro hybrid mass spectrometer (Thermo Fisher Scientific) with nano electrospray source; 240°C capillary temperature; spray voltage, 2200 V. MS spectra were acquired in profile mode by FTMS at resolution of 30.000 (at m/z 400). Top 20 most intense multiply charged ions from were selected with an isolation window of 2.0 and fragmented in the linear ion-trap by collision induced dissociation with normalized collision energy of 30. Dynamic exclusion of sequenced peptides for 60 s and charge state filtering disqualifying singly charged peptides were activated and predictive AGC was enabled. Centroid mode was used for CID MS/MS.

2.3. Database Searches and Data Interpretation

Obtained data files were analyzed by Proteome Discoverer 1.4 (Thermo Fisher Scientific) and the search algorithm SEQUEST was used against UniProt release 2013_09 Homo sapiens database. Carbamidomethylation of cysteine residues was set as static modification, and phosphorylation of threonine and serine and oxidation of methionine were set as variable modifications. Trypsin was used as the enzyme with 2 missed cleavages allowed. The precursor mass tolerance was set to 10 ppm with 0.6 Da fragment mass tolerance. Proteins were identified with at list 2 peptides of rank 1 with high score and target false discovery rate 0.01.

2.4. SERCA-1 and Calsequestrin analysis

Muscle biopsies were heat stabilized using Stabilizor T1 device (Denator AB, Sweden). The biopsies were homogenized in lysis buffer (Protein Simple, Santa Clara, CA) and by sonication 3 × 10 s. The samples were stored for 2 h at 4°C and then centrifuged for 1 h at 4×10^4 *g* at 5°C. The supernatant was then removed to a new tube. Total protein concentrations were measured by 2-D Quant kit (GE Healthcare) according to the manufactures recommendation. Samples were diluted to adjust protein concentration to 1 μg/μl in 5 μl with sample buffer. The quantification of expression level of SERCA-1 was performed by a novel nanovolume capillary electrophoresis based protein analysis, Simple western size/charge assay instrument Peggy™ [31]. The lysate samples were mixed with a master mix containing 80 mM DTT, fluorescent molecular markers and heated at 95°C for 10 minutes. The samples, blocking reagents, primary antibody, secondary antibody and chemiluminescence substrate were loaded onto a 384-well plate according to the user manual provided by the company (Protein Simple, Santa Clara, CA). The primary antibody (SERCA-1; Abcam, Calsequestrin;Novus Biological) was diluted in antibody diluent in a 1:50 dilution. The chemiluminescence signals were digitized using a charge coupled camera (CCD). The digital images were analyzed with Compass software (ProteinSimple), and the quantified data of the detected protein were reported as molecular weight and peak area.

2.5. Statistical analysis

The proteomic data was analyzed using a multivariate projection model, projections to latent structures with discriminant analysis (PLS-DA) [32]. PLS-DA is a supervised linear regression method used to find a relationship between X variables (spot volume ratios) and a binary Y vector by using class membership data (MYA/CON). The spot volume ratios were mean centered and scaled for unified variance. The number of PLS-DA components was determined by full cross-validation (SIMCA-P version 12, Umetrics AB, Umeå, Sweden). The PLS-DA model was also validated by both response permutation tests and CV-ANOVA on the residuals [33]. To identify spot of interest variable of importance value (VIP)>1 was used.

For the traditional statistical analyses IBM SPSS (version 22) was used. Mann-Whitney U test was used for the group comparisons of SERCA-1 and CSQ. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Myosin light chains

Proteins of interest in this analysis are MLC proteins and adjacent protein spots in the 2D gel analysis presented in figure 1A. Previous studies have presented the 2D-gel protein pattern of human skeletal muscle [34] and the possible correlation between a regulatory MLC and myalgia [2]. PLS-DA of the 2D- protein pattern from 12 female cleaners with work-related trapezius myalgia and 12 pain free female cleaners reveals the importance of the ten investigated proteins in separating between MYA and CON. Proteins with VIP>1 were, two fast regulatory MLC, *H3BN54* and *H3BML9*) (VIP 1.41 and 1.04 respectively) and one slow MLC *Q6IB42* (VIP 1.09) (figure 1B). The other proteins showed VIP<1. The ten protein spots (figure 1A) were analyzed by mass spectrometry. The results showed that the ten spots were different proteins with different molecular weight and pI. The sequences of each individual protein isoform, obtained from the LC-MS analysis are here presented as supplement information. Phosphorylated peptides were detected at one position in spot 4-5 (table 1). There were no phosphorylated peptides in spot 6-10. The

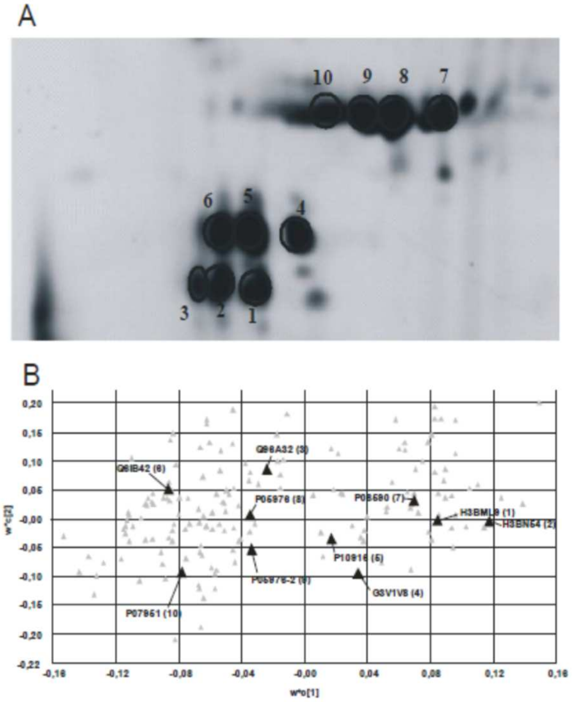

Figure 1. A) The typical 2D-gel image of muscle protein extract enlarged at 4.9-5.4 pH (theoretical) range. The marked spots are referred to the spot numbers in table 1. B) PLS-DA weight plot (w*c[1]/w*c[2]) with proteins involved in the separation of MYA (right) and CON (left). PLS-DA model: (Q^2(cum) = 0.65, (R^2(cum) =0.29, component 1: 16%, component 2: 13 % explained variance). Identified myosin light chain proteins included in the analysis are marked with larger black triangles. Smaller gray triangles are other proteins within the PLS-DA model (2).

immunological investigation of phosphorylation of the ten proteins could not detect any tyrosine phosphorylation on the proteins. No significant difference in threonine phosphorylation was detected on spots corresponding to the different MLCs.

3.2. Proteins of calcium regulation

The immunological automated western blot detection of SERCA-1 and CSQ-1 showed that the level of SERCA-1 was significantly higher (p = 0.017, figure 2A) in myalgic muscle compared to healthy, indicative of an increased activity in calcium channel activity connected to calcium release ad uptake actions in the SR of the muscle regulating the contractile actions of the muscle. The level of CSQ was decreased (p = 0.010, figure 2B) in myalgic muscle compared to healthy muscle, suggesting a decreased calcium buffering capacity in the myalgic muscle. These significant results support the results from the myosin light chain analysis suggesting an altered calcium regulation in the myalgic muscle.

4. Discussion

The performed LC-MSMS analysis allowed us for the first time to pinpoint and characterize 9 myosin light chain isoforms (table 1) previously named as differentially phosphorylated isoforms of MLC. Surprisingly, no difference in phosphorylation between spots was detected according to present MS-analyses, additionally confirmed both by serine and tyrosine phosphorylation immunodetection on 2D gels. Phosphorylation of myosin light chain proteins has in previous studies been attributed to contractile function of the myosin molecule [11], additional results are presented showing phosphorylated and dephosphorylated muscles develop the same isometric tension at full Ca^{2+} saturation [35]. In rats an increased phosphorylation due to increased neuronal activity is observed when the muscle transitions from slow to fast muscle [36, 37]. Altered phosphorylation of MLC may also be indicative of disease [38]. Transformation of fast isoforms of MLC to slow isoforms has shown to occur together with MHC transformation, according to proteomic profiling of senescent muscle [4-6]. This has also been evidential in senescent muscle rat models [4] where increases of MLC slow has been shown. Together the MHCs and the MLCs dictate the contractile function of the myosin molecule. Laboratory experiments on rat conclude that hybrid fibers, fibers expressing different isoforms of MHC and MLC, appear in muscle transition when a muscle remodel from slow to fast. Recent studies indicate an increased remodeling of the myalgic muscle due to an increased amount of satellite cells and metabolites connected to muscle remodeling [39, 40].

To further elucidate the potential difference in muscle Ca^{2+} regulation the abundance of a calcium regulatory protein analysis of the Ca^{2+} channel protein SERCA-1 [22] and CSQ, a protein of Ca^{2+} storing [41] were performed. The influence of Ca^{2+} levels and the pathophysiological mechanisms of trapezius myalgia have been discussed by Gissel et al 2005 [25] presenting the Ca^{2+}accumulation theory [26]. Gissel et al. have in laboratory animal models shown that low force static work induces Ca^{2+} accumulation in the muscle subjected to long term stimulation, causing a leakage of lactate dehydrogenase that will induce a pain potentiation. Lactate is a substrate of anaerobic metabolism which has been a marker of muscle damage [42] but recent studies have shown that it is an energy substrate of anaerobic metabolism being transported out of the cell by the lactate shuttle acting as fuel elsewhere [42-46]. The synthesization of glycogen is also increased in anaerobic conditions [47, 48] and increased glycogen has been shown to have an impact on Ca^{2+} release in the skeletal muscle [49, 50]. In coherence, enzymes active in the glycogen transformation, metabolizing glycogen in the glycolysis, has been shown to occur with a higher abundance in myalgic muscle compared to healthy [2].

Table 1. Myosin light chain (MLC) protein isoforms identified from trapezius muscle by 2D gel and following mass spectrometry analyses. The spot numbers are referred to the marked spots in figure 2. Sequenced phosphorylated peptides are shown. VIP = variable of importance in projection from the PLS-DA analysis (figure 1B).

Spot no.	Identified protein	Uniprot. Acc. no.	pI #	Mw # (kDa)	Seq.Cov. (%)	Phosphorylated peptides	VIP
1	Fast skeletal MLC2 regulatory	H3BML9	5.21	15.6	72.9	TVEGGsssVFsMFDQTQIQEFK	1.04
2	Fast skeletal MLC2 regulatory	H3BN54	5.01	15.6	73.3	TVEGGsssVFsMFDQTQIQEFK	1.41
3	Fast skeletal MLC2 regulatory	Q96A32	4.90	15.6	77.5	TVEGGsssVFsMFDQTQIQEFK	0.56
4	Slow MLC, ventricular/ cardiac isoform	G3V1V8	5.31	18.0	67.1	AGGANsNVFsMFEQTQIQEFK	0.65
5	Slow MLC, ventricular/cardiac isoform	P10916	5.21	18.0	67.5	AGGANsNVFSMFEQTQIQEFK	0.26
6	Slow MLC, ventricular/ cardiac isoform	Q6IB42	5.10	18.0	48.6		1.09
7	Ventricular/ slow twitch MLC alkali	P08590	5.41	22.1	57.4		0.88
8	MLC 1/3, skeletal muscle isoform	P05976	5.35	22.1	76.8		0.41
9	MLC3 of MLC 1/3, skeletal muscle isoform	P05976-2	5.32	22.1	80.0		0.45
10	Tropomyosin beta chain	P07951	5.30	22.1	74.9		0.99

 # Calculated.

In the present study, myalgic muscle exhibits a significantly increased abundance of SERCA-1 compared to healthy muscle (figure 2A). The SERCA-1 protein has shown to exist in both types of fibers but in a considerably higher abundance in type 2 fibers [51]. The increased abundance of SERCA-1 in myalgic muscle indicates a difference in the contractile characteristics of myalgic muscle compared to healthy muscle. Previous studies have shown an impaired ability to relax the myalgic muscle during the pauses that are occurring in a standardized dynamic work [20]. Since the SERCA-1 proteins are determinants of the Ca^{2+} influx and *efflux* of the muscle sarcolemma, where the efflux is crucial for the muscle relaxation rate. In this material where there is no difference in fiber type content [52], though the high amount of SERCA-1 is indicative of fast twitch characteristics of myalgic muscle as it resequesters Ca^{2+} enabling a fast relaxation of the muscle. When CSQ subsequentially binds Ca^{2+} it will buffer and keep the Ca^{2+} comparatively low and still have a large amount of Ca^{2+} ready to be released to produce muscle contraction [23, 41]. SERCA also enables leakage out of the SR and since the Ca^{2+} uptake by SERCA is energy consuming where a higher concentration of ADP will reduce Ca^{2+} uptake in the SR [24].

