World Journal of Microbiology and Biotechnology (2020) 36:6
https://doi.org/10.1007/s11274-019-2780-0

ORIGINAL PAPER

# Anhydrobiotic engineering for the endophyte bacterium *Kosakonia radicincitans* by osmoadaptation and providing exogenously hydroxyectoine

**Mauricio Cruz Barrera[1] · Desiree Jakobs-Schoenwandt[2] · Marcus Persicke[3] · Martha Isabel Gómez[1] · Silke Ruppel[4] · Anant V. Patel[2]**

Received: 13 February 2019 / Accepted: 30 November 2019 / Published online: 12 December 2019


## Abstract

This study presents an anhydrobiotic engineering approach aiming at conferring a high degree of desiccation tolerance to the Gram-negative endophyte *Kosakonia radicincitans*. In particular, pre-conditioning of bacteria under high salinities provides a remarkable positive influence on drying survival. The endophytic bacteria accumulate exogenous hydroxyectoine > 500 µmol $g^{-1}$ dry weight cells exerted by osmotic stress at 4% NaCl. Microfermentation research demonstrated that hydroxyectoine provides positive effects on reducing the lag phase duration and alleviates the dissolved oxygen consumption under high salinity conditions. Beyond the amassing of hydroxyectoine, this work provides evidence supporting the notion that hydroxyectoine can produce significant changes in the endogenous bacterial metabolome during the exponential growth phase at high-osmolarity. Metabolome changes include alterations on tricarboxylic acid cycle, novo-synthesis of specific intracellular metabolites such as mannitol, myo-inositol and trehalose, and fold changes on amino acids such as L-leucine, L-asparagine, L-serine, L-methionine and L-proline. The significant fold change of L-aspartate suggests a potential acidic proteome at high-osmolarity environments, extending the knowledge of salt-stressed bacterial endophytes. Thus, these findings place the metabolic salt stress response and the hydroxyectoine accumulation by *K. radicincitans* into a physiological context, paving the way into the interaction between cellular phenotype associated with salt stress tolerance and drying survival capacity of Gram-negative endophytes.

**Keywords** Drying survival · Pre-conditioning · Metabolic response · Plant growth-promoting endophytes · Salt stress

## Introduction

Plant growth-promoting bacterial endophytes (PGPBE) can offer various benefits to the host plant, particularly growth promotion and protection from pathogens (Santoyo et al. 2016). These microorganisms can enhance plant growth and defense by constructing soil microbiomes, supporting phytohormones synthesis, precursors of secondary metabolites and fixing atmospheric nitrogen (Brader et al. 2014; Oteino et al. 2015; Bacon and White 2016). In general, these endophytes offer many options for biotechnological use as biological control agents, biofertilizers or biostimulants (Lodewyckx et al. 2002; Oteino et al. 2015). However, culturable endophytes application through formulates in crop production systems by sprays, seed coatings, granules, and capsules still pose a range of scientific and biotechnological challenges (Santoyo et al. 2016).

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s11274-019-2780-0) contains supplementary material, which is available to authorized users.

✉ Mauricio Cruz Barrera
fcruz@agrosavia.co

[1] Corporación Colombiana de Investigación Agropecuaria (AGROSAVIA), Mosquera, Colombia. Km 14 Bogotá-Mosquera, Mosquera, Colombia

[2] WG Fermentation and Formulation of Biologicals and Chemicals, Department of Engineering Sciences and Mathematics, Bielefeld University of Applied Sciences, Bielefeld, Germany

[3] Microbial Genomics and Biotechnology, Center for Biotechnology, Bielefeld University, Bielefeld, Germany

[4] Leibniz-Institute of Vegetable and Ornamental Crops, Grossbeeren, Germany

Considering that preservation of microorganisms by desiccation is the preferred method in formulates for long-term storage (Berninger et al. 2018), one of the main concerns for PGPBE from the laboratory to industrial development comprises drying tolerance. The challenge is higher when the bacterium presents physiological disadvantages for counteracting desiccation stress as in Gram-negative bacteria. Besides, there is limited knowledge regarding strategies aiming at enhancing drying survival for non-sporulating PGPBE. To increase drying resistance by using integrated novel cultivation-formulation approaches could be the key for further popularization and application of PGPBE.

*Kosakonia radicincitans* DSM 16656$^T$ is a Gram-negative PGPBE showing an ability to increase the growth and yield of different crop plants, such as wheat, corn, maize, tomato and radish (Remus et al. 2000; Berger et al. 2015, 2018), containing plant –promoting gene clusters unique to this species (Becker et al. 2018). These bacterial cells can fix atmospheric nitrogen (Ruppel and Merbach 1995), to solubilize rock phosphate (Schilling et al. 1998), induce plant immune responses (Brock et al. 2013), produces phytohormones such as auxins and cytokinins (Scholz-Seidel and Ruppel 1992), alters the plant secondary metabolite composition (Schreiner et al. 2009) and even product quality (Berger et al. 2017). Despite the potential use of *K. radicincitans* as a commercial biostimulator, this Gram-negative bacterium presents a low resistance for drying, and it is still unknown how the organism adapts to highly osmotic environments.

Several studies claimed that exposing bacterial cells as *Lactobacillus* sp. or *Pseudomonas* sp. to sub-lethal conditions lead supports resistance to deleterious effects caused by abiotic stresses such as drying (McIntyre et al. 2007; Cabrefiga et al. 2014; Shao et al. 2014; Barbosa et al. 2015). Thus, studies on epiphytic bacteria suggested that tolerance against environmental factors could improve by eco-physiological manipulation of growth conditions, cells pre-conditioning or through compatible solutes accumulation. These compatible solutes are low-molecular-weight compounds that primarily accumulate under hyperosmotic stress. Considered also as osmolytes, commonly share the properties of being polar, highly water-soluble, do not interact with proteins and do not carry a net charge at physiological pH (da Costa et al. 1998; Kempf and Bremer 1998; Sevin et al. 2016a). In addition to their function to equilibrate intracellular osmotic balance, compatible solutes operate as useful agents in bacterial cells by working as enzyme functions stabilizers, then protecting whole cells against high temperature, desiccation, salinity, freeze–thaw procedures and even drying (Lippert and Galinski 1992; Sleator and Hill 2002; Manzanera et al. 2004). Noteworthy, both environment variations during growth may lead to greater phenotypic plasticity (Schulz and Boyle 2005). Thereby, earlier studies discussed the beneficial effects on bacterial endophytes caused by salt stress and the uptake of hydroxyectoine, including metabolic reordering and enhancements of phosphatases activity (Barrera et al. 2019). Hence, osmoadaptation by compatible solute gathering as a pre-conditioning mechanism may drive a feasible alternative to strength PGPBE cells before drying.

Hydroxyectoine is an intracellular compatible solute in halophytic bacterial pools (del Moral et al. 1994; Ono et al. 1998), acting as a protein-protecting agent. Its hydroxylated nature has properties superior to its precursor ectoine in many applications (Wang et al. 2006). So far, few studies have dealt with the anhydrobiotic engineering in PGPBE to confer tolerance for drying (Berninger et al. 2018). Indeed, the influence of exogenously compatible solutes during the osmoadaptation in bacterial endophytes and ensuing drying survival is unexplored. Besides, exiguous studies on metabolic profiling responses by exogenous addition of compatibles solutes in bacteria have been carried out, including the osmotic-induced L-proline amassing by *Tetragenococcus halophilus* for revealing alterations in TCA cycle and amino acid profiles (He et al. 2017), and the insights into metabolic osmoadaptation of the ectoine producer *Chromohalobacter salexigens* (Piubeli et al. 2018). However, to the best of our knowledge, there are no studies regarding Gram-negative bacterial endophytes and metabolic profiling upon salt stress and exogenously supplied hydroxyectoine. Furthermore, the protective mechanisms of osmolytes such as hydroxyectoine are partly understood. Therefore, to elucidate the desiccation protective effects and the re-routing of metabolic flux upon hydroxyectoine addition and salt stress, the metabolic responses of hydroxyectoine-added *K. radicincitans* cells were analyzed, using a high-throughput analytical gas chromatography-mass spectrometry (GC–MS) approach.

Here, the research hypothesis states that cells pre-conditioning by osmoadaptation and providing exogenously hydroxyectoine in culture media, protects bacterial cells by shifting metabolic profiling and increasing the drying survival. Thus, this research aimed at determining the influence of *K. radicincitans* cells pre-conditioning on the drying survival and metabolic response.

