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A metabolomics approach to assess the effect of storage conditions on metabolic processes of New Zealand surf clam (*Crassula aequilatera*)

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ABSTRACT

Temperature fluctuations during the live storage and transport of bivalves are known to be one of the most important stressors for these markets. However, the biological mechanisms that induce these stresses and the immunological responses to stressors are not currently understood. In this study, a gas chromatography–mass spectrometry (GC–MS based) metabolomics approach was used along with flow cytometry to characterize the responses of clams during aerial exposure in two different storage conditions (ice and no ice) and storage periods (1 h and 6 h). The results showed a significant increase in haemocyte mortality and ROS production in clams exposed to room temperature (no ice) and after 6 h compared to low temperature (with ice) and 1 h, respectively. The metabolite profiles of clam haemolymph in all groups revealed changes of many metabolites between the different temperatures and sampling times. Among them, lactic acid, succinic acid, malic acid, fumaric acid and glutamic acid were identified as significantly affected by both storage condition and period. These results indicate that clam storage stresses produced biological disturbances related to energy metabolism, which was switched from aerobic to anaerobic metabolism. These findings provide insights regarding the specific metabolic consequences of bivalve live storage and transport, which may lead to improved product handling along the supply chain. In addition, this study demonstrates the effective use of metabolomics and flow cytometric approaches on bivalve haemolymph as a non-destructive shelf life assessment method.

1. Introduction

There is a growing demand for live seafood products, including bivalves (e.g., clams, oysters, mussels) throughout the world. Different methods for storage and transportation of live bivalves have been developed in order to deliver good quality products from capture or aquaculture sources to consumers (Barrento et al., 2013a; Lee et al., 2008; Overaa, 1999). Dry transport and storage with adjusted low temperature conditions has been an accepted common practice due to the lower costs and mortality compared to water submersion conditions (Barrento et al., 2013b; Chen et al., 2015; Cordeiro et al., 2017; Overaa, 1999). While these conditions are recognized to have inherent stress levels, and some degree of mortality is expected, specific quantification of associated stressors, such as temperature, moisture, oxygen and pressure, need to be appropriately evaluated to improve freshness and profitability (Lee et al., 2008; Overaa, 1999). Among these stressors, temperature is the most crucial factor that strongly impacts on metabolism and physiological processes during live storage and transport (Anacleto et al., 2013; Ellis et al., 2014; Matoo et al., 2013; Teaniniuraitemoana et al., 2016). Lower temperature during dry transport has been known to decrease oxygen consumption and ammonia excretion, and it is a common practice at the expense of metabolic performance and sometimes consumer safety (Ali and Nakamura, 1999; Barrento et al., 2013b).

Metabolomics is the qualitative and quantitative analysis of metabolites within cells, tissues or biofluids with huge applicability in all aspects of aquaculture production and supply, including larval production, nutrition and diet, disease and immunology and post-harvest quality control (Alfaro and Young, 2018; Young and Alfaro, 2016). Due to advances in high throughput analytical techniques, metabolomics has increased applicability as a very sensitive and strongly predictive tool to understand endogenous metabolic changes caused by environmental stimuli. Likewise, innovative flow cytometric analyses now afford a myriad of possibilities for highly rigorous haemolymph samples and non-destructive sampling. Thus, we applied GC–MS-based metabolomics and flow cytometry approaches to characterize the stress responses of clams within different storage and transport conditions that mimic supply chain conditions.

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2. Materials and methods

2.1. Experimental design

Adult clams were obtained from a local seafood supplier and acclimatized for one week in a re-circulation system (fresh 5 μm filtered seawater; temperature = 16 ± 0.5 °C; salinity = 34 ppt; pH = 8.26). A pool of 80 healthy clams were selected and randomly divided into two equal groups. One group was placed in four polystyrene boxes with ice at the bottom, but not in contact with the clams (10 clams/box). Another group was kept in four polystyrene boxes without ice. Temperatures inside the boxes were measured by red spirit thermometers (Interlab®, New Zealand). All animals were sampled at 1 h and 6 h after storing in the two temperature treatments. One ml of haemolymph from each animal was extracted from the anterior adductor muscle by opening the valves and inserting a needle (23 gauge × 1.5″) attached to a 3-mL sterile syringe (Terumo, Japan). Immediately after withdrawal, haemolymph samples were diluted with 1 mL of cold artificial sea water (ASW) in 2 mL Eppendorf tubes and kept on ice. 600 μL of haemolymph were subsequently pipetted into 2 mL Cryovials (BioStor™), immediately flash-frozen in liquid nitrogen, and stored at −80 °C until metabolite analyses were carried out. The remaining haemolymph samples were kept at 4 °C for flow cytometric analyses. All clams were maintained in the corresponding treatments for four days to record clam mortalities, although blood samples were only taken the first day (1 and 6 h).

2.2. Metabolomics analyses

Metabolite extractions of haemolymph samples were conducted following Nguyen et al. (2018c). Briefly, stored mussel haemolymph samples were slowly thawed on ice and mixed with 20 μL of d4-alanine (10 mM). The mixture was then re-frozen at −80 °C prior to drying in a SpeedVac Concentrator with a Refrigerated Vapor trap (Savant™ SC250EXP, Thermo Scientific) for 4 h (0 °C, vacuum level = 3). Dried samples were extracted with 500 μL of cold (−20 °C) 50% MeOH:H_2O solution. The mixture was vortexed vigorously for 1 min, re-frozen on dry ice and then thawed again. Extracts were cold (−6 °C) centrifuged at 94*g* for 10 min (Centrifuge 5424, Eppendorf AG, Hamburg, Germany) and the supernatants from the extractions were collected in 2 mL plastic vials placed on dried ice. Similarly, the second extraction was carried out with 500 μL of cold (−20 °C) 80% MeOH:H_2O. The supernatant was collected and mixed with the first supernatant prior to drying in the SpeedVac concentrator specified above. Simultaneously, two blank samples were also prepared using the sample protocol.