Fast twitch fiber normally generates force in a rapid manner, in comparison to slow twitch type 1 fibers. Fast twitch fibers hence needs to have a substantial amount of Ca^{2+} stored in the SR to be released rapidly upon stimulation [53] in order to saturate the calcium binding sites on troponin C. Recent laboratory investigations have shown a reduced rate of torque development in patients suffering from trapezius myalgia [17, 18]. CSQ is a protein that buffers Ca^{2+} in order to upon strong stimuli release Ca^{2+} to enable muscle contraction. Type 2 fibers have been shown to be more dependent on CSQ availability compared to type 1, due to their specific contractile properties [54]. CSQ knockout mice show a reduced ability to quickly generate maximal force during muscle contraction [54-56], similar the muscular traits of chronic muscle myalgia [17, 18, 20]. Also, CSQ could be connected to an increased fatigability of the myalgic muscle. The lower abundance of CSQ in myalgic muscle compared to healthy muscle may explain the muscular traits of the myalgic muscle although this specific correlation has not yet been presented.

Also, a proposed pain inducing calcium accumulation could be obtained by a lack of buffering capacity in the SR and energy deficits disturbing ATP consuming SERCA

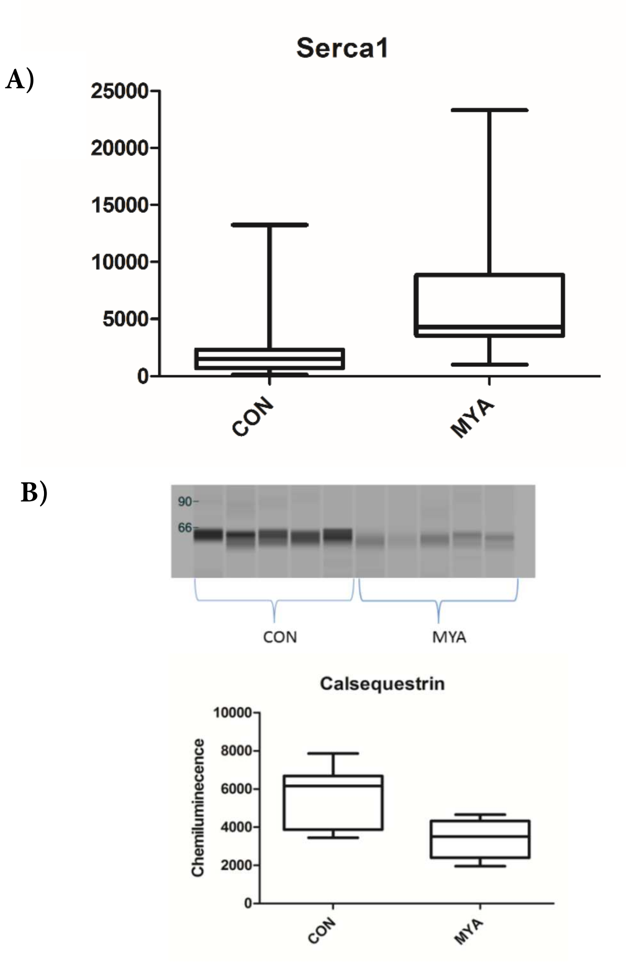

Figure 2: A) SERCA-1 significantly more abundant in myalgic muscle (MYA n=11) compared to healthy (CON, n=11) p=0.017. B) CSQ is significantly less abundant in myalgic muscle (MYA) compared to healthy (CON) p=0.010, bars describe variation, and boxes median values. The abundance of CSQ illustrated by gel from western blot analysis of myalgic patients (MYA) and healthy (CON).

activity. Though, a more comprehensive analysis needs to be performed with specific antibodies analysis of the different CSQ isoforms and protein abundance in individual muscle fibers. In addition the rate at which SERCA moves Ca^{2+} across the SR membrane can be controlled by the regulatory proteins phospholamban (PLB) [57] and sarcolipin [58], reducing the rate of Ca^{2+} movement. Future studies are needed to elucidate the relationship between the different Ca^{2+} regulatory proteins and their inhibitors in myalgic muscle to understand the mechanisms of muscle pain.

In conclusion, our results show an increased abundance of fast regulatory MLC, a higher abundance of SERCA-1 proteins and a lower abundance of CSQ in myalgic muscle compared to healthy. On the basis of previous knowledge, the phosphorylations of the proteins are analyzed showing no difference in phosphorylation between individual MHC protein 2D-gel spots. Instead the MLC proteins are characterized presenting different isoforms of the MLC. The presented results indicate differences between healthy subjects and subjects with chronic trapezius myalgia with respect to contractile molecular events. The results have to be confirmed in other studies and also elucidating if the cause e.g. pain per se, different activation pattern or deconditioning/disuse. Such knowledge will be of importance for designing targeted treatment interventions for patients with chronic myalgia. Muscle stiffness and weakness, increased muscle tension and a reduced capacity to rapidly generate force are some of the muscular symptoms of chronic work related muscle pain. Increasing the understanding regarding the biochemical mechanisms of muscle myalgia will render increased knowledge concerning the concrete muscular symptoms of myalgia.

5. Concluding Remarks

The present work contributes to the understanding of the complexity of mechanisms of muscular adaptation and especially with relevance for contractile properties due to work related muscle pain. In this study we suggest that the physiological mechanisms behind the previously shown differences in force output and endurance in muscle contraction in myalgic patients may have a relation to the pathological alterations of proteins connected to the calcium regulation of the muscle. When investigating other proteins of calcium regulation there is an evident significant difference in their abundance comparing healthy and myalgic muscle. The increased abundance of the calcium ion channel protein SERCA-1 suggests an increased cellular exchange of calcium in the myalgic muscle. In addition there is a lower abundance of the calcium buffering protein CSQ in myalgic muscle which considering previous evidence are a protein prone to cause the intrinsic contractile properties of chronic work related myalgia. Further investigations regarding calcium content in the muscle needs to be done before any conclusion regarding the calcium activity and its relation to the contractile properties of myalgic muscle can be made.

6. Supplementary material

Supplement information: Protein sequences and phosphorylated sites of detected proteins presented in table 1.

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A DIGE proteomic analysis of wheat flag leaf treated with TERRA-SORB® foliar, a free amino acid high content biostimulant

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ABSTRACT

The flag leaf is the most important source of carbohydrate during wheat kernel filling. Around a 75% of all sugars stored in the kernel come from carbon fixed by this leaf. Terra-Sorb® foliar is an L-α-amino acid-based product from enzymatic hydrolysis for foliar application with a high ratio of free to total amino acids. Previous agronomical studies carried out on grassy, horticultural and tree crops have shown that the application of Terra-Sorb® increases photosynthetic plant activity and chlorophyll content, promotes rapid recovery from stress and improves fruit set.

In this work, we have undertaken a proteomic approach to explore molecular mechanisms potentially involved in the stimulating effect of Terra-Sorb® Foliar on wheat yield when applied in commercial fields. Wheat plants at the flag leaf stage were treated, and a DIGE approach was used to compare the proteomes of treated vs. control plants in four biological replicates.

Thirty-seven protein spots were found to change in abundance (ANOVA $p<0.05$) out of which 8 were down-regulated and 29 up-regulated in treated leaves. Twenty protein spots ($1.2<$fold change <1.9) encoded by 11 different genes were successfully identified by nLC-ESI-MS/MS and NCBInr database search.

The deregulated proteins identified were mainly related to the life cycle of Rubisco. Importantly, two proteins involved in the positive regulation of Rubisco activity, namely Rubisco activase, and the large subunit of Rubisco binding protein, were found up-regulated in treated plants, suggesting a better performance of Rubisco. Down-regulated proteins were of metabolic and anti-stress enzymes, including Cu/Zn superoxide dismutase that protects photosystem II from photooxidation.

In conclusion, significant changes were shown to occur in the wheat flag leaf proteome upon Terra-Sorb® Foliar application. The deregulated proteins identified are directly or indirectly involved in the CO_2 fixation which may correlate with the known stimulating effect of Terra-Sorb® Foliar of wheat yield, although further functional experiments are needed to validate the proposed hypothesis.

Keywords: DIGE, leaf, plant biostimulant, proteomics, quantitative, wheat.

Abbreviations

ATP-CF1-A: ATP synthase CF1**; BBCH-scale:** Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie**; CS:** citrate synthase**; DIGE:** differential gel electrophoresis**; EF-Tu:** elongation factor Tu**; EF-Tu Chl:** elongation factor Tu chloroplastic**; GA3PDH:** glyceraldehyde-3-phosphate dehydrogenase**; HSP-90:** heat shock protein 90**; nLC-ESI-MS/MS:** nano-liquid chromatography coupled to tandem mass spectrometry; **Rubisco:** Ribulose-1,5-bisphosphate carboxylase/oxygenase; **RBA:** Rubisco activase; **Rubisco LBP A and B:** Rubisco large-subunit binding protein, subunit alpha and beta; **Rubisco-L:** Rubisco large subunit; **SOD:** superoxide dismutase.

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1. Introduction

The exponential intensification of agricultural practices over the last decades, forced a widespread use of fertilizers and pesticides which has caused an increasing impact on the quality of ground and surface waters and food products [1]. Fertilization sources include inorganic salts, mainly chlorures, sulfates, nitrates and organic or synthetic chelants, used to introduce ions through the foliar cuticle layer [2]. Nitrogen fertilization of crops by a high content of nitrates (NO_3^-) leaves behind 50% of nitrogen applied as a residue that can move throughout the soil into groundwater [3]. The rise of nitrates is a potential hazard to human and animal life [4].

Nowadays, healthy food and environmental protection have been top priorities in scientific research. In recent years, innovations in production have been evolving toward low-cost, organic, sustainable, and environmentally friendly systems that must ensure the high yield and quality of crops. Some authors have proposed the use of biostimulants in plant nutrition instead of inorganic fertilizers to improve the nutrient uptake or their utilization by plants [5].

Terra-Sorb® foliar is an L-α-amino acid-based product from enzymatic hydrolysis of proteins of animal origin that has been specially developed by the R&D Department of Bioiberica, S.A. It is composed of 9.3% of free protein amino acids, namely Asp, Ser, Glu, Gly, His, Arg, Thr, Ala, Pro, Cys, Tyr, Val, Met, Lys, Ile, Leu, Phe and Trp which are the most abundant.