## Materials and methods

Compatible solute standard hydroxyectoine (H-ectoine) was acquired from Sigma Aldrich (Cat: 70709, Sigma Aldrich Corporation, Darmstadt, Germany). All other materials used in this study were provided by Carl Roth GmbH (Karlsruhe, Germany) and concentrations are given as (w/w).

### Bacteria and growth conditions

*Kosakonia radicincitans* DSM 16656$^T$ [Ref: 6554: Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen

und Zellkulturen GmbH, Collection DSM 16656] was provided by Leibnitz Institute of Vegetable and Ornamental Crops in Grossbeeren, Germany. Chemically defined growth medium (DM) was routinely used composed by: (g $l^{-1}$) glycerol (15), yeast extract (8), $K_2HPO_4$ (2.74), $KH_2PO_4$ (1.31), $MgSO_4{\cdot}7H_2O$ (0.5), $FeSO_4H_20$ (60 ppm), $MnSO_4$ (10 ppm) at pH 7.4.

## Osmoadaptation and effect of hydroxyectoine: BioLector procedure

The influence of different NaCl concentrations [0, 1, 3, 4%] and the addition of hydroxyectoine on *K. radicincitans* kinetic growth was monitored online in microtiter plate cultivations (MPCs). These cultivations were carried out in a novel microbioreactor, the RoboLector-BioLector system (m2p-labs, Baesweiler, Germany). Hydroxyectoine was sterilized separately by filtration through a 0.2 µm membrane filter (Durapore® 0.2 µm PVDF, Millipore, Ireland), and added at 1 mM final concentration to the DM media. The microtiter plate (MTP) assays were conducted in 48-well flower plates, and the plates were enclosed with an adhesive gas-permeable membrane (Thermo Scientific, Dreieich, Germany) (Huber et al. 2009). The BioLector instrument was used for non-invasive online assessment of scattered light (signal representing the biomass formation) during cultivations for 48 h. Signals were acquired by irradiating each well with a light of a defined wavelength in a filter (excitation) and detecting and interpreting the reflected/scattered light. The monitoring of all BioLector cultivations used the following adjustments: Scattered light (filter 620 nm, Gain 20), $pO_2$-optode (filter 500 nm, Gain 33). The experiments were carried out at 30 °C under constant stirring (1200 rpm, shaking diameter = 3 mm, orbital) in 48-well MTP-48-BO flower- plates, Lot No: 1711 (mp2-labs, Baesweiler, Germany) with an adjusted volume of 1000 µl DM. Each treatment was composed of three replicates. High densities of bacterial cells were necessary to correlate scattered light intensities and biomass concentrations in BioLector. A high cell-density starter culture of *K. radicincitans* (~5 g dry matter $l^{-1}$) was diluted in DM and measured at the same operating conditions as in the cultivation assessments. (Kensy et al. 2009).

## Detection of hydroxyectoine accumulation

The intracellular and extracellular hydroxyectoine concentration was carried out by previously reported methods (Teixido et al. 2005). Briefly, samples of *K. radicincitans* cells grown in DM 4% NaCl plus hydroxyectoine [1 mM] at 190 rpm, 30 °C were centrifuged for 10 min at 10,000 rpm and 20 °C (Mikro HT 200R, Hettich GmbH & Co. KG, Tuttlingen, Germany). The supernatant fraction served for further high-performance liquid chromatography (HPLC) analysis. The bacterial pellets were re-suspended in HPLC grade water and centrifuged to discard residues of culture medium. Subsequently, approx. 50 µl of concentrated biomass was extracted for quantitative evaluations with 570 µl of an extraction solution (methanol/chloroform/water 10:4:4, v/v) by intense shaking for 5 min followed by the inclusion of equal volumes (170 µl) of chloroform and water (Kunte et al. 1993). After shanking for 10 min, the phase separation was ensured by centrifugation (5 min at 10,000 rpm). The hydrophilic top layer containing compatible solutes was recovered. Hydroxyectoine quantification was achieved by HPLC using an EC 150/4.6 NUCLEODUR® 100-5 $NH_2$-RP column and a UV-detector at 215 nm, at a flow rate of 1 ml $min^{-1}$ at 30 °C accompanied by a column heater and using a solvent gradient established between eluents A and B (80% ACN in HPLC water). The peak areas were calculated and compared with calibration curves created with standards of each solute [0.1-1 mM]. Results were expressed as *µmol* compatible solute $g^{-1}$ (dry weight *K. radicincitans* cells). All results are the mean of four replicates bacterial samples per stress condition and incubation time.

## Drying survival assessments

Bacteria suspensions for drying survival assessments were prepared as follows: DM (100 ml) was poured into 250 ml baffled Erlenmeyer flasks that were autoclaved at 121 °C, 1.5 atm, for 30 min. The initial inoculum concentration in media was adjusted at $10^6$ cells $ml^{-1}$. The cultures were maintained at 190 rpm in a rotary incubator at 30 °C (IKA KS 4000 ic control, Staufen, Germany). Actively growing cells were harvested at exponential phase after 20 h ($OD_{600}$ 0.6) by centrifugation at 5252×*g* for 15 min (Mikro HT 200R, Hettich GmbH & Co. KG, Tuttlingen, Germany), and the obtained pellet of bacteria was washed and centrifuged twice with a corresponding NaCl solution [0, 1, 2, 3 4%] to maintain the osmotic pressure. The bacteria were stored in the same NaCl solution adjusted at $OD_{600}$ ~ 1.0 (~ $1.0 \times 10^{10}$ CFU $ml^{-1}$) for the ensuing drying test. 100 µl of each bacterial suspension was spread evenly as a thin layer onto culture microplates-6 wells (VMR 10062-892, Stockholm, Sweden). Samples were allowed to dry under oxic conditions during 2 h in a sterile cabinet at 25 ± 1 °C, an airflow at 0.4 m $s^{-1}$ with relative humidity at 45 ± 2%. After drying, dried bacteria cells were recovered from microplates by adding 5 ml of NaCl solution [0, 1, 2, 3, 4%] to wash off the dried biofilm in a rotatory shaker at 120 rpm, 20 °C for 1 h. For assessment of the viability, serial dilutions were plated on standard nutrient agar media (Merck, Darmstadt, Germany), incubated at 30 °C for 24 h and counted to determine colony forming units (CFU). Bacterial cells with added hydroxyectoine were treated with the same procedure.

## Biomass samples preparation for metabolic profiling

The relative levels of metabolites in the *K. radicincitans* cells during the exponential phase were assessed in an untargeted approach. Intracellular metabolites extraction and gas chromatography-mass spectrometry (GC–MS) analyses were conducted as follows: 2 ml culture volume of bacteria at exponential phase ($OD_{600}$ ~ 0.6) were harvested by fast centrifugation for 1 min at 15,000 rpm (Mikro HT 200R, Hettich GmbH & Co. KG, Tuttlingen, Germany). Further, the pellet was rapidly quenched in liquid nitrogen until processing. After quenching, samples were freeze-dried (Christ GmbH, Osterode am Harz, Germany) overnight. Metabolites extraction was conducted according to the procedure described by Plassmeier et al. (2007). In particular, ~ 5 mg of dried biomass was added to 0.5 g zirconia/silica beads (0.5 mm diameter, BioSpec Inc., OK, USA). Further, 1 ml 80% MeOH was added with ribitol (10 µmol $l^{-1}$) as an internal standard. Disruption of biomass was performed for 3 × 60 s at 6200 $min^{-1}$ in a homogenizer (Precellys 24, Bertin instruments, Montigny-le-Bretonneux, France). The obtained lysate was centrifuged at 19,000×*g* for 5 min (Centrifuge 5424, Eppendorf AG, Wesseling- Berzdorf, Germany) and 650 µl of the supernatant was transferred to 1 ml micro reaction vessels (Supelco Inc., CA, USA). In parallel, one vessel containing 1 ml 80% MeOH/10 µM Ribitol was used as a blank.

Evaporation of the solvent in samples was ensured at 37 °C and nitrogen gas contact for 80 min (Reacti Therm heating and stirring module, Thermo Fisher Scientific Inc., MA, USA). The derivatization of dried extracts was carried out with the addition of 75 µl of methoxyamine (20 mg $ml^{-1}$ in pyridine) for 90 min at 37 °C [Sigma- Aldrich GmbH (VWR International GmbH, Darmstadt, Germany)]. Later, 75 µl *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) (Macherey–Nagel GmbH & Co. KG, Dueren, Germany) was added for a second derivatization step and the reaction was stirred for another 30 min. Finally, the derivatization reaction was centrifuged at room temperature at 4000 rpm for 5 min, (Centrifuge 5810R, Eppendorf AG) and the supernatant fractions were transferred to HPLC vials with 100 µl inlays (VWR International GmbH) before loading into the GC–MS autosampler.

