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# Adaptive laboratory evolution to hypersaline conditions, of lactic acid bacteria isolated from seaweed

Eleftheria Papadopoulou[a], Mari Cristina Rodriguez de Evgrafov[b], Argyro Kalea[a], Panagiotis Tsapekos[a], Irini Angelidaki[a]

[a] Department of Chemical and Biochemical Engineering, Technical University of Denmark, Kgs. Lyngby DK-2800, Denmark

[b] The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kgs. Lyngby DK-2800, Denmark

*Corresponding author.

E-mail address: iria@kt.dtu.dk (I. Angelidaki)



## Abstract

Seaweed biomass has been proposed as a promising alternative carbon source for fermentation processes using microbial factories. However, the high salinity content of seaweed biomass is a limiting factor in large scale fermentation processes. To address this shortcoming, three bacterial species (*Pediococcus pentosaceus, Lactobacillus plantarum, and Enterococcus faecium*) were isolated from seaweed biomass and evolved to increasing concentrations of NaCl. Following the evolution period, *P. pentosaceus* reached a plateau at the initial NaCl concentration, whereas *L. plantarum*, and *E. faecium* showed a 1.29 and 1.75-fold increase in their salt tolerance, respectively. The impact that salt evolution had on lactic acid production using hypersaline

seaweed hydrolysate was investigated. Salinity evolved *L. plantarum* produced 1.18-fold more lactic acid than the wild type, and salinity evolved *E. faecium* was able to produce lactic acid, while the wild type could not. No differences in lactic acid production were observed between the *P. pentosaceus* salinity evolved and wild type strains. Evolved lineages were analyzed for the molecular mechanisms underlying the observed phenotypes. Mutations were observed in genes affecting the ion balance in the cell, the composition of the cell membrane and proteins acting as regulators. This study demonstrates that bacterial isolates from saline niches are promising microbial factories for the fermentation of saline substrates, without the requirement of previous desalination steps, while preserving high final product yields.

Keywords: Adaptive laboratory evolution, Lactic acid bacteria, Brown seaweed, Salt tolerance

Abbreviations:
ME, Medium-evolved; N.C., Negative control; P.C, Positive control; SD, Standard deviation; WT, Wild type; ALE, adaptive laboratory evolution; LAB, lactic acid bacteria



## Introduction

The European Commission has proposed climate neutrality by 2050, setting as a mid-term goal a 55.00% decrease in greenhouse gases emission by 2030 [1]. To achieve this target, society should expect to replace fossil-based fuels and chemicals with more sustainable alternatives. The biorefinery concept supports this initiative through the production of platform chemicals using renewable biomass [2].

Platform chemicals, such as lactic acid, are used as intermediate substrates for the production of other valuable products, such as polylactic acid [2]. To increase the production of these chemicals using biorefineries, production costs should be equal to or lower than fossil-based processes. To decrease the price of the process, the characteristics of the carbon substrate and the microbial host used for its utilization in a fermentation approach should be chosen carefully [3]. Seaweed [4], food waste of various consistency [5], and agricultural residues, such as molasses [6], are alternative biomass options that can serve as carbon sources for the fermentative production of lactic acid. Despite their promise, these substrates have been largely avoided due to their salt content, which can cause osmotic stress to the bacterial factories [6]. At non-toxic concentrations, increased salinity can, on the other hand, benefit the fermentation procedure by minimizing contamination risk by invaders, while increasing organic acid yields. For example, one study [6] showed that elevated salinity levels boosted the growth of lactic acid bacteria (LAB) communities, while adversely affecting *Clostridium*, a main contaminant of lactic acid fermentation. LAB are potentially good microbial cell factories for production of platform chemicals as they are exceptionally robust. They include members of the Enterococcus, Lactobacillus and Pediococcus genera. LAB can grow in high salt concentrations, low pH, low nutrient environments and a wide range of temperatures. Additionally, they are capable of metabolizing different carbons sources and produce lactic acid as metabolite [3].

To optimize the biorefinery concept, versatile microbial cell factories should be detected and implemented in fermentation systems. Hence, there have been previous attempts to isolate and explore LAB as starter cultures for lactic acid production. For example, Lactobacilli and Pediococci are widely distributed in nature and are currently used in biorefinery processes as starter cultures [3]. *Lactobacillus plantarum* and *Pediococcus pentosaceuous* are well studied

members of this genera [3,7] and are categorized as Generally Recognized As Safe (GRAS) microorganisms. *L. plantarum* has been used in lactic acid fermentation processes [8,9]. Some *L. plantarum* strains have been reported to be resistant to osmotic stress and capable of withstanding 50 to 120 g/L NaCl [10,11]. The *P. pentosaceus* strain HN10 was isolated from fermented foods, which achieved maximum growth rate at 50 g/L NaCl [12]. Another LAB detected and isolated from various environmental niches, such as soil [13], or food [14] sources is *Enterococcus faecium*. Three *E. faecium* strains isolated by fermented soybean paste containing 25.50 g/L NaCl, have been reported to grow in up to 70 g/L NaCl [15]. Additionally, *E. faecium* has been reported as a starter culture for lactic acid fermentation [16,17]. For example, in one study [16] 207.30 g/L L-lactic acid were produced using a starchy waste from rice noodles in a 10-L bioreactor, while in another [17] 44.60 g/L lactic acid were achieved by initially 60.00 g/L corn-steep water eluent, using different strains of *E. faecium* in both cases.