In this report, we studied the effect of the TerraSorb® Foliar on wheat (*Triticum aestivum* L.) flag leaf. A critical stage in the development of wheat plants is the emergence of the final leaf called the flag leaf. At this point, the emphasis of management needs to shift to its protection, as the flag leaf is critical for attaining high grain yields. The flag leaf is the most important source of carbohydrate during wheat kernel filling. Around a 75% of all sugars stored in the kernel come from carbon fixed by this leaf [6]. Plants invest an important amount of the photosynthetic energy in biosynthesis of amino acids for protein synthesis. The application of exogenous amino acids via foliar has been shown in previous agronomical studies carried out on grassy, horticultural and tree crops to result in an increase of photosynthetic plant activity and chlorophyll content, promotion of rapid recovery from stress and improvement of fruit set [7, 8, 9].

Proteomics has been shown as a successful approach to analyze the response of plants to external stimulus [10]. In this work, we have undertaken a proteomic quantitative approach based on DIGE technique to explore molecular mechanisms potentially involved in the stimulating effect of Terra-Sorb® foliar on wheat yield when applied in commercial fields.

2. Material and Methods

2.1. Plant material

Leaf samples from wheat (*Triticum aestivum* L.) were sampled at 39 BBCH stage [11] in eight 2 x 10 m plots in an experimental field in Totana (Murcia, Spain). Four plots separated 5 m from control plots were treated with the biostimulant TerraSorb® foliar (Bioibérica S.A). The product was applied at 500 g-ml/ha real doses. After 48h and 72 h of exposure, wheat flag leaves were harvested.

2.2. Protein extraction

Wheat leaves (0.3 g) were pulverized by liquid nitrogen. The frozen powdered was resuspended in 0.5 ml ascorbic acid 10 mM and 0.1 ml protease inhibitor cocktail. Then, 9 ml of extraction buffer (TCA 10% (w/v), 0.07% DTT (w/v)) were added, vortexed and incubated at -20ºC overnight. This homogenate was centrifuged at 15000 x g during 20 min at 4ºC, the supernatant was discarded and the pellet resuspended in wash buffer (0.07% (w/v) DTT in acetone), vortexed and incubated at least 1 h. This procedure was repeated once. Then, washes were performed with ethyl acetate: ethanol (1:2) and then in TCA 10% (w/v) and finally in TCA 10% (w/v) in acetone and finally the pellet was dried at 4ºC. Subsequently the washed pellet was homogenized in 3 ml of extraction buffer (0.7 M sucrose, 0.1M KCl, 0.5M Tris-HCl pH 7.5, 50 mM EDTA, 1% (w/v) PVPP, 1% (w/v) DTT and a cocktail of protease inhibitors) and incubated shaking during 40 min at 4ºC. An equal volume of Tris-saturated phenol pH 7.5 (AppliChem, Darmstadt, Germany) was added and the mixture incubated for 20 min at 15000 x g at 4ºC. The upper phenol phase was recovered and the aqueous phase was submitted to a second phenol extraction. Both phenol phases were pooled and washed twice with an equal volume of phenol washing buffer adjusted to pH 7.0 (0.7 M sucrose, 0.1 M KCl, 0.5 M Tris, 50 mM EDTA, 1% DTT, and a cocktail of protease inhibitors). The recovered phenol was precipitated with 5 vols of 0.1 M ammonium acetate (w/v) in methanol overnight. The precipitate was washed three times in 0.1 M ammonium acetate (w/v) in methanol and twice in 80% acetone (v/v).

2.3. Labeling of proteins with CyDye

The precipitated and air-dried proteins were solubilized in labeling buffer (7 M urea, 2 M thiourea, 30 mM Tris-HCl, 4% CHAPS pH 9.0). Insoluble material was pelleted by centrifugation (12000 x g, room temperature, 5 min). The protein concentration in the supernatant was measured using the RCDC method (Bio-Rad, Madrid, Spain).

Two hundred micrograms of protein was diluted to 1 µg/µl with labeling buffer and further cleaned with the Ettan 2D Clean-up kit (GE Healthcare, Madrid, Spain) according to the manufacturer's recommendations. The recovered precipitated protein was solubilized in 30 µl of labeling buffer, the pH was adjusted to 8.5 using NaOH (100 mM), and the protein content was measured again. For labeling of the proteins, 400 pmol of CyDye in 1 µl was mixed with 18 µl of the sample containing 50 µg of protein and incubated on ice for 30 min in the dark. The labeling reaction was terminated by adding 1 µl of 10 mM lysine. Each sample was covalently labeled with a fluorophore, either with Cy3 or Cy5. A mixture containing equal amount of proteins from every sample in the experiment was labeled with Cy2 and used as internal standard.

2.3.1. 2-DE and image scanning

For analytical 2-D DIGE analysis 20 µl each of Cy3-, Cy5-, and Cy2-labeled sample (150 µg of protein) were combined, mixed with 60 µl of sample buffer 1 (7 M urea, 2 M thiourea, 30 mM Tris-HCl, 4% CHAPS, 100 mM DTT), and incubated for 10 min on ice. Then, samples were diluted to 150 µl with sample buffer 2 (7 M urea, 2 M thiourea, 30 mM Tris-HCl, 4% CHAPS, 50 mM DTT) and submitted by cup-loading to IEF as the first-dimension separation. IPG strips, pH 4-7, 24-cm (GE Healthcare) were swollen overnight in 450 µl of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 0.5% IPG buffer, 0.005% bromophenol blue). IEF was performed at 20ºC on an IPGphor Unit (GE Healthcare/Amersham Biosciences) using the following settings: 3 h at 300 V, 6 h gradient from 300 to 1000 V, 3 h gradient from 1000 to 8000 V, 5 h at 8000 V until an accumulated voltage of 77 kVh was achieved. Strips were equilibrated after focusing in two steps: 30 min in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol (v/v), 2% SDS (w/v)) supplemented with 1% DTT followed by 30 min in equilibration buffer supplemented with 1.25% iodoacetamide. SDS-PAGE was carried out as the second-dimension separation in 12.5% acrylamide gels in an Ettan Dalt-six (GE Healthcare/Amersham Biosciences) vertical unit. The separation was run overnight: first step at 80 V, 10 mA/gel, and 1 W/gel for 1 h, second step at 150 V, 12 mA/gel, and 2 W/gel until bromophenol blue line reached to the bottom of the gel. Images of the Cy3-, Cy2-, and Cy5-labeled samples were acquired in a Typhoon 9410 scanner (GE Healthcare/Amersham Biosciences) according to the manufacturer's recommendations.

2.3.2. DIGE image analysis

DIGE images were analyzed using the Progenesis SameSpots v3.0 software (Non-linear Dynamics, Newcastle, UK). First, the images were aligned. The most prominent spots were used to manually assign vectors to the digitized images within each gel and then the automatic vector tool was used to add additional vectors, which were manually revised and edited for correction when necessary. These vectors were used to warp and align gel images with a reference image of one internal standard across and within each gel. After automatic spot detection, spots were manually revised with edit tools for correct detection. Gel groups were established according to the experimental design and spot normalized volume was used to select statistically significant (fold-change, ANOVA, false discovery rate) differentiated spots between conditions analyzed in the experiment. The abundance patterns of the selected spots were analyzed and grouped by hierarchical clustering and assessed by principal component analysis implemented in Progenesis SameSpots. Spot picking and tryptic digestion Preparative 2-D gels loaded with 1.8 mg of protein were used for spot picking. After that 2-DE the gel was stained with the colloidal Coomassie blue G250 method [12], scanned in a transmission-light densitometer (Image Scanner; GE Healthcare), and aligned with the DIGE reference image with SameSpots to outline the spots of interest selected in the previous analysis. The spots were excised using a manual spot picker (The Gel Company, Tübingen, Germany) with a 1.5 mm diameter picker head. Trypsin in-gel digestion [13] was carried out in a Progest (Genomic Solutions, Huntingdon, UK) automatic in-gel protein digestor according to the manufacturer's recommendations for colloidal Coomassie Brilliant blue-stained samples. The gel plugs were extensively washed to remove dye and SDS impurities with 25 mM ammonium bicarbonate, in-gel reduced with 60 mM DTT and S-alkylated with excess iodoacetamide followed by digestion with modified porcine trypsin (Promega, Madison, WI, USA) at 37 ºC for 6 h. Peptides were extracted into ammonium bicarbonate, then into 70% acetonitrile (ACN) and finally into 1% formic acid (FA). Extracted peptides were dried down in a speed-vac bench-top centrifuge and resuspended in 0.1% FA (typically 10 µl).

2.3.3. MS-based protein identification

The tryptic fragments were analyzed by LC-MS/MS using an Agilent 1100 series nano-HPLC system lined on an XCTplus ion trap mass spectrometer (Agilent) equipped with a nano-ESI source. Sample concentration and desalting were performed on a Zorbax 300SB-C18 trap column (0.3 x 5 mm, 5 µm) at 0.3 µl/min while peptide separation was achieved on a Zorbax 300SB-C18 analytical column (75 µm x 15 cm, 3.5 µm) using a 30-min linear gradient of 5-35% ACN containing 0.1% (v/v) FA at a constant flow rate of 0.3 µl/min. MS and MS/MS spectra were acquired in the standard enhanced mode (26 000 m/z per second) and the ultrascan mode (8100 m/z per second), respectively. Mass spectrometer settings for MS/MS analyses included an ionization potential of 1.8 kV and an ICC smart target (number of ions in the trap before scan out) of 400 000 or 150 ms of accumulation. MS/MS analyses were performed

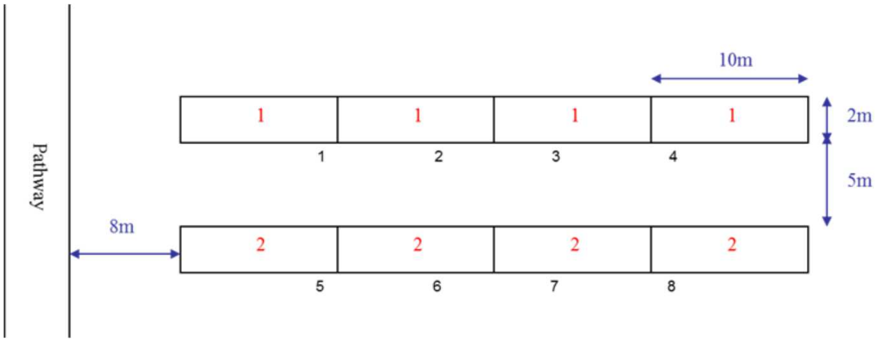

Figure 1. Wheat plants were cultivated in field (Totana, Albacete). This scheme represents an overview of plots disposal where plots 1 untreated a considered as control plants and plots 2 were treated with TerraSorb® foliar. Numbers 1, 2, 5, 6 were plots where plants were harvested two days post-treatment and 3, 4, 7, 8 plots were plots where plants were harvested three days post-treatment.

using automated switching with a preference for doubly charged ions and a threshold of 105 counts and 1.3 V fragmentation amplitude. Each MS/MS spectra dataset (1200 spectra/run) was processed to determine monoisotopic masses and charge states, to merge MS/MS spectra with the same precursor ($\Delta m/z<1.4$ Da and chromatographic $\Delta t<15$ s) and to select high-quality spectra with the Extraction tool of the Spectrum Mill Proteomics Workbench (Agilent) (SMPW). The reduced dataset was searched against the NCBInr in the identity mode with the MS/MS Search tool of the SMPW using the following parameters: trypsin, up to two missed cleavages, fixed modification S-carbamidomethyl, and a mass tolerance of 2.5 Da for the precursor and 0.7 Da for product ions. Peptide hits were validated first in the peptide mode and then in the protein mode according to the manufacturer's recommended score settings. Briefly, identities interpreted for individual spectra were automatically designated as valid by applying the following scoring threshold criteria to all spectra, protein details mode: protein score > 20, peptide details mode (score, Scored Percent Intensity, delta rank1-rank2) in the following order: peptide charge +2 (> 6, > 60 %, > 2), peptide charge +1 (> 6, > 70 %, > 2), peptide charge +3 (> 8, > 70 %, > 2), peptide charge +4 (> 8, > 70 %, > 2), peptide charge +5 (> 12, > 70 %, > 2) and peptide charge +2 (> 6, > 90 %, > 1). Validation was edited to filter out those peptides with equal scoring in the direct and reversed search and Δmass (|observed - expected|) > 15 ppm. Validated files were summarized in the protein mode to assemble peptides into proteins in order to generate the minimal protein list that best explains the matched peptides. A protein was considered identified with a minimum of two different peptides and a score above threshold marked for the SMPW search engine.