## GC–MS assessments

GC–MS analysis was conducted on a TraceGC gas chromatograph connected with a PolarisQ ion trap mass spectrometer and an AS2000 autosampler (Thermo Finnigan GmbH, Dreieich, Germany) (Plassmeier et al. 2007; Krell et al. 2018). Briefly, supernatant fractions were injected at 1 µl volume and 250 °C through a 30 m × 0.25 mm Equity-5 column with a 0.25 µm 5% diphenyl/95% dimethylsiloxane coating (Supelco Inc.). The temperature was maintained at 80 °C for 3 min. Further, the temperature profile was settled at 5 °C $min^{-1}$ to 325 °C, acquiring the mass spectra at 4 scans $s^{-1}$ with a range of 50–550 m/z. Previous to the next injection procedure, the temperature was adjusted to 80 °C and maintained for 5 min. The integration of peaks in chromatograms was performed using Xcalibur 2.0 (Thermo Finnigan GmbH). Samples were inspected for the existence of a ribitol peak (m/z: 217). Metabolite relative contents are expressed in arbitrary units (semi-quantitative determination). Moreover, metabolite verification was performed via commercially available standards and the NIST 98 database (NIST, MD, USA). Peak integration was conducted automatically and normalized to dry biomass weight and ribitol area.

## Metabolome data processing

The intracellular metabolite levels were determined to extend the understanding of the physiological adaptations of *K. radicincitans* to support hyperosmotic salt stress. Metabolic data processing and statistical analysis were performed through MetaboAnalyst 4.0 workflow (http://www.metaboanalyst.ca). The data followed normal distribution after data examination and normalization (internal standard ribitol). Unsupervised Principal Component Analysis (PCA) and supervised Partial Least Square-Discriminant Analysis (PLS-DA) were used as multivariate approaches, for exploring and selecting essential features. Performance of PLS-DA model was elucidated using two criteria, R2 and Q2 and cross-validated by permutation test (Xia et al. 2009, 2012, 2015). The level of significance for the contribution of metabolites was identified using the variable importance in projection (VIP), which was computed on the weighted sum of the squares for the partial least squares (PLS-DA) loadings (Farres et al. 2015).

## Statistical analysis

Data were analyzed using the SPSS Statistics v.22 software (SPSS, Chicago, IL). Data were inspected for normality and homogeneity of variance using Shapiro–Wilk's and Levene's test, respectively. Means were tested for significant differences by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. The level of significance was set at $p < 0.05$. Percentage data on drying survival were arcsine transformed before statistical analysis.

## Results

### Osmoadaptation and effect of hydroxyectoine: BioLector procedure

To investigate the osmotic pressure influence on the kinetic growth response of *K. radicincitans,* a high-throughput microfermentation strategy using a novel microbioreactor system was followed. The BioLector scattered signal demonstrated that *K. radicincitans* showed growth variability under different $a_w$ [0.97, 0.96, 0.955, 0.95] conditions generated by the ionic solute NaCl at 0, 1, 3 and 4% in DM, respectively. Interestingly, bacterial cells were able to grow in media with a low $a_w$, simulating environmental stress conditions (Fig. 1a). At 16 h, *K. radicincitans* in DM 1% NaCl (0.96 $a_w$) reached a similar cell density relative to the control without NaCl addition in DM at 0.97 $a_w$. Relative to the biomass (X) evolution with the highest point value of Ln (X/Xo) data, the maximum growth of 88.91% ± 1.49% and 75.99% ± 1.82% compared to the control was obtained at 19.3 h and at 33 h for 0.955 $a_w$ and 0.95 $a_w$ respectively (Fig. 1a). However, at 0.95 $a_w$ bacteria proliferation was strongly impaired. Thus, further kinetic parameters were also affected by increasing the salinity in media such the maximum specific growth rate ($\mu_{max}$). The $\mu_{max}$, which was taken at the point of highest slope or maximum exponential growth stage in the range at 7–10 h, 10–12.3 h, 12–14.3 h and 17–20 h for NaCl at 0, 1, 3 and 4% in DM, respectively (Fig. 1a). The $\mu_{max}$ for non-amended media and 1% NaCl were 0.334 ± 0.004 $h^{-1}$and 0.361 ± 0.008 $h^{-1}$, decreasing significantly to 0.2849 ± 0.015 $h^{-1}$ in DM at 3% NaCl and 0.1529 ± 0.0026 $h^{-1}$ in DM 4% NaCl ($F_{3, 11} = 319.5$, $p < 0.001$). *K. radicincitans* was not able to grow at $a_w$ lower than 0.94. Glycerol as the main C-source was depleted after 18 h and 30 h in DM 1% and 4% NaCl respectively (data not shown).

Dissolved oxygen tension (DOT) signal displayed no oxygen limitation throughout the whole cultivation in DM ($a_w = 0.97$) (DOT ≥ 60%). DOT curves dropped likely until complete glycerol consumption, after 10 h, 19.3 h and 21.3 h for DM without salt and supplemented with 1% and 3% NaCl respectively (Fig. 1b). Interestingly, DOT curve at 4% NaCl showed an extended plateau in the range of 20 h to 47.3 h, at levels lower than 8% of air saturation, indicating a high oxygen consumption rate during the exponential and early stationary phases.

*Kosakonia radicincitans* osmoadaptation improved by adding hydroxyectoine at 1 mM. Hence, exogenously provided hydroxyectoine can extend the upper growth limit of *K. radicincitans* under high-salinity conditions. Thus,

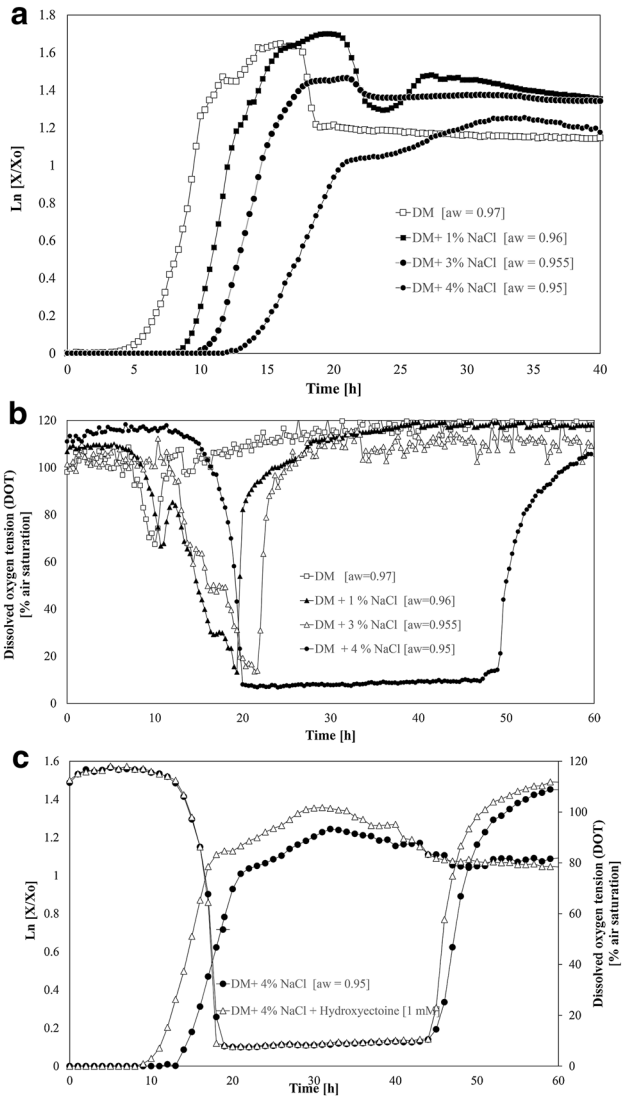



**Fig. 1** **a** *K. radicincitans* osmoadaptation at different NaCl concentrations in defined media (DM). **b** Effect of NaCl concentration in DM on dissolved oxygen tension (DOT). **c** Effect of hydroxyectoine addition at 1 mM on *K. radicincitans* kinetic growth during osmoadaptation in DM at $a_w$ 0.95 (4% NaCl). Ln [X/Xo] values were calculated by calibrating scattered light intensities and biomass (X) concentration curves of cultivations in a MTP in the BioLector system. Mean values, n = 3

after 24 h of incubation, the biomass with hydroxyectoine supply increased significantly by 15.18 ± 3.82%, compared to that obtained at DM 4% NaCl. Furthermore, the lag-phase in DM 4% NaCl [$a_w$ 0.95] lasted 14 h, and it shortened by 3.1 h with the inclusion of hydroxyectoine (Fig. 1c). The specific growth rate was also significantly higher in hydroxyectoine-added cells at 0.1808 ± 0.004 $h^{-1}$ (range at 18–20 h) in comparison to DM at 4% NaCl at 0.1562 ± 0.004 $h^{-1}$ ($F_{1, 5} = 18.98$, $p = 0.0121$).