Despite the advantages of using naturally evolved strains, robust microorganisms are typically obtained by applying the tools of metabolic engineering to well-studied model organisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*. Lack of genetic information and the tools needed for metabolic engineering on LAB minimizes their use in biorefineries [3]. To address this shortcoming, we have isolated three LAB species from *Alaria esculenta*, a brown seaweed, with the aim of obtaining a microbial candidate able to metabolize saline fermentation substrates while sustaining the lactic acid yield achieved in low salinity media. The isolated strains were then evolved toward higher salinities using adaptive laboratory evolution (ALE) techniques. Isolates of the evolved populations were sequenced to identify genetic changes triggered by osmotic stress. This is the first attempt to study and evolve seaweed environmental

isolates in high salinity media and characterize the evolution effect on the production of lactic acid.

## Materials and Methods

### Sample collection and reagents

All bacterial cultivation was performed following the DE Man, Rogosa and Sharpe (MRS) [18] protocol for MRS culture media, prepared by adding each chemical (MERCK A/S, Søborg, Denmark) individually to the solution. Glucose was filter sterilized, using sterile, 0.20 μm pore size syringe filters (MERCK A/S, Søborg, Denmark) and added to the solution externally, after the autoclaving process, to avoid degradation. The media was set at pH 6.50. For the NaCl rich media, 200 g/L NaCl (MERCK A/S, Søborg, Denmark) was added to the MRS broth.

The brown seaweed *Alaria esculenta* was used as substrate for the bacterial isolation. *A. esculenta* was farmed and collected in May 2020 in BANTRY Marine Station (BMRS), Cork, Ireland. The microbial isolation was conducted by preparing enrichment cultures, where seaweed tissue was added as inoculum (1% w/v) and cultivated anaerobically with MRS media, in serum bottles (50 ml). Serial dilutions of the bacterial culture were prepared (0, $10^{-2}$, $10^{-4}$, $10^{-6}$), using phosphate-buffer saline (PBS), (MERCK A/S, Søborg, Denmark). The diluates were then stroked on MRS agar plates, including 0.04% bromocresol purple (Thermo Scientific, Roskilde, Denmark), as acid indicator. Plates were incubated in anaerobic jars at 37˚C, for 48 h. In total, 13 isolates were selected according to morphological differences (shape and color) of the microbial colonies. The isolates were preserved in 20% autoclaved glycerol, at -80°C. All processes took place under sterile conditions.

For the isolate identification, DNA was extracted using the Zymo Research D4068 Quick-DNA Miniprep Plus Kit (Zymo Research, 50 Preps/Unit, Nordic BioSite Aps, Copenhagen, Denmark). The 16S rRNA gene was amplified with the primer set 27F/1492R by polymerase chain reaction (PCR). The purification step was performed using QUIAquick PCR Purification Kit (QIAGEN, Vedbæk, Denmark). Microplate reader (Thermo Scientific, Roskilde, Denmark) and the SkanIt™ Software for Microplate Readers (Thermo Scientific, Roskilde, Denmark) were used for qualitative and quantitative DNA estimation. Premixed samples, prepared with a Mix2Seq kit (Eurofins Genomics, Ebersberg, Germany) were sequenced by the Sanger method (Eurofins Genomics, Ebersberg, Germany). The raw nucleotide reads were blasted in NCBI [19,20], and three LAB species were identified; *Pediococcus pentosaceus* (two strains), *Lactobacillus plantarum*, and *Enterococcus faecium*.



**$IC_{50}$ determination**

The wild type (WT) half maximal inhibitory concentration ($IC_{50}$) was evaluated before beginning the evolution experiments for all the bacterial species. WT microorganisms were plated on MRS agar plates and grown anaerobically for 48 h. Four individual colonies of each microorganism were randomly selected and were re-cultured in fresh MRS media for 8 h. The optical density (OD) was measured at 600 nm ($OD_{600}$) using a 96-well cell culture plates (Corning96 Well TC-Treated Microplates) and a microplate reader (Biotek) using BioTek Gen5 software (Agilent, Glostrup, Denmark) and dilutions of $10^3$ cells/ml were prepared in fresh MRS media.

Tests for each isolate were performed in two 24-deep well plates, with 5 ml final culture volume. Eight different NaCl concentrations were tested (18; 25; 35; 50; 71; 100; 141 and 200

g/L NaCl) using a 1.41-fold gradient. Each plate included positive and negative controls. All the conditions were tested in triplicate. Each well was inoculated with $10^3$ cells/ml. After inoculation, the plates were covered with a Breath-Easy sealing membrane (MERCK A/S, Søborg, Denmark) to avoid contamination and placed in an anaerobic box, using an anaerobic atmosphere generation bag (MERCK A/S, Søborg, Denmark) to create anaerobic conditions. The anaerobic box was incubated at 37°C, without shaking, for 16 h. After that time, the $OD_{600}$ was measured using a microplate reader , using BioTek Gen5 software.