3. Results

3.1. Selection of plant material for differential proteomic analysis

Wheat plants (*Triticum aestivum* L.) were cultivated in field under not controlled conditions in an experimental land in Totana. When wheat plants reached the flag leaf stage (39 BBCH) were exposed to TerraSorb® foliar biostimulant as described in Materials and Methods. After two and three days following foliar spraying treatment according to the scheme in Figure 1, flag leaves were sampled for further proteomic analyses.

Protein extractions were performed from the whole wheat flag leaf without pod considering one leaf as a single individual sample. Four biological replicates were considered in the experiment. Each leaf had an estimated fresh weight of 0.3 g and was processed for protein extraction using a modified method based on the robust phenol/ammonium-methanol precipitation according to Martínez-Esteso et al., (2011) [14]. The amount of protein extracted was statistically independent (ANOVA<0.01) from the applied treatment obtaining a protein yield of 32.4 ± 3.5 mg protein/g fresh tissue for treated samples and 39.9 ± 6.5 mg protein/g fresh tissue for control samples.

Table 1. Experimental design of 2-D DIGE experiment.

Gel number	Cy2 (Standard)	Cy5 (sample plot/days post-treatment)	Cy3 (sample plot/days post-treatment)
1	Control+treatment	Control plot 1/2	Treatment plot 7/3
2	Control+treatment	Treatment plot 5/2	Control plot 3/3
3	Control+treatment	Treatment plot 6/2	Control plot 4/3
4	Control+treatment	Control plot 2/2	Treatment plot 8/3

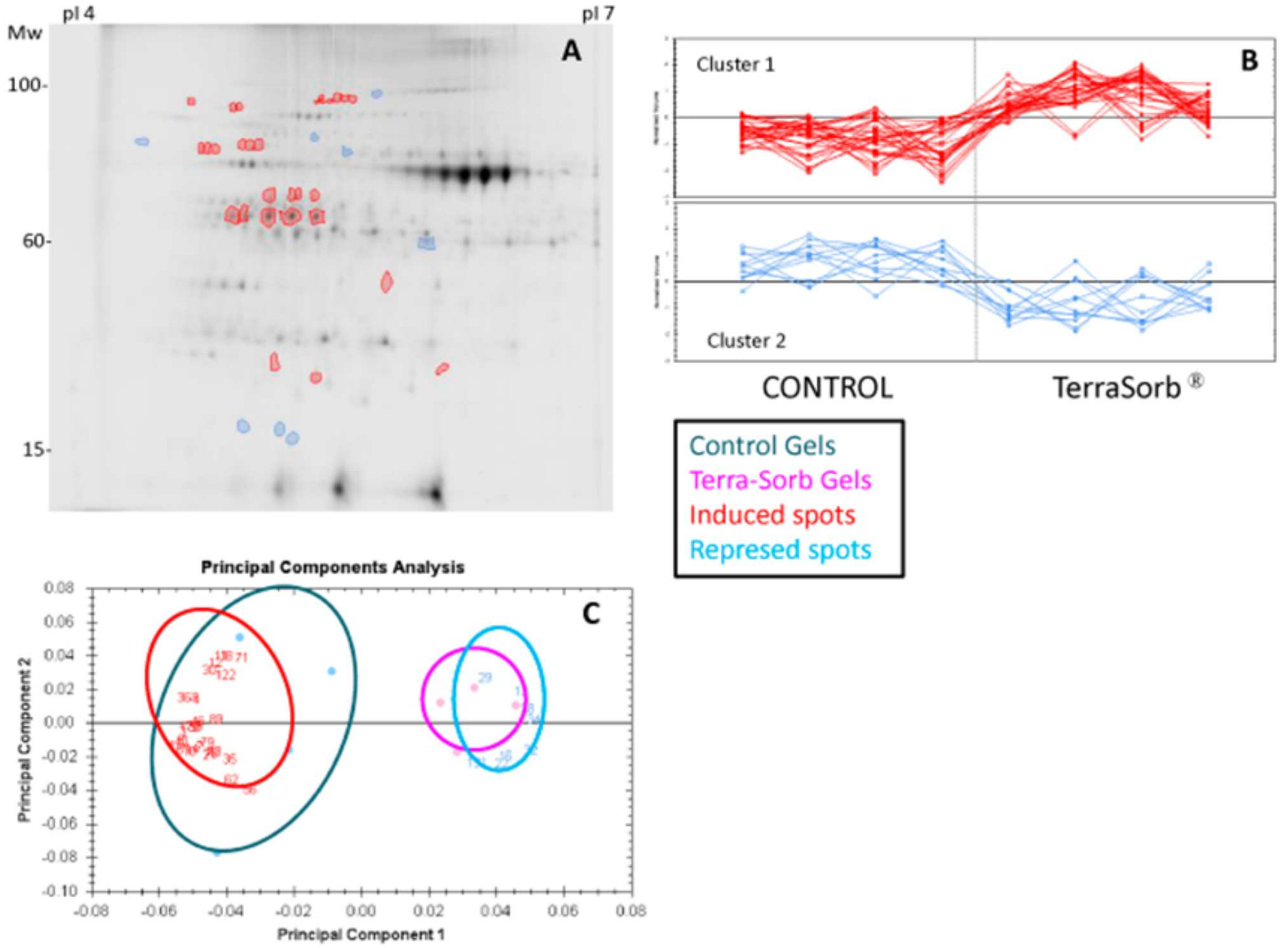

Figure 2. 2D DIGE analysis of TerraSorb ® treated vs. Control wheat flag leaves. A) 2D gel reference image showing the location of the selected spots and pertaining to a cluster by a color code. B) Standardized expression profiles of the spots in four replicates across the control and the treatment. C) PCA bi-plot of the two first principal components. Together they both explained 75.49% of the selected spots variability, and the third principal component only explained an additional 9.35%.

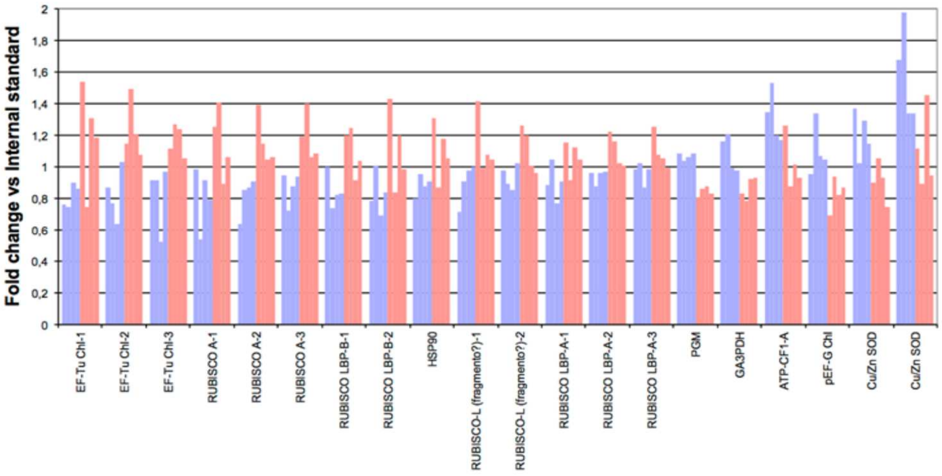

Figure 3. Proteins de-regulated in wheat flag leaf treated with TerraSorb® Foliar. Shown is the relative abundance in each of the four biological replicates of control and treated individuals. NCBInr accession numbers for each protein are: 125540125 EF-Tu Chl, 12643756 Rubisco A, 2493650 Rubisco LBP-B, 556673 HSP90, 548677 Rubisco-L, 134102 Rubisco LBP-A, 32400802 PGM, 15222111 GA3PDH, 14017569 ATP-CF1-A, 1568639 Cu/Zn SOD.

3.2. Protein abundance patterns of wheat flag leaf in response to TerraSorb® treatment

To detect proteins differentially accumulated in the TerraSorb® treatment, 2-D DIGE patterns from control and the corresponding treated samples were compared according to the experimental design shown in Table 1. 2-D DIGE gel maps resolved approximately 918 unique spots across the compared control vs. treatment samples. The comparative analysis by SameSpots software revealed 37 spots (4 % from the number of total spots) above the threshold settings (ANOVA p-value<0.05). Figure 2A shows the reference gel image in which the selected spots are outlined. After a hierarchical clustering analysis of the abundance patterns, the selected spots were classified into two groups (Figure 2B): up-regulated in treatments with TerraSorb® (red), down -regulated in treatment with TerraSorb® (blue). The same color code was used to outline the corresponding spots in the reference gel image (Figure 2A) and the unsupervised PCA bi-plot of spots and gels (Figure 2C). The latter shows a gel grouping (colored dots) that agrees with the experimental design. Twenty-nine of these spots increase their abundance and eight spots shown a decreased accumulation in the treated plants when compared to the controls (Figure 2B). The fold-changes determined were moderate for all the proteins, occurring the highest change of 1.83-fold for the spot 56.

3.3. Identification of differentially expressed proteins

Protein identity was successfully achieved for 26 of the selected spots following a typical protein identification workflow (spot excision, trypsin in-gel digestion, nLC-MS/MS and database search). This number of proteins represents a 70 % from the total of differentially accumulated proteins detected due to TerraSorb® treatment. The twenty-six protein spots were encoded by 13 different genes according to the top hit accession in NCBInr, but in quantitative terms we only considered twenty protein spots whose fold change was above 1.2, which are shown in Table 2. Supplementary File 1 provides details of proteins identification. The values shown in the 'Fold Change' column represent the average increase or decrease of protein abundance related to control with a significance of > 95 % in t-student, and in brackets are given the normalized spots volumes in % of the total spot volume. Those data with a significance level < 95 % are assumed to be due to biological variation and excluded from the analysis. Figure 3 summarizes the results of identification pertaining to the deregulated spots. The identified proteins which abundance increased can be clearly seen in Figure 3 where red bars related to the normalized volume in the treated samples versus internal standard stick out the blue bars corresponding to the control samples. The increased spots are mainly related to the life cycle of Rubisco [15] including six spots of Rubisco large-subunit binding protein, subunit alpha (Spots 8, 11, 12) and beta (Spots 18, 30, 122) (Rubisco LBP A and B) involved in its synthesis and assembly, three spots of Rubisco activase (RBA) (Spots 5, 9, 89) involved in its catalytic activity and two spots of an N-terminal fragment of Rubisco large subunit (Rubisco-L) (Spots 56, 62) which may be related to the degradation of the protein. The rest of increased spots are related to protein synthesis and folding including a spot of heat shock protein 90 (HSP-90) (Spot 71) and three spots of elongation factor Tu (EF-Tu) chloroplastic (Spots 10, 14, 73). The identified proteins which abundance decreased include proteins involved in central metabolism: a spot of phosphoglycerate mutase (PGM) (Spot 32), a spot of glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) (Spot 34) and a spot of ATP synthase CF1 (ATP-CF1-A) (Spot 29), and stress, including two spots of Cu/Zn superoxide dismutase (SOD) (Spots 13, 131). Also, a fragment of a precursor of EF-Tu was found decreased (Spot 22). Most of the spots changing have undergone a moderate increment of its abundance and those proteins down-regulated are spots of medium to low abundance.