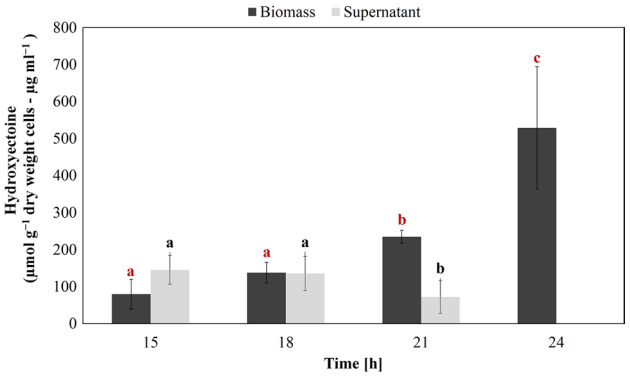



**Fig. 2** Accumulation of intracellular hydroxyectoine in *K. radicincitans* cells grown in DM modified with 4% NaCl ($a_w$ 0.95) and hydroxyectoine [1 mM] at 30 °C, 190 rpm for 24 h. Different letters indicate significant differences within time-samples in biomass and supernatant fractions, according to Tukey post hoc test at $p < 0.05$ (mean ± SD, n = 4)

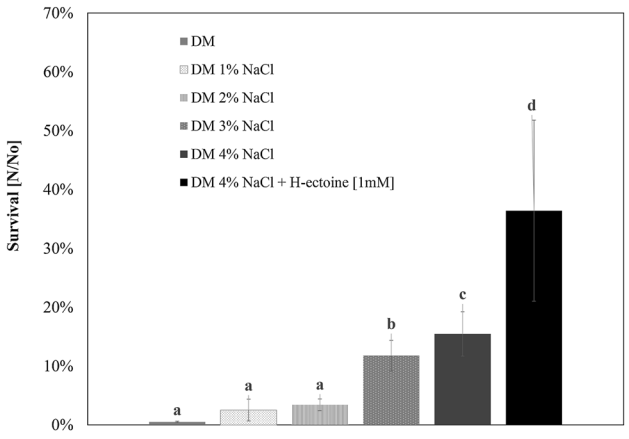



**Fig. 3** Survival of *K. radicincitans* cells after 2 h of drying (25 ± 2 °C, RH 45 ± 3.5%). **a** Effect of pre-conditioning by osmoadaptation in DM NaCl amended media and the addition of hydroxyectoine [1 mM] to 4% NaCl pre-conditioned cells. Columns with different letters are significantly different ($p < 0.05$) according to Kruskal–Wallis post hoc test (mean ± SD, n = 4)

## Hydroxyectoine accumulation

The bacterial hydroxyectoine uptake was detected after 15 h in response to high salinity during the exponential growth phase in DM at 4% NaCl (Fig. 2). The culture age caused a significant effect on the hydroxyectoine accumulation ($F_{3, 15} = 36.45$, $p < 0.001$), since osmolyte content in *K. radicincitans* cells increased over time, reaching more than 500 µmol per gram of dry biomass at 24 h. No hydroxyectoine was detectable in cells grown in DM in the absence of salt.

## Drying survival

The survival of *K. radicincitans* fresh suspension after desiccation was influenced significantly by salt concentration increments in the culture media DM. Thus, bacterial cells pre-conditioned in DM 4% NaCl showed the highest drying tolerance in comparison to non-preconditioned cells. Interestingly, exposing bacteria to persistent high osmolarity surroundings during cultivation at 4% NaCl, resulted in a nearly two orders of magnitude higher survival rate (Fig. 3) with 15.5 ± 3.7% of living cells recovered, compared to 0.51 ± 0.12% in control media DM. The inclusion of hydroxyectoine at 1 mM in DM 4% NaCl provided a further desiccation tolerance in comparison to cells pre-conditioned with DM 4% NaCl. Hence, the combination of ionically amended media and hydroxyectoine significantly increase drying survival to 36.42 ± 1.53% ($Chi^2 = 16.01$; df = 18; $p = 0.00681$, Fig. 3).

## Metabolome responses to hyperosmotic salt stress

To elucidate the physiological changes conferring enhancement on drying survival in bacterial cells after osmotic stress, a non-targeted metabolomics was performed. More than 70 metabolites were identified by comparing to their corresponded mass spectra database and their retention time values. The spectrum of identified metabolites encompassed sugars, organic acids, sugar alcohols, polyamines, and amino acids. Principle components analysis (PCA) for the intracellular metabolites showed a significant alteration caused by the osmotic stress (Fig. 4a). No outliers were detected by PCA at 95% confidence. The PLS-DA scores for each replicates disclosed 3 clusters well separated, where the unstressed conditions at 0% NaCl controls had negative t1 scores and the cells subjected to extended osmotic stress at 4% NaCl had positive t1 scores (Fig. 4b). The model was significant ($R^2 = 0.9817$; $Q^2 = 0.84722$) indicating sizable changes in the metabolic pools under salt stress. Metabolic profiling analyses revealed that prolonged exposure of *K. radicincitans* to osmotic stress at 4% NaCl, resulted in substantial changes in cytoplasmic energy metabolism-associated metabolites. Changes included up-regulation of pyruvate and organic acids such as fumaric acid and L-malic acid, along with sugars and polyols such as galactose, trehalose, mannitol and myo-inositol (Fig. 5a).

The contribution of the variables was determined by interpreting the variable importance in projection (VIP) score, which is estimated from the weighted sum of the square for each PLS-DA loadings for each component. Within the twenty most important variables identified by VIP scores (> 1.9), L-aspartate, malate, trehalose and mannitol were

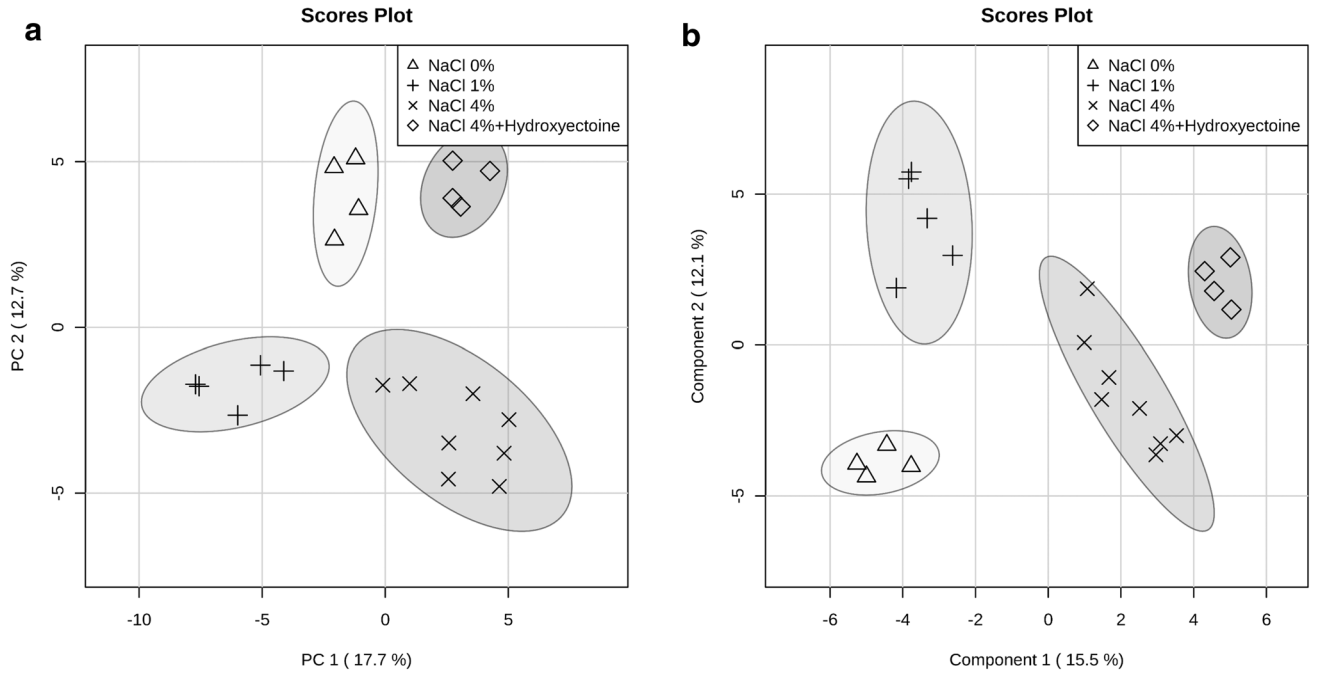



**Fig. 4** **a** PCA scores plot and **b** Partial least squares discriminant analysis PLS-DA score plot for the detected metabolites during osmoadaptation and by adding hydroxyectoine [1 mM]