To calculate $IC_{50}$ values, the $OD_{600}$ measurements of the negative control were subtracted from those of the individual strains and the positive control, and the growth values for the individual strains were normalized according to the positive control, following the equation below.

$$Equation\ a.\ 1 - \frac{OD_{600_{Growth}}}{OD_{600_{Positive\ control}}}$$

The $IC_{50}$ values were then calculated using dose response–inhibition function in GraphPad Prism 9.3.1., with the function $log_{(inhibitor)}$ vs normalized response–variable slope analysis.

**Evolution of NaCl-resistant isolates**

All evolution experiments took place in two 24-deep well plates with each column representing a different NaCl dilution (35; 50; 71; 100; 141; 200 g/L NaCl) and each row a different evolution lineage (**Figure 1**). Each well contained 5 ml total volume, including the growth media, MRS with the corresponding NaCl dilution, and 0.13 ml inoculum (2.50% of the total volume). All WT strains were evolved in four replicates. Positive controls (0 g/L NaCl media, with the addition of bacterial inoculum), and negative controls (0g/L NaCl media, without

the addition of bacterial inoculum) were included. The positive-control isolates are referred as medium-evolved (ME) lineages.

To begin the evolution process, single WT colonies were selected from a solid culture and grown overnight in liquid MRS media, without the addition of extra salt. All the wells except for the negative control were inoculated with 0.13 ml inoculum. The plates were then covered with Breath-Easy sealing membranes and placed in an anaerobic box with an anaerobic atmosphere generation bag (MERCK A/S, Søborg, Denmark). The box was incubated at 37°C, without shaking, for 16 h. After the time, the OD at 600 nm ($OD_{600}$) was measured using a microplate reader (BioTek), supported with a BioTek Gen5 software (Agilent, Glostrup, Denmark). The strains with no more than 50% inhibition were selected as inoculum for the next evolution experiment. The inhibition value was calculated according to equation a. This process was repeated every 16 h, for 11 d. Samples from the chosen inoculum were selected and stored in 20% glycerol, at -80°C. On the final experimental day, the strains obtaining a 50% inhibition value at the highest possible NaCl concentration were collected and plated on agar plates, and a single colony of each was randomly selected. The chosen colonies were cultured in liquid media and stored for further analysis.



**Post-evolution testing**

The most evolved lineages and the medium evolved (ME) lineages for each bacteria species were plated on non-saline MRS media for 24 h. Three colonies from each plate were randomly selected and cultured in non-selective liquid cultures for 6 h. The isolates were tested for their individual resistance to different salinity levels, following the experimental design,

described above. For this experiment the salt concentrations were increased by 1.20-fold per step (50; 60; 72; 87; and 104 g/L NaCl).

**Fermentation of evolved strains in seaweed hydrolysate**

Hydrolysate from mixed samples of brown seaweed was prepared using the enzymes Cellic®CTec2 (Novozymes A/S, Kalundborg, Denmark), and alginate lyase (MERCK A/S, Søborg, Denmark) at a concentration of 15% (Enzyme/Substrate level, % weight), and 0.10% w/w, respectively. The enzymatic hydrolysis took place in a 2 L bioreactor (Sartorius BIOSTAT®, Göttingen, Germany), with 100 g/-dried biomass/L biomass load, pH 4.8, and 50˚C for 24 h. The hydrolysate was centrifuged (10000 rpm, 15 min), MRS media, except for glucose, were added, and the mixture was autoclaved for 15 min at 121˚C. The carbohydrate source for the fermentation was the sugars contained in the hydrolysate mix. The total sugar content was 29.48 g/L, with 20.93 g/L glucose and 8.54 g/l mannitol. Fermentation media of two different salinity levels were prepared, using the seaweed hydrolysate. In the first condition, salinity remained at 35 g/L NaCl (i.e. background from hydrolysate), and in the second condition additional salt was added to reach a final concentration of 71.35 g/L NaCl. The WT, ME, and the most tolerant strain of the evolution process, for each bacteria species, were used as fermentation inocula. Fermentations took place in triplicate anaerobic flasks (50 ml), with inoculum concentration $10^6$ cells/ml, pH 6.5, 37˚C, without shaking, for 44 h. Lactic acid titer (g/L) was measured at the end of the fermentation process. To compare the obtained results with previous studies, relative lactic acid production (Equation b) was used as a performance parameter.

$$\textit{Equation b.}\ \frac{Practical\ LA\ concentration}{Theoretical\ LA\ concentration} \times 100$$

**Analytical methods**

The quantification of lactic acid as titer (g/L) was carried out by High Performance Liquid Chromatography (HPLC). The samples were prepared by centrifuging at 10,000 rpm for 10 min. The supernatant was 4-fold diluted and filtered through a 0.22 μm syringe filter (MERCK A/S, Søborg, Denmark). The samples were analyzed by HPLC directly after preparation. The HPLC was equipped with a refractive detector, a Bio-Rad HPX-87H (300 mm × 7.8 mm) column, and 12 mM $H_2SO_4$ eluent operating at flow rate of 0.60 ml/min, with column oven temperature 63°C. For quantification of the compounds, a calibration curve was included with different dilutions (1, 2.5, 5, 7.5, 10 g/L) of a mix solution with glucose, succinic acid, lactic acid, formic acid and acetic acid. To determine the sugar concentration (mg/L) included in the substrate of interest, Ion Chromatography was used (IC; Dionex ICS-6000 HPIC System). The sample preparation process was identical to that for HPLC. After the preparation the samples were analyzed by IC equipped with a Dionex™ CarboPac™ PA20 column (3 × 150 mm). The quantification was by applying a calibration curve (1; 2.5; 5; 7.5; 10; 15; 20 mg/L), able to identify mannitol, fucose, rhamnose-arabinose, galactose, glucose, xylose, and mannose. The salinity, expressed as NaCl content was measured with as CDM230 conductivity meter (MeterLab, Hach, Brønshøj, Denmark).