4. Discussion

The proteins whose abundance increases upon TerraSorb® treatment carry out functions related to the positive regulation of carbon fixation and protein synthesis. In contrast, proteins whose abundance decreases are involved in functions related to energy metabolism and oxidative stress. The effect of TerraSorb® in the proteome of the flag leaf is clearly different from control samples. Having just the proteomic profile obtained in this experiment, it can be tentatively assigned an explanation at molecular and physiological level. Nevertheless, this general overview suggests that the effects are specific and in consequence related to particular biological processes.

The in bibliographic information about the effect of amino acid-based biostimulants on plant growth at molecular level is scarce. These reports were focused on the expression of a specific set of genes and activity of enzymes being deregulated by the treatment, underlying a targeted approach. Transcripts and activities for the enzymes involved in carbon metabolism (malate dehydrogenase isocitrate dehydrogenase, citrate synthase) and N reduction and assimilation (nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase, aspartate aminotransferase) were monitored in maize and were found to increase in response to the treatments [5]. In the model plant *Arabidopsis thaliana* the protecting effect against abiotic stresses of an amino acid-based biostimulant product was studied by monitoring the expression of selected genes related to abiotic stress response, in particular salt, drought and low temperature. An increased expression of these stress resistance genes was shown thus concluding that the product stimulates endogenous plant defense response to both biotic

Table 2. Proteins differentially expressed in DIGE of treated and control samples. Selection and quantification of spots of interest was assisted by Progenesis SameSpots. Spots were excised from the gel, trypsin in-gel digested, and the eluted peptides were analyzed by mass spectrometry (ESI-MS/MS). Proteins were identified by MS/MS search against NCBInr protein database using SpectrumMill. [a]Score: statistical probability of true positive identification of the predicted protein calculated by Spectrum Mill and Mascot Score (*). [b]SC: percentage of predicted protein sequence covered by matched sequences. [c]Tmw/Emw: molecular mass of predicted protein/molecular mass of protein on gel. [d]TpI/EpI: pI of predicted protein/pI of protein on gel.

Ref. Spot	Protein Information							Fold Change (Norm. Vol.%)	
Ref. Spot	Accession No.	Protein name	Score[a]	SC (%)[b]	Tmw/Emw[c] (Da)	TpI/EpI[d]	Identified peptides/ matched spectra	Con-trol	Terra-Sorb
73	125540125	translational elongation factor Tu	72.18	10	63685.4/39616	5.74/5.35	4/6	1 (0.051)	1,41 (0.072)
10	125540125	translational elongation factor Tu	37.26	5	63685.4/38898	5.74/5.22	2/2	1 (0.074)	1,37 (0.101)
14	125540125	translational elongation factor Tu	56.93	8	63685.4/39855	5.74/5.26	3/4	1 (0.043)	1,30 (0.056)
18	2493650	RuBisCO large subunit-binding protein subunit beta, chloroplast	291.89	48	53412/55747	4.88/4.99	17/26	1 (0.038)	1,26 (0.048)
122	2493650	RuBisCO large subunit-binding protein subunit beta, chloroplast	369.36	62	53412/54382	4.88/5.05	20/33	1 (0.046)	1,23 (0.057)
8	134102	RuBisCO large subunit-binding protein subunit alpha, chloroplast precursor	271.12	40	57520.9/52828	4.83/4.81	15/19	1 (0.019)	1,4 (0.026)
12	134102	RuBisCO large subunit-binding protein subunit alpha, chloroplast precursor	205.89	34	57520.9/52921	4.83/4.77	11/15	1 (0.019)	1,33 (0.026)
11	134102	RuBisCO large subunit-binding protein subunit alpha, chloroplast precursor	271.12	40	57520.9/53014	4.83/4.74	15/19	1 (0.013)	1,28 (0.017)
56	548677	RuBisCO large subunit. Ribulose bisphosphate carboxylase large chain, N-terminal domain.	48.32	7	52183.6/20446	6.09/5.36	3/11	1 (0.027)	1,83 (0.050)
62	548677	RuBisCO large subunit. Ribulose bisphosphate carboxylase large chain, N-terminal domain.	41.34	7	52183.6/20979	6.09/6.06	3/3	1 (0.005)	1,61 (0.008)
5	12643756	RuBisCO activase A chloroplast precursor	50	7	51074.3/35650	8.04/5.36	3/4	1 (0.442)	1,43 (0.632)
9	12643756	RuBisCO activase A chloroplast precursor	50	7	51074.3/35797	8.04/5.23	3/4	1 (0.718)	1,41 (1.015)
89	12643756	RuBisCO activase A chloroplast precursor	81.61	24	51074.3/35843	8.04/5.10	5/5	1 (0.617)	1,27 (0.787)
71	556673	Heat shock protein 90kDa	238.80	22	88117/73511	4.90/4.67	14/20	1 (0.005)	1,47 (0.007)
32	32400802	phosphoglycerate mutase	51.29	20	29578.7/57149	5.44/5.35	3/5	1 (0.015)	-1,26 (0.012)
34	15222111	glyceraldehyde-3-phosphate dehydrogenase	82.43	17	42847.0/31638	8.16/5.96	5/5	1 (0.146)	-1,27 (0.115)
29	14017569	ATP synthase CF1 alpha subunit	29.01	4	55295.8/51750	6.11/5.52	2/2	1 (0.025)	-1,27 (0.020)
22	125549171	Fragment of putative Elongation factor G, chloroplast precursor (EF-G).	70.54	7	84912.5/55747	5.43/4.43	4/4	1 (0.005)	-1,29 (0.004)
131	1568639	Cu/Zn superoxide dismutase	84.15	25	20323/18765	5.35/5.15	4/6	1 (0.020)	-1,34 (0.015)
13	1568639	Cu/Zn superoxide dismutase	43.65	2	20323/18856	5.35/4.96	2/2	1 (0.008)	-1,48 (0.005)

and abiotic stress factors [16]. Physiological experiments have pointed out that the exogenous application of the amino acid-based biostimulant foliar (BioIberica S.A., Barcelona) improves photochemical leaf efficiency in ryegrass plants grown at 36ºC [17]. A definite cause and effect relationship has not yet been proved and the molecular mechanism that might be related to the improvement of the plant performance after exposure to abiotic stresses is still unknown.

In the present study, the application of a hypothesis-free approach has led to a discovery of deregulated proteins not anticipated, up to date, in plants treated with an amino acid-based biostimulant, thus providing clues on mechanisms of action.

4.1. Positive regulation of Rubisco

Different proteins which have a role in regulating the Rubisco protein were identified. Rubisco activase was found up-regulated by treatment with TerraSorb® foliar in four spots. RBA produces ATP-dependent conformational changes in Rubisco, the enzyme responsible for carbon fixation during photosynthesis, making the inactivated protein re-enter the catalytic cycle. Rubisco can be inactivated during its catalytic cycle by the strong binding of phosphate sugars such as ribulose-1,5-bisphosphate or by the inhibitor 2-carboxyarabinitol 1-phosphate. The activation occurs through a mechanism dependent on ATP in which RBA provokes conformational changes in the Rubisco enzyme which promotes the release of the inhibitory sugar phosphates from the catalytic site [15]. Besides RBA, some polypeptides related to Rubisco regulation were found up-regulated. On the one hand, two subunits of Rubisco LBP, A and B, that form a macromolecular complex that assists to the correct folding of Rubisco. Altogether, these results indicate the need for validation and further study of the role that RBA and Rubisco LBP could have in promoting carbon fixation through the increase of the overall Rubisco activity. On the other hand, we also found that two low molecular weight fragments of Rubisco-L N-terminal domain that are known to fold as a ferredoxin are up-regulated. Although, it has been reported the presence of Rubisco-L fragments with unknown function as a consequence of thermal stress [18], we cannot presently foresee any potential role for this fragment found in the treated wheat plants.

4.2. Protein synthesis stimulation

The elongation factor Tu involved in protein biosynthesis in the chloroplast was detected up-regulated by TerraSorb® foliar treatment. Moreover, HSP-90, a component of the folding machinery of proteins, was detected to be up-regulated. Interestingly, pre-EF-Tu has been demonstrated to reduce thermal aggregation of RBA [19], which is known to display an exceptional sensitivity to thermal aggregation, and constraints photosynthesis at high temperature [20, 21]. The profiles of EF-Tu and RBA are parallel in the response to the biostimulant TerraSorb® foliar. EF-Tu is thus another target of interest for further studies, as it could play a role beyond protein synthesis in the stabilization of RBA, although other experiments are needed to test this hypothesis.

4.3. Down-regulation of metabolic enzymes and anti-stress proteins

Four proteins were found to be down-regulated: ATP-CF1 -A, PGM, G3PDH and a Cu/Zn SOD. For the former three, the level of decrease could be consistent with a lower consumption of the photosynthetic product 3-phosphoglycerate catabolism, that is, to obtain energy for the oxidative processes. Decreased levels of Cu/Zn SOD would be consistent with a lower oxidative state. Cu/Zn SOD is specific and its role is to eliminate superoxide generated by the photoreduction of O_2 in the photosystem I. Although, the reasons for the effect of TerraSorb® in the reduction of oxidative stress at the photosynthetic level cannot be explained by the proteomic data alone obtained in this experiment, the result is consistent with the finding that a biostimulant based on porcine hemoglobin hydrolysate lessened the harmful effects caused by the intense cold and heat treatment in lettuce [8].

5. Concluding Remarks

Our proteomic study provides information about deregulated proteins that were identified in wheat flag leaf after treatment with TerraSorb® foliar. The obtained information provided molecular targets for further validation and a hypothesis for the molecular mechanism underlying the effect of TerraSorb® foliar on wheat flag leaf. However, the confirmation of the effects of this amino acid-based biostimulant at molecular level has shown, that the discovery proteomics approach will need to be included in the future design of targeted studies focused on these biological processes.

6. Supplementary material

Supplementary material 1. Peptide table. For each identified protein a list of matched peptides is given. Besides, charge state and precursor ion m/z data is given for LC–MS/MS analyzed proteins.

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Vaginal bacterial microbiota of an endangered donkey breed: a comparison between *Miranda* donkey breed (*Equus asinus*) jennies with and without reproductive problems

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ABSTRACT

This study provided an overview of the vaginal bacterial microbiota of *Miranda* donkey breed jennies with and without reproductive problems. This Portuguese autochthonous donkey bred is in danger of extinction. Bacteria isolated were predominantly Gram-positive and belonging to the genera of *Bacillus* spp, *Corynebacterium* spp., *Lactobacillus* spp., *Staphylococcus* spp. and *Streptococcus* spp.. The species found in the vaginal microbiota were diverse and didn't differ significantly between the jennies with and without reproductive problems. However the isolates of *Streptococcus zooepidemicus* of jennies with reproductive problems presented a higher number of the studied genes encoding virulence factors then the isolate without reproductive problems. This is the first study reporting vaginal bacterial microbiota of *Miranda* donkey breed jennies. Since there are few studies regarding the vaginal microbiota of equines, especially in this donkey breed, these results can be an asset for future studies.