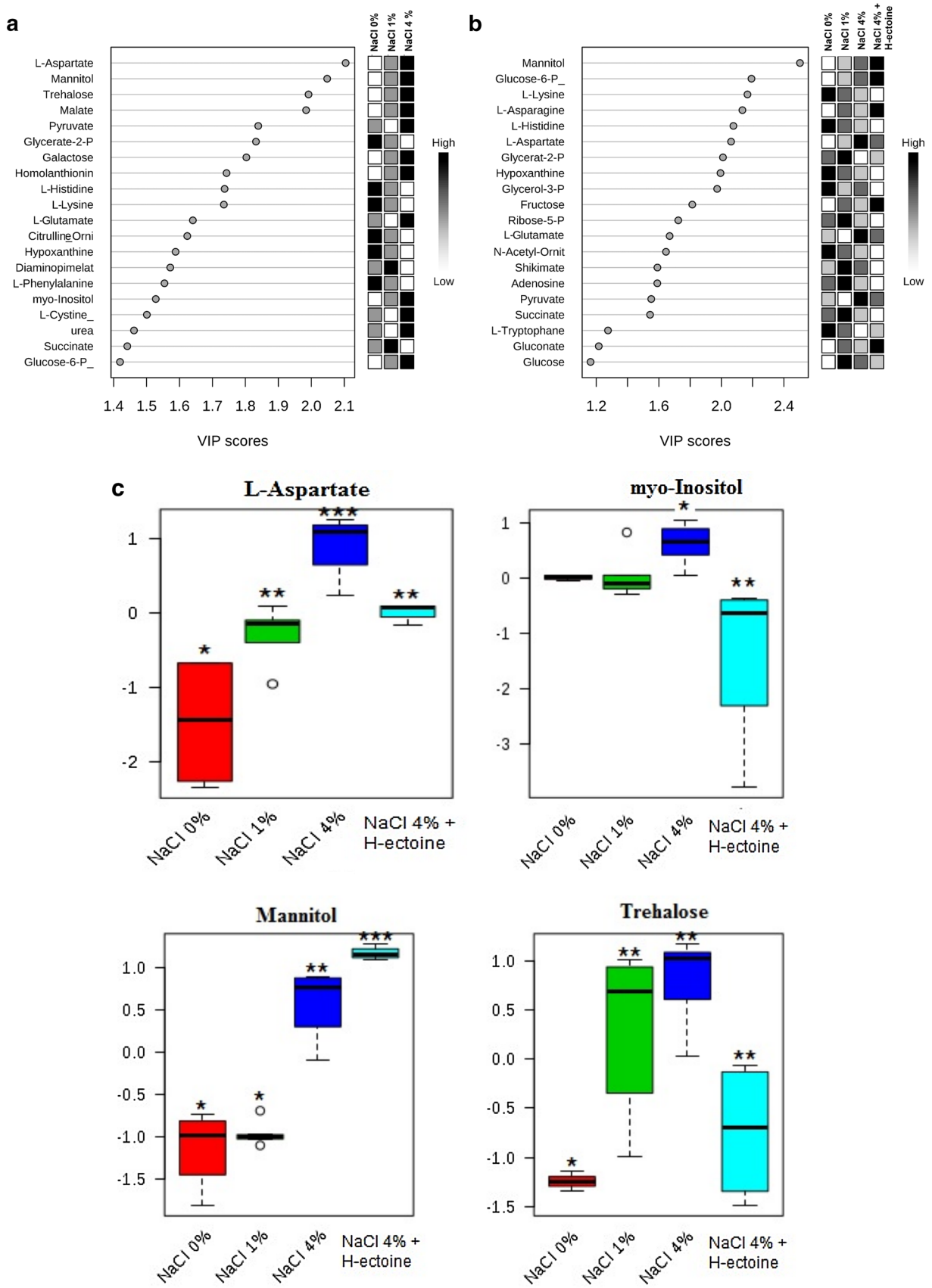
established as metabolites that contributed significantly to the class separation of osmotic stress levels (Fig. 5a).

The alterations in the cellular levels of central metabolic pathway metabolites were analyzed for all *K. radicincitans* cultivations to elucidate a unified response to osmotic stress. The trend observed for these putative altered metabolites and related pathways during salt-stress is summarized in Table S1. Particular attention was paid in investigating the levels of compatible solutes and their precursors. Thus, following the increased extracellular osmolarity, the relative abundance of L-aspartate, mannitol and trehalose increased significantly ($p < 0.05$) to 11.07, 66.84 and 65.12-fold respectively, in the *K. radicincitans* cytoplasm (Fig. 5c). Interestingly, the relative abundance of TCA cycle intermediates such as pyruvate, fumarate, malate and citrate tend to increase upon salt stress (Fig. 6). Besides, cells raised within high osmotic-induced media, greater pool contents of L-asparagine and L-glutamine with 3.49 and 2.98-fold changed were measured. Conversely, at DM 4% NaCl, for some aromatic amino acids such as L-histidine, L-tyrosine, L-phenylalanine and L-tryptophan a considerable drop in the relative abundance was detected.

## Metabolome responses to hyperosmotic salts stress in the presence of hydroxyectoine

The presence of hydroxyectoine during the osmoadaptation altered the abundances of metabolites throughout their metabolic networks. PCA plot detected no outliers at 95% confidence (Fig. 4a). The addition of hydroxyectoine led to a new cluster within this PCA plot (4% NaCl + hydroxyectoine), which is distinguished from the cluster with 4% NaCl mainly by positive PC2 scores. Similarly, the PLS-DA plot revealed four separated clusters, corresponding to the media amended with NaCl [0, 1, 4%] and the additional treatment with hydroxyectoine. Here relative to 4% NaCl, the direction of separation by addition of hydroxyectoine is along component 1, suggesting that cells with the presence of the osmolyte were metabolically different (Fig. 4b). Looking at the VIP scores (Fig. 5b), mannitol, L-lysine, L-asparagine and L-histidine were identified as the main metabolites contributing to class separation. Remarkably, L-aspartate showed a high VIP score in combination with a significant reduction in relative abundance with the inclusion of hydroxyectoine to the medium at 4% NaCl.

The uptake of hydroxyectoine during bacteria growth at DM 4% influenced the relative abundance of the majority of TCA intermediates, such as pyruvate, malate, succinate and citrate (Fig. 6). Conversely, significant changes ($p < 0.05$) in most of the detected intracellular amino acids in *K. radicincitans* cells were observed upon hydroxyectoine addition. Thus, exogenously supplied hydroxyectoine led to a significant fold change in the pools of L-asparagine, L-proline, L-serine, L-glutamate, L-homoserine, L-methionine, L-leucine and the urea cycle intermediates citrulline–ornithine–arginine (Figs. 7, 8a, b). However, the addition of


a
NaCl 0%
NaCl 1%
NaCl 4 %
L-Aspartate
Mannitol
Trehalose
Malate
Pyruvate
Glycerate-2-P
Galactose
Homolanthionin
L-Histidine
L-Lysine
L-Glutamate
CitrullineOrni
Hypoxanthine
Diaminopimelat
L-Phenylalanine
myo-Inositol
L-Cystine_
urea
Succinate
Glucose-6-P_
High
Low
1.4 1.5 1.6 1.7 1.8 1.9 2.0 2.1
VIP scores
b
NaCl 0%
NaCl 1%
NaCl 4%
NaCl 4% + H-ectoine
Mannitol
Glucose-6-P_
L-Lysine
L-Asparagine
L-Histidine
L-Aspartate
Glycerat-2-P
Hypoxanthine
Glycerol-3-P
Fructose
Ribose-5-P
L-Glutamate
N-Acetyl-Ornit
Shikimate
Adenosine
Pyruvate
Succinate
L-Tryptophane
Gluconate
Glucose
High
Low
1.2 1.6 2.0 2.4
VIP scores
c
L-Aspartate
myo-Inositol
Mannitol
Trehalose
NaCl 0%
NaCl 1%
NaCl 4%
NaCl 4% + H-ectoine

◂**Fig. 5** Variable importance in projection (VIP) plot displays the top 20 most important metabolite features identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study. **a** During osmoadaptation from non-amended media DM to 4% NaCl. **b** Upon addition of hydroxyectoine [1 mM] at 4% NaCl amended media. **c** Box-whisker plots for relative abundance concentrations of important metabolites in PLS-DA model: L-aspartate, mannitol, trehalose and myo-inositol. Asterisks indicate the level of statistical significance ($p < 0.05$; $n > 4$)

hydroxyectoine induced a drop in relative abundance levels of aromatic amino acids such as L-phenylalanine and L-tryptophan, as well as the reduction of the arginine-proline metabolism intermediate N-acetylornithine. Besides, it was notable that levels of L-lysine, L-aspartate and L-glutamine decreased in the hydroxyectoine-added cells (Figs. 7, 8a, b).