**Statistical analysis**

GraphPad Prism 9.3.1 was used for the statistical analyses of the data. The results are given as mean values of three replicates, except where differently stated, and the error bars represent standard deviations (SD). One-way analysis of variance (ANOVA) combined with

Tukey's multiple comparison test were applied to detect statistically significant differences between the results. An asterisk represents data with P value <0.05.

**Whole genome sequencing**

The WT strain, the ME, and the most salt-tolerant populations from the post-evolution test for each bacterial species were plated on non-specific MRS media for 24 h. In total, 6 single cell colonies (1 WT, 1 ME, 4 lineages evolution strains) were selected and cultured in liquid media for 8 hours. Genomic DNA was isolated using Zymo Research D4068 Quick-DNA Miniprep Plus Kit (Zymo Research, 50 Preps/Unit, Nordic BioSite Aps, Copenhagen, Denmark). Extracted DNA samples were sent to the University of Padua (Veneto, Italy) for whole genome sequencing, using Illumina NextSeq 500, with an average of 420-fold coverage.

**Analysis of whole genome sequencing**

The whole genome sequencing data was analyzed using the online workspace Pathosystems Resource Integration Center (PATRIC) 3.6.12 [21]. All sequences were trimmed with minimum contig length 300 and contig coverage 10X. The WT genome of each species was used as genome of reference. The genome was first assembled and annotated using RAST tool kit (RASTtk), [22]. Variation analysis feature in PATRIC was used to identify the sequence variations between the WT, the ME, and the evolved strains. The aligner BWA-mem [23], and the single nucleotide polymorphism (SNP) caller FreeBayes [24] were used to detect point mutations. SNPs existing in the ME lineages were excluded by the study. The DNA sequences are available on the National center for biotechnology information (NCBI) database [20] under the accession no **PRJNA916087.**

## Results and Discussion

This study aimed to acquire salinity tolerant microorganisms, able to metabolize saline substrates and produce high titer lactic acid. LAB isolated from the seaweed *A. esculenta* were tested for salinity resistance in natural seaweed salinity (35g/L NaCl), or hypersaline conditions (up to 200g/L NaCl), and they were further evolved to NaCl to improve salinity resistance, using ALE techniques. The genetic alterations caused by salt, the stress factor introduced to the bacterial growth media, were detected by whole genome sequencing, and characterized.

As far as we are aware, ALE experiments have not been previously applied to native seaweed LAB isolates to increase resistance in hypersaline conditions. Previous studies [10,25] conducted on salinity resistance of LAB focused mainly on their use as probiotics and as starter cultures of cheese fermentation, but not on their application in biotechnical processes, such as lactic acid production for industrial use. Lactic acid derives from the anaerobic fermentation of carbohydrate sources which can be inexpensive residue streams or biomasses with various NaCl contents. High salt concentrations have been previously found to inhibit microbial processes [26], even though they could actually protect the fermentation process from external microbial contaminations [6].



### Extent of salinity tolerance of environmental isolates

Three bacterial species were isolated from seaweed. The species were identified as *P. pentosaceus* (two different strains), *L. plantarum*, and *E. faecium* based on 16S sequence analysis. The salt tolerance of each environmental isolate was tested (**Figure 2**). *P. pentosaceus* (strain 2) showed the highest salinity resistance with half the bacterial population able to tolerate

69.20 g/L NaCl. *P. pentosaceus* (strain 1), and *L. plantarum* noted an $IC_{50}$ of 56.10 and 55 g/L NaCl, respectively, while the population of *E. faecium* was inhibited at 40.60 g/L NaCl. *P. pentosaceus* (strain 2), (GenBank accession no. **SAMN32411881**), *L. plantarum* (GenBank accession no. **SAMN32411876**), and *E. faecium* (GenBank accession no. **SAMN32411886**) were selected for the evolution process. Previous studies [3] on *P. pentosaceus* and *L. plantarum* showed that on average 46.67% and 78.33% of the bacterial population could grow in 50g/L NaCl, compared to the population in the control-no stress media (no salt addition). In the present study, these values were deermined as 89.14% for *P. pentosaceus* and 72.47% for *L. plantarum*. Comparing the findings with the same study, no growth was detected at 100 g/L NaCl. Differences in resistance to stress factors between strains of the same species can be explained, due to isolation from different environmental niches. Numerous studies have evaluated the halotolerance of LAB, as salt is an important stress factor in food fermentation processes. *P. pentosaceus* isolated from Kombucha leaves was tested for salt resistance in NaCl concentrations between 25-115 g/L. The results showed that growth ($OD_{600}$) of the strain in 50g/L NaCl was at the same level as in the control (0g/L NaCl), which agrees with this study. Likewise, when the NaCl concentration rose to 75.00 g/L, the $OD_{600}$ values were close to zero [27]. Another study investigating the microorganisms participating in the fermentation process of jalapeño peppers under saline conditions, showed that *L. plantarum* had an $IC_{50}$ value of 66.00 g/L NaCl, 1.18-fold higher than that reported in this study [28]. The salt concentration measured at the bacterial isolation source was 57.50 g/L, compared to the 35.00 g/L NaCl measured in the seaweed. Similarly, in a previous study [29], strains of *E. faecium*, *E. faecalis* and *T. halophilus* were studied for growth in media containing 35, 70, and 140g/L NaCl. All bacteria showed resistance in the two first conditions, but only *T. halophilus* survived in 140g/L NaCl. *E. faecium* could