Keywords: Equids; Donkey; *Streptococcus zooepidemicus*; Vaginal bacterial microbiota; Virulence factors.

1. Introduction

The *Miranda* donkey breed is the only Portuguese autochthonous donkey breed native of northeast of Portugal and it is currently endangered of extinction. Until 2012, it was reported that there were only 725 animals alive. The potentially reproductive population at the end of 2012 comprised 545 females and 72 males, alive and younger than 20 years old. Only around 15% of the females were foaling each year, a reported low fertility rate [1,2].

Commensal microbiota is a complex community of microorganisms that colonizes several biological systems in animals. So far, equine native microbiota has not been extensively characterized. These microbial communities are related to normal functions of different organisms and its characterization may provide important data about beneficial microbes useful for the promotion of equine's health. Many uterine pathogens inhabit the vagina. The knowledge of bacterial biota inhabiting the genital tract of the mare would be important to develop therapeutic strategies to control the setting of the disease [3].

Bacterial infections of genital tract are known to be an important cause of reproductive problems in equines. Bacteria involved in equine endometritis are, for the most part, considered to be opportunistic pathogens. They are capable of colonizing the lower genital tract as well as a variety of extragenital locations in the animal; yet they are usually barred from ascending to the cervix and uterus by

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host defences. Previous studies showed that the most common bacterial causes of uterine infection include *Streptococcus zooepidemicus*, *Escherichia coli*, *Staphylococcus aureus, Klebsiella pneumonia* and *Pseudomonas aeruginosa*. Bacteria which are capable to establish endometritis are considered to differ from the physiological genital microbiota by several tracts that promote colonizing and causing damage to equine endometrium. The concept that some virulence mechanisms are sufficient to explain why a bacterium can cause endometritis may oversimplify the complex nature of the disease [4,5,6,7].

Taylorella equigenitalis is a Gram-negative bacterium responsible for contagious equine metritis (CEM), a sexually transmitted infection of horses that was first reported in 1977. In 2001 it was confirmed the existence of a second species within the genus *Taylorella* which was named *Taylorella asinigenitalis* and isolated from genital tract of donkeys. Until now *T. asinigenitalis* is not associated with CEM [8,9].

Uterine infections caused by *S. zooepidemicus* have been established as the leading cause of bacteria-induced endometritis in equines. The pathogenesis of endometritis induced by *S. zooepidemicus* is based on interactions between bacterial virulence factors and the infected host tissues. Understanding the role played by virulence factors can help elucidate the understanding of the pathogenesis of infection and can be used to identify points of treatment or vaccination. Known virulence factors of *S. zooepidemicus* include M-like proteins and superantigens [4,10,11,12,13] The designation M-like protein usually refers to proteins that bind fibrinogen and have antiphagocytic activity. The antiphagocytic of these proteins appears to be associated with their ability to inhibit deposition of the complement component C3b on the bacterial surface and their ability to bind fibrinogen which then inhibits the phagocytosis. Superantigens interfere with the development of a protective immune response, by disrupting antigen-specific T cell responses and inhibiting the production of antibodies [14,15].

The main aim of this study was to identify and characterize bacteria from vaginal samples of eight Miranda jennies with reproductive problems (included the females with an infertility story) and nineteen *Miranda* jennies without reproductive problems. The analysis of the presence of genes encoding virulence factors in *Streptococcus zooepidemicus* isolates (*sze*F, *sze*N, *sze*P, *szm* and *szp*) was also an aim of our study.

2. Material and Methods

2.1. Samples and bacterial isolates.

Fifty four vaginal exudates (two samples per animal) were collected between October of 2014 and February of 2015. Before collecting each sample, the genital zone of each jenny was washed with water to eliminate fecal contaminations. One of the samples collected from each jenny was transported refrigerated in Amies Transport Medium with charcoal (Remel) to preserve the viability of bacteria present in the sample. The other sample collected was transported at room temperature in Nutrient Broth (Oxoid), in order to preserve the viability of *Mycoplasma* spp..

For the samples transported in Amies Transport Medium with charcoal, a dilution in saline solution was prepared before performing streak plate method in the following culture mediums: BHI agar (Liofilchem) with 5% Sheep Blood, Cetrimid agar (Merck), Chromocult Coliform agar (Merck), Columbia CNA agar (BBL) with 5% Sheep Blood, Columbia (Oxoid) Chocolate agar with 5% Horse Blood and streptomycin, Columbia (Oxoid) Chocolate agar with 5% Horse Blood, Amphotericin B and Violet Crystal and MacConkey (Merck) agar. These plates were incubated for 24-48h at 37ºC, with exception of Columbia Chocolate agar plates that were incubated for 5-7 days at 37ºC in a jar with microaerophilic environment. It was also used 0.5 ml from the dilution made to inoculate in a tube of Rappaport-Vassiliadis Enrichment Broth (Oxoid), to test if the samples presented *Salmonella* sp.. The tubes with Rappaport-Vassiliadis Broth were incubated in a water bath at 37ºC for 24-48h. On the other hand, the samples transported in Nutrient Broth were centrifuged at 3000 rpm for 5 min; the supernatant was aspired with a syringe and then filtered to the surface of Mycoplasma agar base with selective Mycoplasma supplement. Spread plate technique was performed and the plates were incubated for 4-14 days at 37ºC in a humid atmosphere. These plates were viewed under the low power objective (10x) of the optical microscope, in order to investigate the presence of typical "fried egg" colonies.

The different bacteria isolated were identified by colony morphology, Gram-staining, catalase test and oxidase test. Identification to the species level was made using API 20 Strep, API 20 E and API 20 NE systems. PCR amplification of the 16S ribosomal DNA was also performed for the isolates that indicated belonging to *Streptococcus* spp. to identify and confirm which isolates were *Streptococcus zooepidemicus*. The sequences of the oligonucleotide primer and the thermal cycler programs are given in table 1.

2.2. Antimicrobial susceptibility test.

Antibiotic susceptibility of *Streptococcus zooepidemicus* isolates was tested by the agar disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2013) for 9 antimicrobials: ampicillin (10 μg), cefepime (30 μg), clindamycin (2 μg), chloramphenicol (30 μg), erythromycin (15 μg), linezolid (30 μg), quinupristin -dalfopristin (15 μg), tetracycline (30 μg) and vancomycin (30 μg).

Table 1 - Oligonucleotide primers and PCR programs used in present study. The oligonucleotide primers were synthesized by Invitrogen™.

Target	Oligonucleotide primer	Sequence (5'–3')	PCR program	Reference
16s rDNA	16SP0	GAAGAGTTTGATCCTGGCTCAG	1x (95°C 5min), 25x (95°C 30s, 56.5°C 30s, 72°C 2min), 1x(72°C 10min)	[21]
	16SP6	CTACGGCTACCTTGTTACGA		
szeF	*szeFF*	CATAAAGTTAGTCGTGCAGAG	1x (95°C 15min), 35x (94°C 30s, 59°C 30s, 72°C 1min), 1x(72°C 10min)	[11]
	szeFR	CGATGACGATGATTCACATCA		
szeN	*szeNF*	GACACCGGTAACATTTCAAGAG	1x (95°C 15min), 35x (94°C 30s, 59°C 30s, 72°C 1min), 1x(72°C 10min)	[11]
	szeNR	GGGTTGACCACTCTTGTAG		
szeP	*szePF*	TCCAGTTGAGAAATCCTGGC	1x (95°C 15min), 35x (94°C 30s, 59°C 30s, 72°C 1min), 1x(72°C 10min)	[11]
	szePR	CCTAAAAATTTCGACATCAAGTG		
szm	*szmF*	ATAAAGAAGTTCCTGTCAT	1x (95°C 2min), 30x (94°C 30s, 55°C 30s, 72°C 1min), 1x(72°C 10min)	[12]
	szmR	CAACAGACAGGAGACTGTTGC		
szp	*szpF*	ACAAAAGGGGAATAAAATGGC	1x (95°C 2min), 30x (94°C 30s, 60°C 30s, 72°C 150s), 1x(72°C 5min)	[13]
	szpR	TTTACCACTGGGGTATAAGGCTT		

Table 2 - Vaginal bacterial microbiota of *Miranda* jennies.

Identified bacteria	Number of isolates (Jennies with reproductive problems) N=46	Number of isolates (Jennies without reproductive problems) N=103	P-value	Genotype of virulence factors detected (if applicable)
Aerococcus viridans	-	3	0.242	-
Aeromonas hydrophila	-	1	0.516	-
Bacillus spp.	13	19	0.143	-
Corynebacterium spp.	12	9	0.076	-
Enterococcus faecium	-	3	0.242	-
Escherichia coli	-	1	0.516	-
Gemella haemolysans	-	1	0.516	-
Lactobacillus spp.	6	26	0.404	-
Micrococcus spp.	1	1	0.520	-
Mycoplasma spp.	-	5	0.115	-
Neisseria spp.	1	2	0.884	-
Oligella urethralis	-	1	0.516	-
Pantoea Agglomerans	-	2	0.349	-
Pseudomonas chlororaphis	-	6	0.242	-
Rhodococcus spp.	5	1	0.028	-
Staphylococcus spp.	4	17	0.461	-
Streptococcus acidominimus	-	1	0.516	-
Streptococcus oralis	2	-	0.026	-
Streptococcus spp.	-	3	0.242	-
Streptococcus zooepidemicus	2	1	0.144	szeF, szeN, szeP (67%) szeF, szp (33%)

2.3. Virulence factor genes.

The presence of genes encoding virulence factors (*sze*F, *sze*N, *sze*P, *szm* and *szp*) for *Streptococcus zooepidemicus* isolates was also analyzed by PCR. The sequences of the oligonucleotide primers and the thermal cycler programs are given in table 1.

2.4. Statistical analysis

Data were first analysed using Shapiro-Wilk normality test to test the normal distribution of the values. To test if there were significant differences between the bacteria isolated from jennies with reproductive problems and bacteria isolated from jennies without reproductive problems an Independent-Samples Kruskal-Wallis test was performed. These tests were done using IBM SPSS Statistics 19. *P*-values less than 0.05 were considered significant.

3. Results

From a total of 54 vaginal exudates from 27 jennies, 149 bacterial isolates were obtained. Among these isolates, 130 were identified as Gram-positive bacteria, 14 as Gram-negative bacteria and 5 bacteria without Gram determined (identified as *Mycoplasma* spp.). The most isolated genera were *Bacillus* spp. (22.15%), *Corynebacterium* spp. (13.42%), *Lactobacillus* spp. (21.48%) and *Staphylococcus* spp. (14.09%). All genera and bacterial species isolated can be found in the table 2. The species found in the vaginal microbiota were diversified and didn't differ much between the jennies with and without reproductive problems. *P*-values only were less than 0.05 in *Rhodococcus* spp. and *Streptococcus oralis* showing significant differences between jennies with and without reproductive problems. All P-values can be found in the table 2. It was not isolated any bacteria that could be compatible to *Taylorella equigenitalis* or *Taylorella asinigenitalis*.

Only one isolate of *Streptococcus zooepidemicus* showed resistance to tetracycline, even though the other two *S. zooepidemicus* isolates were found to have an intermediated resistance phenotype to this antibiotic.

On both two isolates of *S. zooepidemicus* from jennies with reproductive problems, the genotype of virulence factors detected was *sze*F, *sze*N, *sze*P, *szp*. In the isolate of *S. zooepidemicus* from a jenny without reproductive problems we only detected the gene *szp* and the gene *sze*F.