## Discussion

Fluctuations in environmental osmolarity and drought tolerance are ubiquitous stress factors encountered by microorganisms during industrial fermentation and further formulation approaches. Therefore, it is necessary to develop and understand efficient adaptation strategies to mitigate the harmfulness of these stressful conditions. Among these strategies, the intracellular compatible solutes gathering and the addition of exogenous osmolytes to protect cells against highly osmolar environments are valuable and effective (He et al. 2017; Czech et al. 2018). Molecular and physiological processes that allow bacterial endophytes such as *Kosakonia* sp. to withstand salt stress are unknown. Herein, lies a microfermentation and metabolic profiling approach, to investigate systematically the causes of the positive effects on drying survival, mediated by grown cells at high salinities and the hydroxyectoine inclusion in amended media.

Microfermentation studies in the BioLector revealed that *K. radicincitans* cells could consider osmoadapted after growing at $a_w$ lower than 0.955, when the osmotic pressure affects considerably the kinetic behavior and the oxygen transfer rate for growing. The bacteria viability at the stationary phase was slightly affected along with imposed hyperosmotic media; however, all treatments reached $10^{10}$ CFU $ml^{-1}$ after 30 h of cultivation (data not shown), indicating that viability and cellular structures of the endophyte cells were not disturbed under high osmotic potentials. Similar results regarding the bacteria endophyte *Sphingomonas* sp. LK11 and high salinities were previously described (Halo et al. 2015).

DOT curves for DM and DM 1% NaCl demonstrated the natural course of oxygen consumption for a non-oxygen limited *K. radicincitans* cultivation. Thus, the DOT curves showed no plateau, which reveals that the growth of cells was not subjected to oxygen limitation at any time (Wewetzer et al. 2015). Nevertheless, at higher salinities such as 4% NaCl, a plateau existence with DOT < 20% suggested an oxygen limitation during ~ 26 h. This shift in oxygen demand indicates that bacterial proliferation requires higher oxygen transfer rates along with the salt increment within the media. Since *K. radicincitans* cells at high osmotic potential can proliferate under low dissolved oxygen tension (DOT), the osmoadapted phenotypes may have intrinsic changes in their intracellular metabolites.

Upon hydroxyectoine addition in DM at 4% NaCl, both accelerated bacterial response to hyperosmotic conditions by the lag phase reduction and the slight DOT curve shifting were elucidated. The lag phase contraction caused by this osmolyte is consistent with other reports in bacteria (Bursy et al. 2008; Tao et al. 2016). Moreover, results proved that exogenously provided hydroxyectoine extend the upper growth limit of *K. radicincitans* under high-salinity environments. Accordingly, the growth profiles permit to theorize that hydroxyectoine has a robust osmoprotective role and curbs water efflux, since the addition of this osmolyte and further active transport through the semi-permeable cytoplasmic membrane, showed a significant effect on kinetic growth at 4% NaCl. Hence, the cytoplasmic acquisition of this compound could counteract the energy demand triggered by the adaptation to the osmotic pressures unbalance. Thereby, it seems that metabolic energy requires priory the cellular homeostasis over growth at $a_w$ 0.95.

Interestingly, hydroxyectoine uptake > 150 µmol $g^{-1}$ dry weight into *K. radicincitans* cytoplasm is sufficient to shift metabolic response for altering pool composition. The amount of accumulated hydroxyectoine agrees in concept with several reports, which demonstrated the uptake of this osmolyte by bacteria from 100 up to 400 µmol $g^{-1}$ dry weight in early and late stationary phase upon salt stress cues (Bursy et al. 2008; Tao et al. 2016). Besides, hydroxyectoine in Gram-negative bacteria such as *Pseudomonas putida* or *Halomonas elongata* offsets the detrimental events of high salinity on cell growth (Grammann et al. 2002; Manzanera et al. 2004).

As demonstrated, *K. radicincitans* cells grown in low $a_w$-media altered ionically with NaCl, pose superior drying tolerance in comparison to cells grown in an unmodified basal medium. An osmolarity threshold can cause significant differences in drying survival, likely between 2 and 3% NaCl levels in media, where bacteria cells could prefer the amassing of ions primarily $K^+$ and $Cl^-$ (salt-in strategy), over the physiologically compliant organic osmolytes (salt-out strategy) (Kempf and Bremer 1998; Czech et al. 2018). This explanation is also supported by the metabolic profiling findings, where high levels of osmolytes such as mannitol, myo-inositol, trehalose, L-glutamate and L-aspartate were detected at elevated salinities.

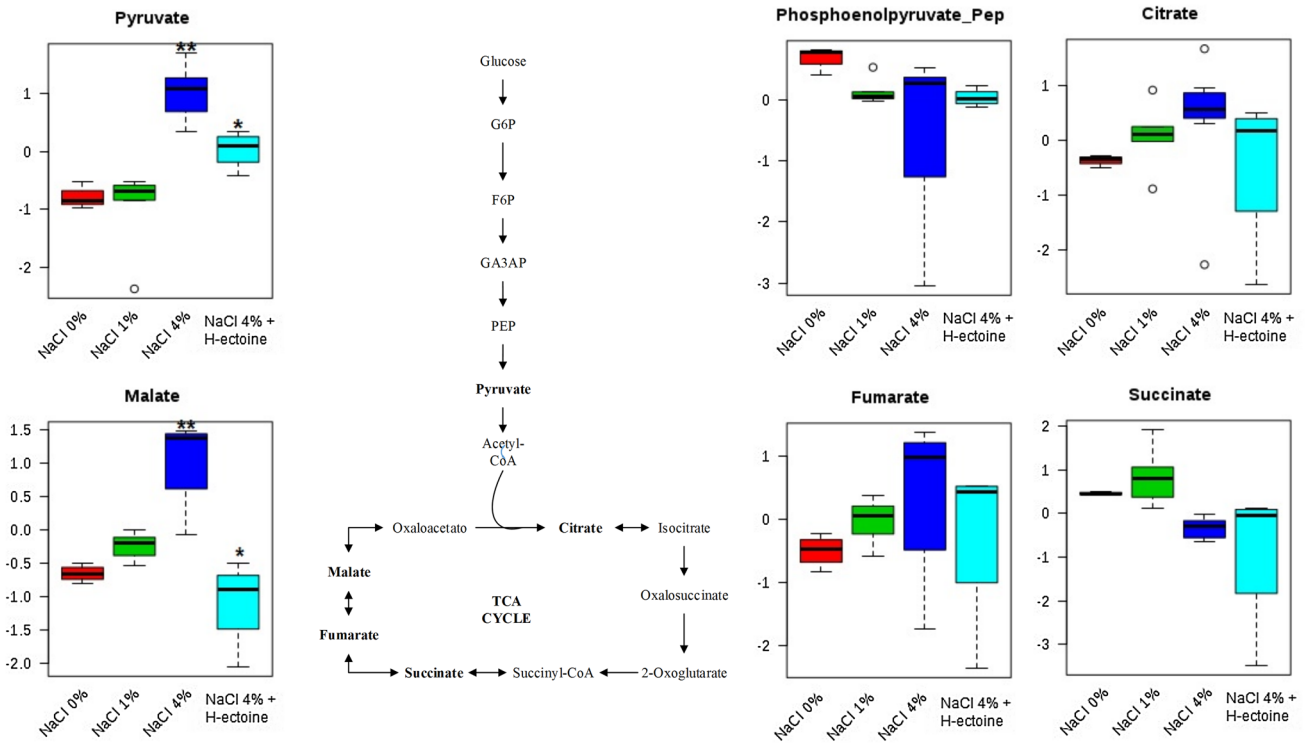



**Fig. 6** Comparison of levels of glycolysis and TCA cycle pathway intermediates in *K. radicincitans* under salt stress and upon addition of hydroxyectoine. Asterisks indicate the level of statistical significance ($p < 0.05$, $n = 8$) in comparison with the control DM amended at 4% NaCl. GA3P, glyceraldehyde 3-phosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; PEP, phosphoenolpyruvate