Extracted metabolites were derivatized based on the protocol described by Nguyen et al. (2018c). Briefly, dried samples were re-suspended in 400 μL of 1 M sodium hydroxide and quantitatively transferred to silanized borosilicate glass tubes (12 × 75 mm) (Kimble™, ThermoFisher, Auckland, New Zealand) containing 334 μL of methanol and 68 μL of pyridine. This was followed by a series of reagent additions and vortexing: 40 μL of MCF reagent – 30 s, 40 μL of MCF – 30 s, 400 μL of chloroform – 10 s, and 800 μL of 50 mM sodium bicarbonate – 10 s. The mixture was centrifuged at 1174*g* on an Eppendorf Centrifuge 5810 R (Eppendorf AG, Hamburg, Germany) for 6 min. The upper aqueous layer was discarded, and a small amount of anhydrous sodium sulphate was added to remove residual water. The chloroform phase containing the MCF derivatives was transferred to 2 mL amber GC glass vials fitted with inserts (Sigma-Aldrich, St. Louis, MO, USA). Two standard amino acid mixtures (100 μL, 20 mM [Merk, Darmstadt, Germany]) were similarly derivatized for quality control (QC) purposes.

The data acquisition was performed on a GC7890-MS5970 system (Agilent Technologies, Inc., US) equipped with a ZB-1701 GC capillary column (30 m × 250 μm id × 0.15 μm with 5 m stationary phase [86% dimethylpolysiloxane, 14% cyanopropylphenyl]) (Phenomenex, Torrance, CA, USA). Helium was used as the carrier gas (flow of 1 mL/min) and the injector temperature was heated to 260 °C. Samples (1 μL) were injected under pulsed splitless mode. The instrumental setup parameters were conducted as previously described (Nguyen et al., 2018c; Young et al., 2017). In addition to samples, a derivatized sample blank containing the internal standard, a derivatized standard amino acid mixture, a non-derivatized standard alkane mixture, and a sample of pure chloroform solvent were also injected at the beginning and after every 6 samples for QC purposes.

2.3. Haemocyte viability and oxidative stress

The remaining haemolymph was used for flow cytometry assays that measured total cell counts, haemocyte cell viability and production of intracellular Reactive Oxygen Species (ROS) using a Muse® Cell Analyzer (EMD Millipore: Hayward, CA, USA). Total haemocyte count and viability parameters were measured using the Muse® Cell Count and Viability Kits (Merck Millipore, USA), following the manufacturer's specifications, as previously described (Nguyen et al., 2018a; Nguyen et al., 2018b; Nguyen et al., 2018c). Intracellular ROS production (namely superoxide radicals) were measured using the Muse® Oxidative Stress Kit (Merck Millipore, USA), following the manufacturer's specifications, as previously described (Nguyen et al., 2018a; Nguyen et al., 2018b; Nguyen et al., 2018c).

2.4. Data analysis

Prior to statistical analysis of results, raw spectra were pre-processed using Automated Mass Spectral Deconvolution and Identification System (AMDIS) software (version 2.66) integrated with a MassOmics R-based package (The University of Auckland). The identified metabolites were then manually checked with the ChemStation software (Agilent Technologies) and AMDIS for confirmation, as previously described (Nguyen et al., 2018c; Young et al., 2017).

Statistical analyses of metabolite data were conducted using Metaboanalyst 3.0 (Xia et al., 2015). Peak intensity data were normalized by the internal standard (d4-alanine) and data were auto scaled to unit variance (mean-centered and divided by the standard deviation of each variable). A two-way ANOVA was used to identify metabolite variabilities between different storage conditions and storage periods. Interactive Principal Components Analysis Visualization (iPCA) was performed to detect overall patterns within the data. Principal Components Analysis (PCA) was used to identify natural groupings of samples at each sampling time.

Statistical analyses for flow cytometric assays were performed using two-way ANOVA with IBM® SPSS® Statistics software (version 23).

3. Results

3.1. Haemocyte viability and oxidative stress

During the experiment, temperatures were 5.7 ± 0.4 °C in the treatment with ice and 16.4 ± 0.9 °C in the treatment without ice (Fig. 1A). Mortality of haemocytes was strongly affected by both storage condition (two-way ANOVA, $F_{1,64} = 12.31$, $p = .004$) and storage period (two-way ANOVA, $F_{1,64} = 16.58$, $p = .002$). No interaction between storage condition and storage period was found (two-way ANOVA, $F_{1,64} = 2.27$, $p = .158$). Cell mortality was significantly higher in clams stored with no ice compared to clams stored in ice, and higher after 6 h compared to 1 h (Fig. 1D). Similarly, there was significantly higher ROS production in haemocytes of clams stored without ice compared to those stored with ice (two-way ANOVA, $F_{1,19} = 30.72$, $p < .001$), and ROS production was higher after 6 h compared to 1 h (two-way ANOVA, $F_{1,19} = 10.55$, $p = .004$) (Fig. 1C). There was a significant interaction of ROS production between storage conditions and storage periods (two-way ANOVA, $F_{1,19} = 5.25$, $p = .034$). The mortalities of clams after four days were 3% and 78% in the ice and no