4. Discussion

Changes in genital bacterial microbiota are a common sign of reproductive disorders. The knowledge of the bacterial microbiota inhabiting a particular physiological niche, in this case the genital tract, would be necessary to develop therapeutic strategies that do not affect the healthy vaginal microenvironment [16, 17].

In this study, different genera of non-pathogenic bacteria and also some considered pathogenic were isolated from the vagina of both healthy and jennies with reproductive problems. Previous studies investigated the vaginal microbiota of mares and concluded that this community can be composed by diverse genera such as *Corynebacterium* spp., *Staphylococcus* spp., *Streptococcus* spp., *Bifidobacterium* spp., *Lactobacillus* spp. or *Bacillus* spp.. On some cases, bacteria like *Escherichia coli*, *Pseudomonas aeruginosa* or *Klebsiella pneumonia*e can also be found to inhabit in the vagina. Our findings are similar to these previous studies suggesting that the vaginal microbiota of *Miranda* jennies does not differ much from the vaginal microbiota of mares [16,18,19].

Streptococcus zooepidemicus is the most frequently isolated pathogen from the uterus of the mare. The prevailing hypothesis is that isolates of *Streptococcus zooepidemicus*, residing in the lower reproductive tract, cause infectious endometritis, by an ascending infection in a random manner, primarily governed by the uterine defence mechanisms of the mare. The clitoral *fossa*, clitoral sinuses and the vagina have been suggested as possible bacterial reservoirs [20]. In this study, we isolated *Streptococcus zooepidemicus* from jennies with and without reproductive problems. The genotype of virulence factors detected from the isolates of the jennies with reproductive problems, which presented more encoding genes of virulence factors, was somehow different from the genotype of jennies without reproductive problems. Despite this, we cannot come to any conclusion since the number of isolates studied is too small to draw definitive conclusions.

5. Concluding Remarks

To our knowledge this is the first study providing an overview of the vaginal bacterial microbiota of the Miranda donkey breed. Bacteria isolated were predominantly Gram-positive and belonging to the genera of *Bacillus* spp, *Corynebacterium* spp., *Lactobacillus* spp., *Staphylococcus* spp. and *Streptococcus* spp.. Since there are few studies on this matter focusing this issue, especially in this autochthonous donkey breed that is near extinction, the results of this study may be an asset for future investigations.

In order to find out the reason of the fertility problems of *Miranda* jennies, it would be important to evaluate in the future, all the points through which the diagnosis of uterine infections must pass, to exclude the possibility of these problems being caused by a bacterial infection. The study of genital microbiota of *Miranda* donkey breed males will also be important to knowledge if it includes pathogenic bacteria which can infect jennies during mating. Other problems not related with microorganisms should also be taken into consideration in the future in order to find out the reason for the reproductive problems and low fertility rates in this breed.

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Implementing effective machine learning-based workflows for the analysis of mass spectrometry data

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Abstract

Mass spectrometry using matrix assisted laser desorption ionization coupled to time of flight analyzers (MALDI-TOF MS) has become very popular during the last decade due to its high speed, sensitivity and robustness for accurately detecting proteins and peptides. This allows quickly analyzing large sets of samples in one single batch and doing high-throughput proteomics. In this scenario, bioinformatics methods and computational tools play a key role in MALDI-TOF MS data analysis, as they are able to correctly handle the large amount of raw data generated with the goal of discovering new knowledge and extracting useful conclusions.

A typical MALDI-TOF MS data analysis workflow consists of three main stages: data acquisition, preprocessing and analysis. Although the most popular use of this technology is to identify proteins through their peptides, analyses that make use of artificial intelligence (AI), machine learning (ML), and statistical methods are of particular interest to conduct biomarker discovery, automatic diagnosis, and knowledge discovery.

In this introductory work, the potential of these techniques is explored and novel solutions based on the application of AI, ML, and statistical methods are reviewed. In addition, an integrated software platform that supports full MALDI-TOF MS data analysis is presented with the goal of facilitating the work of proteomics researchers without advanced bioinformatics skills.

Keywords: Computational mass spectrometry, Machine learning, MALDI-TOF MS data, intelligent analysis workflow.

1. Introduction

In the last years, high-throughput proteomic data analysis using matrix assisted laser desorption ionization coupled to time of flight analyzers based mass spectrometry (MALDI-TOF MS) has been an active research area due to its high speed, sensitivity and robustness for detecting proteins and peptides. Within this technique, large sets of samples are analyzed quickly in one single batch. In this context, bioinformatics methods and computational tools play a key role in MALDI-TOF MS data analysis, since they can handle the vast amount of raw data generated, supporting the application of complex analysis with the goal of finally extracting new knowledge and useful conclusions [1].

A common MALDI-TOF MS data analysis workflow is characterized by three main stages: (*i*) data acquisition, (*ii*) preprocessing, and (*iii*) analysis. This standardized workflow starts with the acquisition and management of raw data that must be preprocessed to obtain clean peak lists, suitable for being used as input of the analysis stage [2]. Despite its apparent simplicity, each of these three main stages is composed by smaller steps, and different solutions and approaches have been proposed to address them in the last years [3]. Regarding the analysis stage, the most popular use of MALDI-TOF MS is to identify proteins through their peptides, a process known as *peptide-mass fingerprinting* (PMF). For this application scenario, the mass spectrum must be preprocessed for obtaining a list of peptide experimental masses, which can be searched against a database to identify target proteins. Nevertheless, analyses that make use of artificial intelligence (AI), machine learning (ML), and

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statistical methods can also be executed in order to perform biomarker discovery, automatic diagnosis and knowledge discovery [1,4,5], taking peak lists as input. AI and ML methods have demonstrated their usefulness when applied to many different biomedical, biological and *omics* problems.

It is also important to note that when working with MALDI-TOF MS data, low quality spectra may be occasionally generated. For example, spectra showing a low number of m/z values in comparison with other spectra, or containing many unique m/z values not present in their sibling replicates. These noisy spectra may easily lead to many different types of errors or most severe incorrect conclusions. To prevent such a scenario, a quality control (QC) step, which may be performed between the preprocessing and the analysis tasks, should be considered.

Based on our previous experience in the field [6–8], the present work reviews the most important aspects for correctly implementing such machine learning-based workflows for the analysis of MALDI-TOF MS data. The core workflow analyzed, shown in Figure 1, is also implemented by Mass-Up [9], an open-source software platform freely available to the scientific community.

2. Preprocessing

Preprocessing of MALDI-TOF MS data is a decisive stage that transforms raw data into a suitable input for further analysis. In this context, inadequate or incorrect preprocessing methods can result in a biased dataset, hindering the process of reaching meaningful biological conclusions [10]. In such a situation, preprocessing becomes critical since raw data contains signals coming from the real peptides/proteins, as well as signals derived from several forms of noise (e.g. chemical, electronic factors, etc.). The specific goals of this phase are (*i*) to remove noisy peaks without discarding any of the true peaks and (*ii*) to determine both m/z and intensity values with the best accuracy [11]. Since there is no standard MS data preprocessing pipeline, some authors proposed different guidelines to establish a design/data analysis protocol [12,13]. After reviewing these guidelines, we proposed the following core preprocessing steps: (*i*) baseline correction, (*ii*) smoothing, (*iii*) peak detection and (*iv*) peak alignment. While the first two steps aims to remove noise, peak detection is a feature extraction process able to select true (i.e. peptide/protein-related) peaks from a given spectrum. Finally, peak alignment (also referred as peak matching) consists on determining which peaks correspond to the same peptide/protein in different samples. As a result of this phase, all the aligned peaks have the same mass values in all spectra and therefore, they are comparable and suitable for further machine learning analyses.

Additionally, our proposed workflow also incorporates a complementary filtering step that is closely related with the matching process. This step allows the creation of a

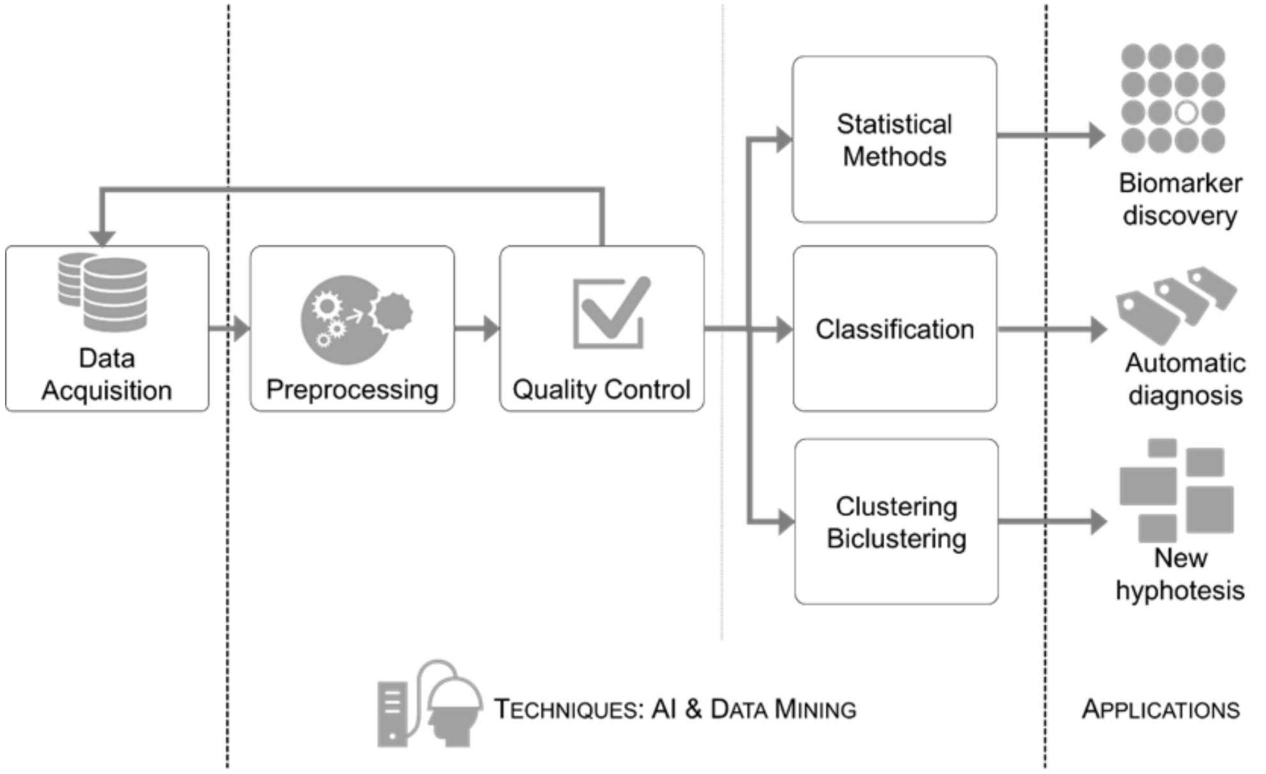


Figure 1. Machine learning-based workflow for the analysis of MALDI-TOF MS data.

consensus spectrum for a sample, which summarizes its replicates in one single spectrum. In our approach, the Percentage of Presence (POP) parameter allows the user to set the number of replicates where an m/z value must be present in order to be considered a valid consensus m/z value.

3. Machine learning-based analyses

Each preprocessed spectrum (or peak list) contains a finite number of peaks. A biomarker analysis can be done by using some adapted statistical methods that led to identify which of those peaks are associated with factors of interest [14].

Automatic diagnosis given a set of previously classified samples, is a supervised ML problem [15]. For example, given an unlabeled serum sample from an individual, which can come from one or more replicates (i.e. spectra), the purpose of classification could be to assign it to a specific diagnostic group (e.g. healthy or diseased). In this case, a classification model is built from a set of labeled samples using the intensity or the presence/absence of the different peaks (m/z) as input features [1]. It is important to note that when intensity values are used, the dataset must be normalized in order to make intensities comparable. Data used to build this model is called *training* data. The model is then used to predict the class of those unlabeled samples.