Beyond the trehalose amassing, an osmotically-responsive and effective intracellular drying protector in bacteria (Tunnacliffe et al. 2001; Reina-Bueno et al. 2012), the high levels of the polyol mannitol was rather surprising. Mannitol may uphold turgor without increasing ionic strength in *K. radicincitans*, since formerly it was found only in a few bacteria to cope with osmotic stress (Sand et al. 2015; He et al. 2017). Thus, mannitol can protect encapsulated bacteria as in *Bifidobacterium animalis* cells envelopes against drying stress (Dianawati et al. 2012). Noticeably, according to the *K. radicincitans* DSM 16656$^T$ genome (Becker et al. 2018), mannitol is probably synthesized by the reduction of fructose 6-phosphate (F6P) via mannitol-1-phosphate 5-dehydrogenase. Thereby, here, the fructose and mannose metabolism and levels of F6P tend to increase along with osmotic stress and by the addition of hydroxyectoine, boosting the relative abundance of mannitol protecting anhydrobiotic-induced damage.

L-Aspartate was found to be the dominant amino acid under regular conditions, whose levels along with L-glutamate increased at higher salinities. It was also disclosed in the pathway analysis, demonstrating alanine, aspartate and glutamate as one of the major pathways impacted by osmotic unbalance (Fig. S1). The accumulation of L-aspartate was also surprising since only a few studies upon Gram-negative bacteria indicated this feature (Joghee and Jayaraman 2014; Yin et al. 2017). High levels of L-aspartate may confer desiccation tolerance to bacteria cells by inserting in the interfacial region of the bacterial plasma membrane, increasing membrane fluidity (Martos et al. 2007). Moreover, L-aspartate is a powerful chelation agent playing a role in controlling concentrations of cations such as $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$ and $Cu^{2+}$ during extended exposure to osmotic stress (Sajadi 2010). Evidence indicates that these cations are involved in the regulation of a range of courses in Gram-negative bacteria, including cell division and gene expression, as a reaction to external stimuli (Malek et al. 2012).

This research found a noticeable increase in the TCA cycle of organic acids levels like pyruvate, malate, and fumarate. These findings are in line with the impact observed upon salt-stress to the pyruvate metabolism, as shown by the metabolic pathway analysis (Fig. S1). Similar results in TCA cycle alteration were found in halophilic bacterial isolates (Joghee and Jayaraman 2014). The excessive TCA cycle activation could drive the high energy demand required to preserve cell homeostasis at high osmotic pressure, where the cells hierarchically prefer the cell homeostasis over growth. Other studies indicate that under stressful conditions, energy pool management is the first concern of cells (Roessler and Muller 2001). The increment in oxygen

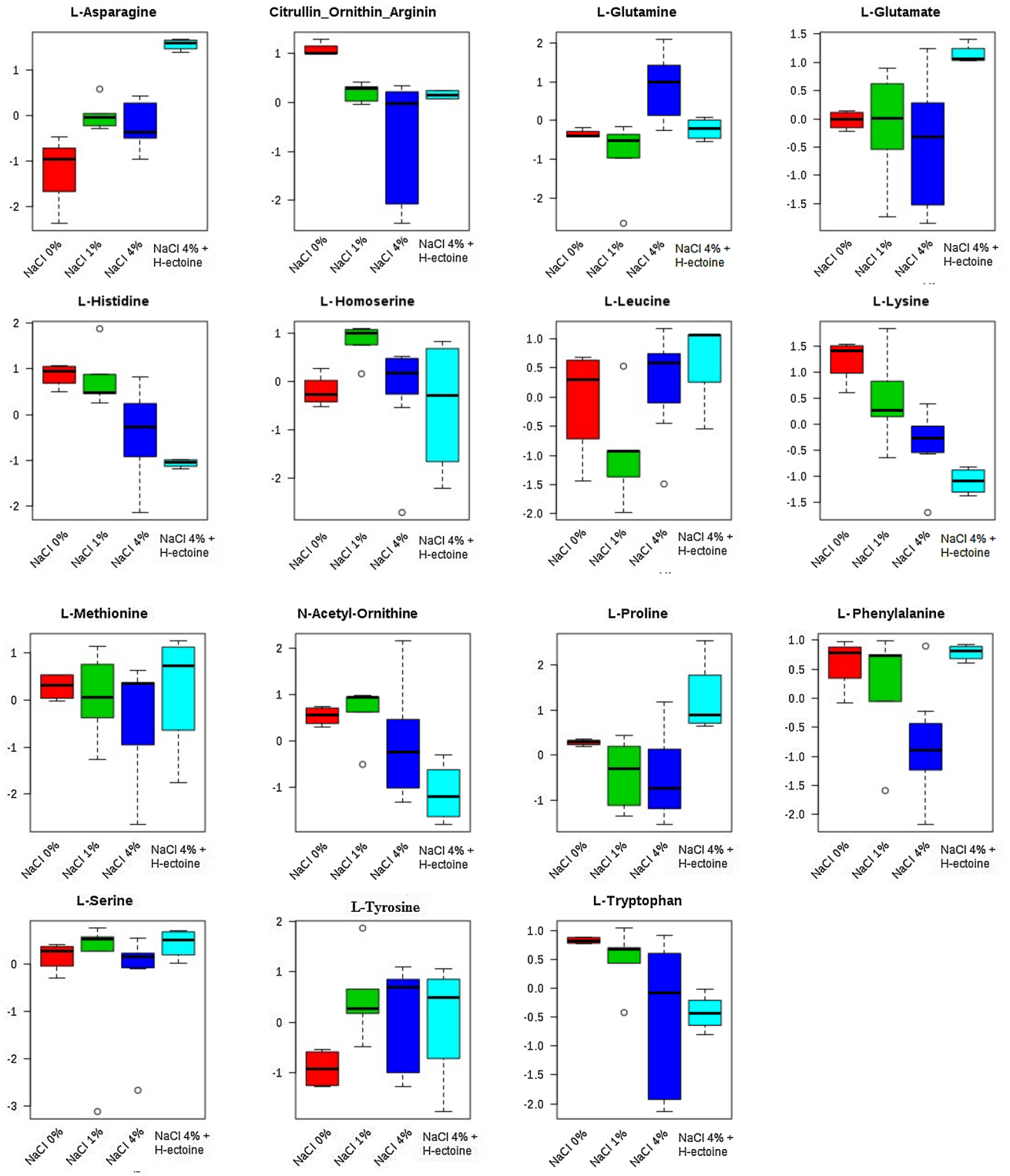



**Fig. 7** Box-whisker plots of selected amino acids with significant changes along with salt stress and the addition of hydroxyectoine

consumption in DOT curves also demonstrates the high energy demand required to sustain proliferation at high salinities.

At high salt concentrations, occurred a significant drop in the relative abundance of aromatic amino acids such as L-histidine, L-tyrosine, L-phenylalanine and L-tryptophan.