grow in 70g/L NaCl, contrary to the findings of this study, indicating however the lowest growth rate among the three species. The findings indicate that salinity resistance of LAB can vary depending on the source of isolation and on the bacterial strain itself [3].

**Evolution leads to resistance improvement in high salinity media**

Species *P. pentosaceus, L. plantarum* and *E. faecium* were evolved in three replicate lineages, designated 1, 2 and 3, to increasing concentrations of NaCl. To account for possible media evolution phenotypes, parallel medium-only experiments were performed using the WT for each species. These strains are referred to as media evolved (ME). Three single, freshly streaked colonies from each bacterial species were grown overnight in MRS medium. An aliquot of each culture was inoculated in MRS media containing 35; 50; 71; 100; 141; 200g/L NaCl and allowed to grow for 16 h at 37°C. At the end of the growth period, the $OD_{600}$ was measured and the replicate with an inhibition of less than 50% was used as the inoculum in the next salinity experiment (Figure 1). The experiment was stopped after 11 days, when the microorganisms were no longer evolving.

The evolution process led the microorganisms towards higher salinity resistance. The three lineages of the high salinity tolerant *P. pentosaceus* (strain 2) reached a plateau in the WT concentration of 71g/L, indicating that this organism could not adapt further. Previous studies on *P. pentosaceus* strains, isolated from chicken gastrointestinal tract [30] and fermented salty fish [31] indicate that this species can naturally tolerate salinity up to 65g/L NaCl. The behavior of *P. pentosaceus* can be attributed to the fact that hypertonic conditions in the bacterial environment caused serious cell wall damages, even though the bacterial density remained stable in further salinity increase as observed in scanning electron microscopy (SEM) images [25].

*L. plantarum* evolved, after 2 days of ALE experiments, and reached a final salinity 1.29-fold higher than the WT strain (from 55 to 71 g/L NaCl). Previous studies on salinity adaptation of an *L. plantarum* strain isolated from fermented food showed that the strain was able to grow in up to 80g/L NaCl, testing the salinity resistance in NaCl concentrations 10; 20; 40; 60; and 80 g/L NaCl [32], while in this study salinity concentrations between 18 and 200 g/L, increasing by 1.41-fold, were applied.

*E. faecium* strains showed the highest adaptation capacity, reaching a final NaCl concentration 1.75-fold higher than the WT one (from 40.60 to 71 g/L). For lineage 1 and 2, the evolution was successful after 5 days whereas for lineage 3, after 8 days. The inoculum effect is a possible explanation of the $OD_{600}$ drop of lineage 2 (**Figure 3**). *E. faecium* has been characterized as a species with high plasticity and environmental adaptability [33]. This statement is based on the finding that 202 environment-specific genes were detected in a comparative genomic analysis of 161 *E. faecium* isolates deriving from human, animal or fermented dairy origins. At the end of the evolution period, *P. pentosaceus*, *L. plantarum* and *E. faecium* populations had the same salinity tolerance, growing in media with 71 g/L NaCl.

The salinity tolerance of individual members of the evolved populations were tested following the evolution period. The isolate results were then compared with the final evolution concentrations of their corresponding populations. The ME strains were also tested for their individual resistance levels to determine the if the media influenced the evolution of each species (**Figure 4**). According to the findings, the resistance levels of individual strains, compared to the respective population, was species dependent. The individual strains of *P. pentosaceus* (strain 2), (GenBank accession no. **SAMN32411883**; no. **SAMN32411884**; and no. **SAMN32411885**) and *L. plantarum* (GenBank accession no. **SAMN32411878**; no**. SAMN32411879**; and no.

**SAMN32411880**) could withstand 1.13- and 1.16-fold lower concentrations of NaCl, compared to their corresponding population (**Table 1**; Figure 4). However, some *E. faecium* (GenBank accession no. **SAMN32411888**; no. **SAMN32411889**; and no. **SAMN32411890**) individual strains showed improved resistance to salt, growing in 1.04-fold higher salt concentration compared to the initial population. This difference was not significant (P >0.05). Generally, it is speculated that the exposure to a stress factor can increase the resistance of the evolved microorganism to future stress events [34]. It was demonstrated [34] that when a strain of *E. faecium* was exposed to stress events, such as sub-lethal concentrations of NaCl, it showed an improved adaptation to a second stress episodeat increased heat levels. The resistance of individual isolates is supposed to be improved, following that one of the bacterial population [35]. However, isolates-outliers with decreased resistance compared to their corresponding populations can be detected because of sample storage at -80°C, before use. Another explanation for this phenomenon could be that a memory-like behavior is expressed at the population level, rather than on the single cell level [36]. Furthermore, the response of a single cell towards a stress factor depends on the its position in the cell cycle.