Common types of ML supervised techniques are, among others: (*i*) *Bayesian classifiers*, such as *Naïve Bayes*, which are based on Bayes theorem; (*ii*) *rule-based learners*, which are based on the creation of human-readable rules that could explain why certain samples belong to a class; (*iii*) *decision trees*, which are based on tree-like structures that organize the knowledge to discriminate between samples and predict their class; (*iv*) *random forests*, which use several decision trees to predict the class of each sample; (*v*) *support vector machines* (SVMs), such as *Sequential Minimal Optimization* (SMO), which are based in the concept of linear separability between classes; and (*vi*) *artificial neural networks* (ANNs), which simulate brain's operation in order to build the model and predict the class of each sample [1]. In algorithms such as rule-based learners or decision trees, it is also possible to consider some specific peaks as biomarkers used to separate the target classes. Despite the fact that these algorithms take peak lists as input, they can still contain noisy, irrelevant or redundant peaks, which can reduce the accuracy of the underlying classifiers. To mitigate these symptoms, *feature selection* can be applied prior to the use of classification algorithms, generating a cleaner dataset on which apply them. Feature selection methods can also be used to discover potential biomarkers.

In contrast with supervised machine learning, in unsupervised classification (or *clustering*), samples do not have associated class labels and they consist in grouping together samples with similar peak profiles. The main clustering approaches are: (*i*) *partition clustering* (e.g. *K-means* algorithm), (*ii*) *hierarchical clustering*, and (*iii*) *mixture models* [15]. These techniques are characterized by the fact that they perform a one-dimensional clustering using samples' attributes. A specific sub-type of clustering, called *biclustering* (or *co-clustering),* is able to perform a two dimensional clustering, that is, clusters are modeled with both samples and samples' attributes. These unsupervised techniques lead to the creation of new hypotheses (e.g. proposed groups) that must be further explored and evaluated.

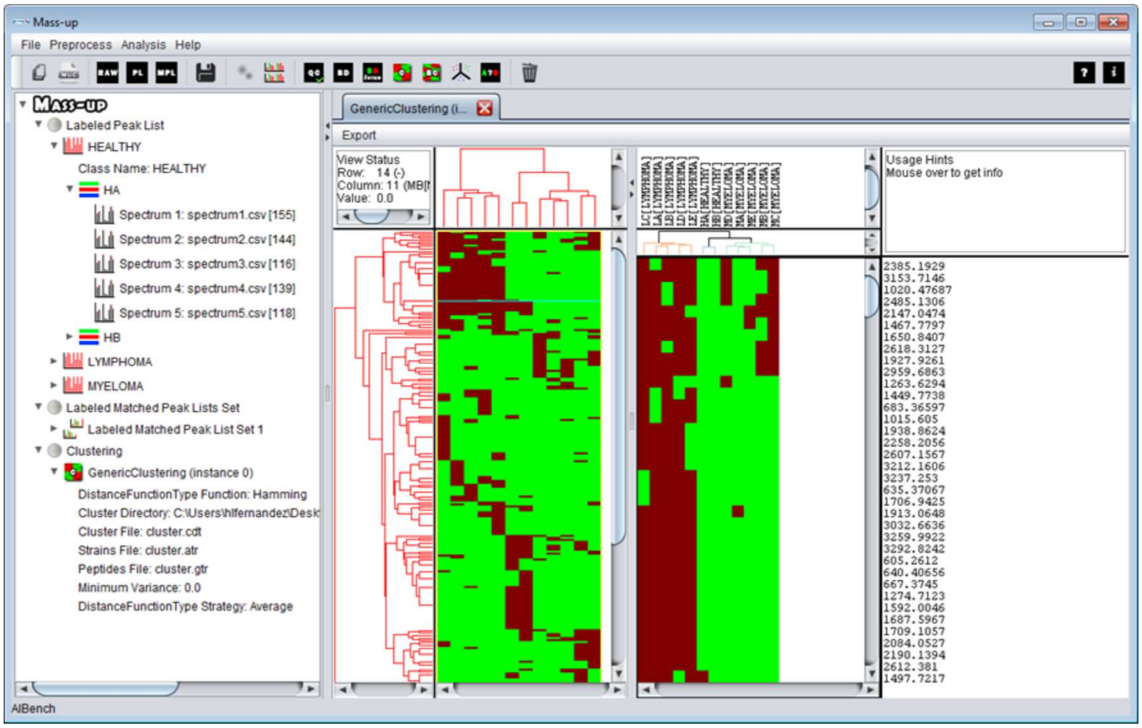
4. Results

The straightforward workflow proposed in this work is implemented by Mass-Up [9], our all-in-one open software development for MALDI-TOF MS knowledge discovery fully covering the whole data analysis workflow. Mass-Up is an AIBench-based application [16] that allows researchers to easily manage and visualize raw data or peak lists, preprocess data, and execute different types of analyses such as (*i*) biomarker discovery, (*ii*) clustering, (*iii*) biclustering, (*iv*) three-dimensional PCA visualization and (*v*) classification of large sets of spectra data. This section briefly outlines the most relevant aspects of each analysis stage, from preprocessing to advanced machine learning-based analysis.

As commented before, preprocessing of raw data is a critical stage needed to generate a suitable input for further analysis in form of clean peak lists. Since inadequate or incorrect preprocessing methods can hinder the achievement of meaningful biological conclusions [8], Mass-Up includes state-of-the-art algorithms supporting the main preprocessing steps: (*i*) baseline correction, (*ii*) smoothing, (*iii*) peak detection and (*iv*) peak alignment. Mass-Up provides *Top Hat*, *SNIP*, *Convex Hull*, and *Median* algorithms for baseline correction from the MALDIquant package [17]. Regarding smoothing, the *moving average window* and *Savitzky-Golay* methods, both from the MALDIquant library, are offered. Additionally, Mass-Up supports two m/z selection alternatives: the *CWT-based* method implemented in MassSpecWavelet and a *SNR-based* method provided by MALDIquant. Concerning peak matching algorithms, Mass-Up includes a sequential procedure based on a sliding window (*Forward*, an in-house development) and a clustering based approach from MALDIquant.

When analyzing MALDI-TOF MS data, low quality spectra can be occasionally obtained (e.g. spectra showing a significant lower or higher number of m/z values in comparison with other spectra). These kind of spectra could lead to the achievement of incorrect conclusions or even hinder them. In order to prevent this possibility, a quality control (QC) step was included between the preprocessing and the analysis tasks. This QC procedure has two targets: *replicates*, a low-level analysis on the replicates of each sample; and *samples*, a high-level analysis with extra information about the intra-sample m/z matching process.

An important aim of MALDI-TOF MS analyses is



Labeled Peak List
HEALTHY
Class Name: HEALTHY
HA
Spectrum 1: spectrum1.csv [155]
Spectrum 2: spectrum2.csv [144]
Spectrum 3: spectrum3.csv [116]
Spectrum 4: spectrum4.csv [139]
Spectrum 5: spectrum5.csv [118]
HB
LYMPHOMA
MYELOMA
Labeled Matched Peak Lists Set
Labeled Matched Peak List Set 1
Clustering
GenericClustering (instance 0)
DistanceFunctionType Function: Hamming
Cluster Directory: C:\Users\hlfernandez\Desk
Cluster File: cluster.cdt
Strains File: cluster.atr
Peptides File: cluster.gtr
Minimum Variance: 0.0
DistanceFunctionType Strategy: Average

View Status
Row: 14 (-)
Column: 11 (MB[I
Value: 0.0

Usage Hints
Mouse over to get info

```
2385.1929
3153.7146
1020.47687
2485.1306
2147.0474
1467.7797
1650.8407
2618.3127
1927.9261
2959.6863
1263.6294
1449.7738
683.36597
1015.605
1938.8624
2258.2056
2607.1567
3212.1606
3237.253
635.37067
1706.9425
1913.0648
3032.6636
3259.9922
3292.8242
605.2612
640.40656
667.3745
1274.7123
1592.0046
1687.5967
1709.1057
2084.0527
2190.1394
2612.381
1497.7217
```

Figure 2. Mass-Up application showing a clustering analysis. The example dataset (available at http://sing.ei.uvigo.es/mass-up/downloads/datasets/Cancer-Dataset.zip) is composed of sera from 5 patients with lymphoma, sera from 5 patients with myeloma, and sera from 2 healthy donors. As the dendrogram illustrates, the three conditions are correctly separated since all the samples of each condition are grouped together.

biomarker discovery, that is, the identification of peptides or proteins of a sample able to differentiate specific conditions such as diseases or infections [18]. Following the recommendations given by McDonald [19], Mass-Up includes four different tests of independence (i.e. Fisher's exact test, Yates' chi-square test, Randomization test, Chi-square test) that allow users to identify those peaks that can be potential biomarkers to differentiate the conditions. The test applied in each analysis depends on both the number of samples and the number of peaks present in the dataset.

PCA is a mathematical procedure that can be applied to reduce the dimensionality of a set of samples containing eventually correlated variables (i.e. m/z values), by creating a set of values of linearly uncorrelated variables called principal components (PC). These PC can be used to represent the samples in a 3-dimensional space. By simply assigning different colors to samples' conditions, users can visually identify if there is a separation between conditions and, therefore, they are distinguishable.

Cluster analysis allows finding groups of samples with similar spectral profiles in the dataset. As an unsupervised technique, it allows discovering hidden or previously unknown subgroups of unlabeled samples. When applied to labeled data, it allows researchers to check if the different conditions previously identified in the dataset are separable by means of this technique (see Figure 2). Mass-Up includes an in-house development of an agglomerative, bottom-up hierarchical clustering algorithm.

In previous studies we have proposed a novel workflow for the application of biclustering to MALDI data [20], a simultaneous clustering on both rows and columns. Mass-Up supports this workflow allowing researchers to apply different biclustering algorithms such as Bimax and BiBit and inspect results in an intuitive biclustering viewer.

Finally, sample classification is the ability to predict the label of a sample given a training set of labeled samples, therefore, the capacity of producing a diagnosis machine [21]. Mass-Up provides an interface adapted from the Weka software allowing users to configure a specific classifier and evaluate its performance using different validation schemes. Through this operation, users can determine which classifier performs best for the dataset under study. As a result, users can: (*i*) analyze the performance of the classifier using different statistical measurements (e.g. accuracy, kappa, precision, recall, etc.) and (*ii*) make ROC analyses per condition.

Mass-Up is freely available at http://sing.ei.uvigo.es/mass-up/, where users can find installers for Windows and Linux/MacOS systems along with detailed tutorials, manuals and sample datasets.

5. Concluding Remarks

In this work, we have explored machine learning-based workflows for the analysis of MALDI-TOF mass spectrometry data. The proposed approach enhances typical

MALDI-TOF MS data analysis workflows by adding a quality control step after preprocessing and, by supporting the application of different ML approaches.

With Mass-Up, a multiplatform open-source tool implementing such workflow is provided to the scientific community. Its usefulness is demonstrated by the increasing number of studies that use our solution [22–24] and by the fact that it has been included in public mass spectrometry software repositories and projects, such as MASSyPup(64), the Mass Spectrometry Live Linux, a Puppy Linux based Live distribution that groups several tools focused on the analysis of MS data. A strength of Mass-Up is that it comes within a friendly graphical user interface designed to allow proteomics researchers analyze MALDI-TOF MS data without the need to be bioinformatics experts.

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