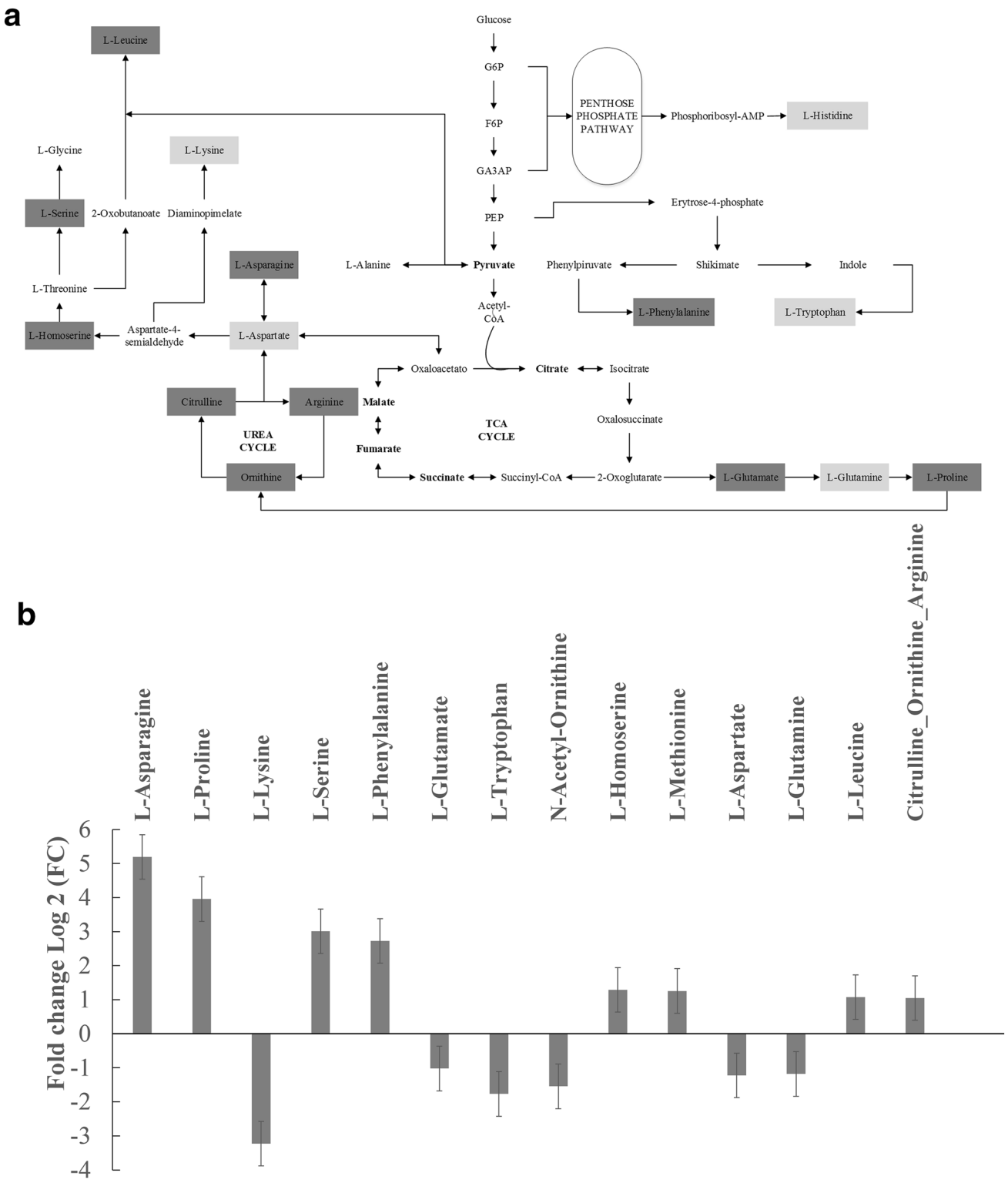



**Fig. 8** Effect of exogenous hydroxyectoine addition on amino acid metabolism in *K. radicincitans* under salt stress. **a** Schematic diagram of amino acid metabolism observed in *K.* radicincitans, dark and light grey boxes indicate positive and negative fold changes after the addition of hydroxyectoine respectively. **b** Metabolic profile of amino acids under salt stress. Fold change represents the ratio of amino acid content in the hydroxyectoine-added cells and control cells in DM at 4% NaCl, ($p < 0.05$; $n > 4$)

Since levels of basic amino acids such as L-lysine and L-histidine drop along with high salinities and acidic amino acids such as L-aspartate and L-glutamate increased, it is suggested that *K. radicincitans* possess intracellular acidic proteins functional at high-osmolarity (Oren 2013). The potential acidic proteome in *K. radicincitans* may be considered to be correlated with the amassing of KCl to contribute to the intracellular osmotic balance (Oren 2013).

The superior drying survival of *K. radicincitans* cells upon adding hydroxyectoine during the osmoadaptation procedure probably occurred due to the physicochemical features of this osmolyte. Thus, hydroxyectoine decreases water activity coefficients (Held et al. 2010) and provides superior glass-forming properties and redox stability (Tanne et al. 2014). Besides, hydroxyectoine is more efficient than ectoine as a water-binder by increasing the hydration and mobility in lipid membranes, giving an advantage for cell membranes to withstand severe surrounding environments like the osmotically-unbalanced, accelerating cellular repair mechanisms (Harishchandra et al. 2010). These properties may confer to Gram-negative bacterial cells more extended life stability, function-preserving in macromolecules, higher desiccation tolerance and protection against drying (Manzanera et al. 2002, 2004; Pastor et al. 2010). However, beyond these findings that gave scarce insights into the metabolic response of hydroxyectoine added-cells, this study presents a different role of hydroxyectoine as a metabolic shift-trigger for providing advantages to cells. Hence, the impact of osmotic stress and hydroxyectoine on *K. radicincitans* was explored in more detail on the levels of carbon metabolites.

The levels of intermediates involved in glycolysis and the tricarboxylic acid (TCA) cycle under salt stress were monitored. Upon salt-amended media, higher levels of glycolytic intermediates (glyceraldehyde 3-phosphate, PEP and pyruvate) and higher contents of TCA cycle intermediates (fumarate, citrate and malate) were detected. A more dynamic central carbon metabolism may provide salt-tolerant bacteria cells with the decisive energy in the form of ATP and precursors constructing bricks to fuel courses conveying salt tolerance, such as the biosynthesis of novo-compatible solutes (Sevin et al. 2016b). Conversely, hydroxyectoine possess stress-relieving properties for alleviating the energy requirements for living at high salinities, since the levels of TCA intermediates tend to decrease. Though osmoadaptation is an energy-demanding process in bacteria, generally at higher salinities, the enrichment of compatible solutes in the cytoplasm is energetically substantially less demanding than their novo-synthesis production (Oren 2011; Czech et al. 2018). Hence, the intracellular levels of trehalose, L-glutamate, L-aspartate and myo-inositol decreased as a response to exogenous hydroxyectoine in amended media.

The hydroxyectoine functionality as an osmotic stress-relieving cytoprotectant does not seem to be based exclusively on its extensive intracellular amassing. Thereby, the hydroxyectoine uptake by *K. radicincitans* leads to an increase in the relative abundance of amino acids such as L-leucine, L-asparagine, L-serine, L-methionine and the aromatic amino acid L-phenylalanine significantly. The increment of amino acid pools may contribute to balancing the vital osmotic gradient across the cytoplasmic membrane and further increase desiccation tolerance. Interestingly, an advantageous amino acid such L-proline increased 15.5-fold in the hydroxyectoine-added cells, enabled by the contraction of L-glutamate gathering requirements, which may lead to cells the glutamic acid-glutamic semialdehyde-proline pathway. The intracellular increment of L-proline may contribute to the desiccation tolerance in bacteria, functioning as chemical chaperone, since this amino acid may provide protein stability and thermodynamic advantages to cells, such as reducing and increasing the entropy and free energy of thermal of unfolding respectively (Prajapati et al. 2007; Mosier et al. 2013). Questions remain regarding how conservative the acidic proteome in *Kosakonia* sp. at high salinities can be, and which genes are involved in such adaptation. Then, further studies, along with other alternative *omics,* would widen the understanding of the underlying cellular mechanisms in osmoadaptation as a pre-conditioning strategy for enhancing drying survival.

To conclude, this study provides proof that substantial alterations in endogenous metabolites pools upon exposure to high salinity, including elevated levels of mannitol and L-aspartate, play a crucial role in delivering desiccation tolerance to the endophyte *K. radicincitans*. Metabolic approaches indicate that *K. radicincitans* adapts to prolonged osmotic stress by altering its amino acid and TCA cycle pools. Thus, to maintain the osmotic balance *K. radicincitans* accumulates hydroxyectoine from amended media and novo-synthesize compatible solutes, increasing intracellular acidic amino acid pools. These meaningful alterations in metabolite pools and eventual acidic signature proteome, induces a phenotypic shift as an osmoadaptation mechanism for conferring survival under desiccation stress. Finally, this study will encourage anhydrobiotic engineering in Gram-negative bacteria endophytes, supporting the exploitation of compatible solutes for developing dryable formulations.

**Acknowledgements** Authors want to thank the University of Applied Sciences in Bielefeld, Leibnitz Institute of Vegetable and Ornamental Crops (IGZ) Grossbeeren, ABiTEP GmbH in Germany; Universidad Nacional de Colombia and AGROSAVIA in Colombia. This research was funded by Departamento Administrativo de Ciencia y Tecnología e Innovación de Colombia -Colciencias with Grant 647.

## Compliance with ethical standards

**Conflict of interest** The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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