The ME strains of *L. plantarum* (GenBank accession no. **SAMN32411877**) *and E. faecium* (GenBank accession no**. SAMN32411887**) also showed a higher resistance in the fermentation media (1.05- and 1.12-fold, respectively) compared to the WT strains, indicating that an adaptation in the specific media also occurred. A collateral adaptation in the culturing media, while testing a specific stress factor, can arise through the introduction of the environmental isolates in media with diverse nutrient characteristics from the initial environment. However, this observation does not apply for the ME *P. pentosaceus* (strain 2), (GenBank accession no. **SAMN32411882**) , as the specific media choice did not promote further

adaptation. Except for the non-significant difference between the individual isolates and the population of *E. faecium*, the rest of the differences presented in this session were statistically significant ($P<0.05$).

**Salinity evolved bacterial strains maintain initial lactic acid titer in hypersaline seaweed hydrolysate**

Seaweed hydrolysate, in initial salinity (35 g/L NaCl), and seaweed hydrolysate amplified with extra NaCl (71 g/L) were used as fermentation substrates for lactic acid production. The WT, ME, and evolved lineages of each bacterial species, *P. pentosaceus*, *L. plantarum*, and *E. faecium*, were used as starter cultures for the two fermentation conditions. The starting inoculum was $10^6$ cells/ml and the fermentations were performed anaerobically in 50 ml serum bottles, at pH 6.5 and 37˚C, without shaking. After 44 h samples were collected and analyzed to determine the final concentration of organic acids and sugars, accumulated in the fermentation broth.

All evolved seaweed isolates were able to grow in natural and hypersaline seaweed hydrolysate and produce lactic acid. In both natural seaweed hydrolysate and hypersaline conditions, the final lactic acid concentration attained was at similar levels ($P\geq0.05$). However, *E. faecium* produced a significantly lower amount of lactic acid compared to the other two species, in both salinity conditions (**Figure 5**). The relative lactic acid concentration obtained by the evolved in hypersaline conditions *L. plantarum* (75.29%, **Table 2**) was comparable with previous studies, where WT strains metabolized seaweed hydrolysates under optimal conditions [8,37,38]. Even though the salinity evolved *L. plantarum* and *E. faecium* presented lower relative

lactic acid concentration, improvement of the substrate and the fermentation conditions may have increased the performance.

The evolved strain of *P. pentosaceus* was less productive than the ME and WT strains by 1.38- and 1.31-fold, respectively, at natural seaweed salinity. No significant differences were observed among the evolved, the ME and the WT strains when the salinity level was increased to 71 g/L NaCl. This result mirrored the evolution experiment, where *P. pentosaceus* did not evolve further in higher salinity (Figure 3, Figure 4). The same finding applied in the cases of *L. plantarum* and *E. faecium*, where less lactic acid was produced by the evolved strains compared to ME and WT ones when growing in media with 35 g/L NaCl. This effect may appear because the evolved strains, having been exposed to stress conditions, changed their metabolism towards a survival mode, rather than growth and enlargement [39]. On the other hand, when the NaCl concentration rose to 71g/L, the evolved *L. plantarum* strains produced on average 1.19-fold more lactic acid compared to the WT ($P \leq 0.05$), and the evolved strains of *E. faecium* produced lactic acid, while the WT strain could not be productive under the same conditions ($P \leq 0.05$). No significant differences were noted between the evolved and the ME strains, indicating that an adaptation to media also affected the behavior of the microorganisms and should be considered for the optimization of a fermentation process. Previous studies, where food waste was used as fermentation substrate, have shown that an increase of NaCl content up to 40 g/L led to an increase in the lactic acid concentration, while an inhibition was observed once the NaCl increased to level higher than 50 g/L NaCl [40].

Previous studies have indicated that NaCl acts as an inhibitory factor in biotechnological processes, such as anaerobic digestion [26], or hydrogen production [41] that are reliant on the performance of robust mixed cultures. However, increased salinity was also associated with

increased solubilization of carbohydrates and subsequently enhanced production of L- versus D-lactic acid in a heterofermentative process using food waste [5]. The above-mentioned contradictory findings mark the importance of studying the impact of NaCl in fermentation media and the microbial factories used for their fermentation. Overall, the microorganisms studied here were able to withstand high NaCl concentrations, while maintaining similar conversion of sugars to lactic acid both in natural seaweed hydrolysate and in hypersaline conditions.

**Intracellular metabolism balance, composition of bacterial cell wall, and strain specific proteins affected by elevated NaCl**

Whole genome sequencing of the WT, ME and evolved strains with the highest salinity resistance was performed to determine the genetic basis for the observed increase in salinity tolerance. Three SNPs were detected in *P. pentosaceus*, and two in *L. plantarum*, whereas broader changes were observed in *E. faecium* (**Figure 6**). Synonymous mutations were not considered in this study.

Adaptation traits caused by stress-related genome changes, are specific to different bacterial strains [39]. An example of strain-specific alterations are the mutations in hypothetical proteins, which were detected in all three studied bacteria. The genome representation of the hypothetical proteins was further investigated using BLASTP, but no information was found. Mutations in genes expressing hypothetical proteins arising as result of adaptation have been previously reported [10,42]. The existence of hypothetical proteins, not yet characterized proteins, indicates that genes unique to the specific strains are regulated by the environmental conditions [42].

In *P. pentosaceus* the function of the RNA polymerase sigma factor RpoD was affected. Sigma factors are proteins connected with cell's metabolism. They are important regulators of stress-defense genes and specifically RpoD controls the house keeping genes [43]. *In L. plantarum* both mutations were detected in intergenic regions. The first SNP concerned the function of an efflux ABC transporter (ATP-binding protein), and the second a putative transcriptional regulator. Both regulators were of unknown function. Generally, ABC transporters are widespread in living organisms, and they function as transmembrane transporters of molecules, or they are responsible for non-transport events, such as DNA elongation and repair during translation. The energy for these actions derives from the hydrolysis of ATP [44]. In the case of *L. plantarum* FS5-5 which was tested for salt tolerance as a strain of probiotic interest, mutations in the ABC-transporters were also detected [45]. Mutations in putative transcription factors has been previously reported as a response to stress events in *Escherichia coli*. Mutations in sigma factor RpoD, ABC transporters, and transcription factors have been previously reported on *L. plantarum* strains exposed to salt stress [10].

*E. faecium* presented the most extended mutations. SNPs for a two-component transcriptional response regulator (OmpR family), and a phosphate ABC transporter (substrate-binding protein PstS) were detected in intergenic regions. The functional areas of the genes regulating amino acid permease family protein, putative peptidoglycan bound protein (LPXTG motif), and mobile element proteins were also affected. The salinity increase caused inhibition of the carbonic anhydrase (alpha class, EC 4.2.1.1) and a deletion on the upstream region regulating the conserved hypothetical protein ArsC.

The amino acid permease family protein, the carbonic anhydrase (alpha class, EC 4.2.1.1), and the conserved hypothetical protein ArsC are proteins regulating the intracellular metabolism balance. The amino acid permeases are the intracellular transporters of amino acids, and the carbonic anhydrases are responsible for the conversion of carbon dioxide to bicarbonate and control the transportation of these molecules in and out of the cell. Carbonic anhydrases are also responsible for the transportation of acid and ions through the cell, while acting as pH regulators, affecting in general the growth of the bacterial cells [46,47]. Under stress conditions LAB use the malolactic fermentation pathway where the $CO_2$ produced is transformed into bicarbonate by carbonic anhydrases to increase the intracellular pH [39]. Finally, ars operons are widely distributed in bacterial genomes, including LAB, such as *Lactococcus lactic* [48]. The protein ArsC is an enzyme reducing arsenate to arsenite, protecting the cell from high concentrations of arsenic [49]. The arsC operon has another function, which is connected with the biosynthesis of arsenobetaine, an osmoprotectant molecule [50].

The composition of the *E. faecium* cell wall was also affected by the salt exposure, changing the composition of the putative peptidoglycan bound protein (LPXT motif Lmo2178 homolog). Changes in peptidoglycan synthesis have been indicated as crucial for the survival of LAB cells, under stress conditions [51]. Osmotic stress also had an impact on magnesium transporter E (MgtE). The accumulation of potassium ($K^+$) in the cytoplasm during osmotic stress, inhibited $Mg^{2+}$ uptake as an osmoadaptation response, in *Bacillus subtilis* [52]. Mutations were also detected in mobile element proteins, proteins expressed by carry genes found in plasmid genome, expressed in LAB [39]. These carry genes are suspected to affect the bacterial plasticity, and boost resistance for specific environmental conditions.

## Conclusions

Environmental bacteria isolated from seaweed were evaluated for their ability to be used as microbial factories for lactic acid fermentation of saline substrates. The isolated and tested species were identified as *Pediococcus pentosaceus*, *Lactobacillus plantarum*, and *Enterococcus faecium*. All three microbial populations were able to tolerate 71 g/L NaCl in the growth media. The results of the evolution experiment were then applied to seaweed hydrolysate, with natural salinity content or hypersaline to determine ability to produce lactic acid. The evolved microorganisms were able to produce higher amounts of lactic acid in higher salinity relative to their WT ancestor strains. Finally, comparative genomics analysis found mutations on genes expressing regulatory factors, cell membrane proteins, ion transporters and strain-specific proteins that were likely responsible for the high salinity tolerance observed in the evolved isolates.



## CRediT authorship contribution statement

**Eleftheria Papadopoulou**: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing – Original draft. **Mari Christina Rodriguez de Evgrafov**: Conceptualization, Methodology, Resources, Writing - Review & Editing. **Argyro Kalea**: Validation, Investigation, Writing - Review & Editing. **Panagiotis Tsapekos**: Conceptualization, Resources, Writing - Review & Editing, Supervision, Project administration. **Irini Angelidaki**: Conceptualization, Writing – Review & Editing, Supervision, Project administration, Funding Acquisition

## Declaration of Competing interest

Declarations of interest: none

## Data availability

Data will be made available on request.

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**Figure and Table Legends**

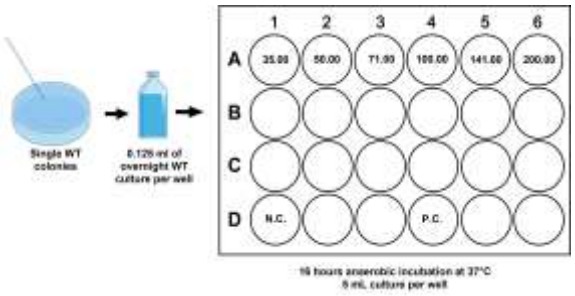


**Figure 1.** Experimental design of the ALE process. Each column (1 to 6) indicates a different NaCl concentration, starting from the lowest (35 g/L NaCl) on the left to the highest concentration (200 g/L NaCl) on the right. Each row represents a lineage (A to C), deriving from a single WT bacterial colony. Triplicates of negative (N.C. - no addition of bacterial inoculum), and positive controls (P.C. - addition of bacterial inoculum) were included on row D. Negative and positive controls were cultivated in non-selective media.

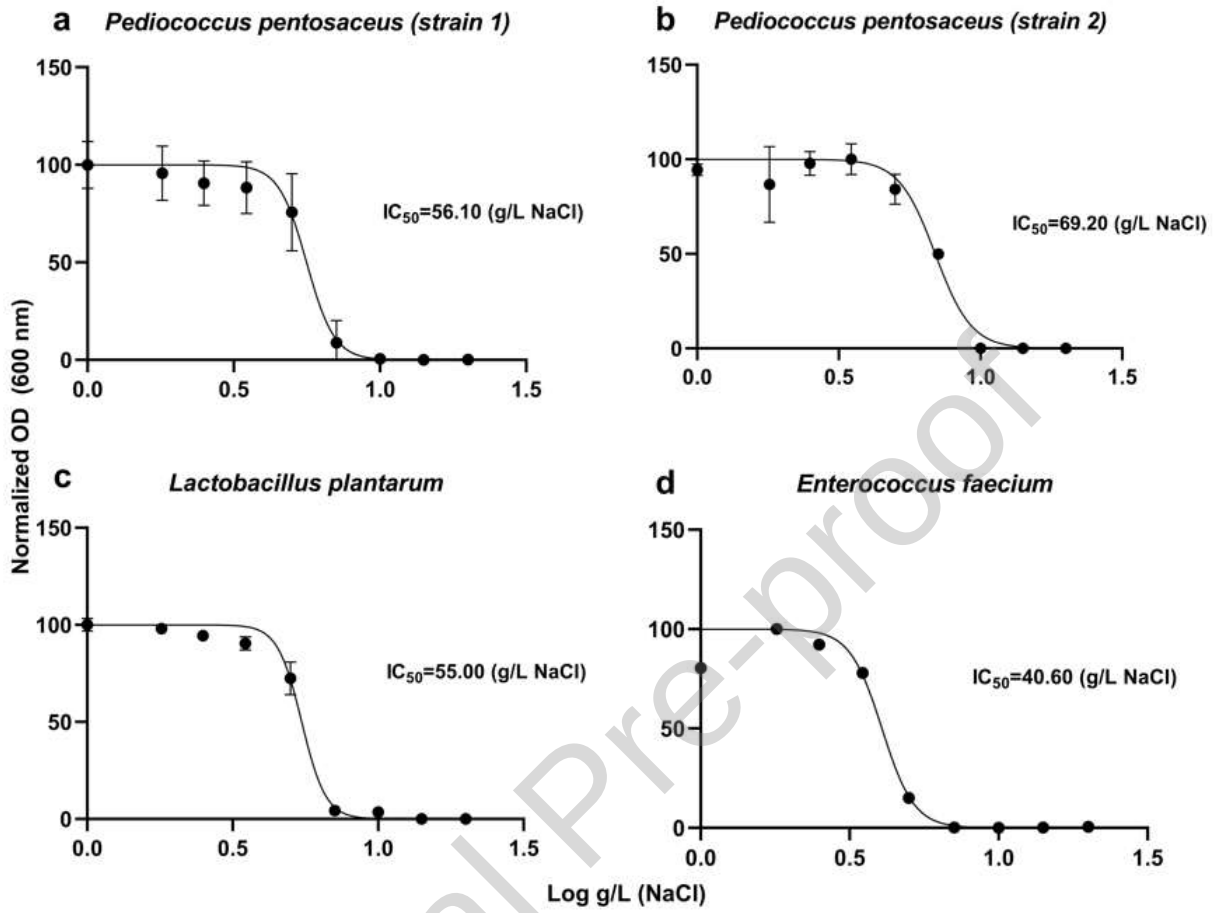


**Figure 2.** Half-maximal inhibitory concentration ($IC_{50}$) plots for four lactic acid producing bacterial isolates. The $IC_{50}$ value shows the concentration of an inhibitor (NaCl) needed to minimize the bacterial growth in half. Each plot is represented by four replicates, except from E. faecium that is represented by 2. Error bars represent a single SD.

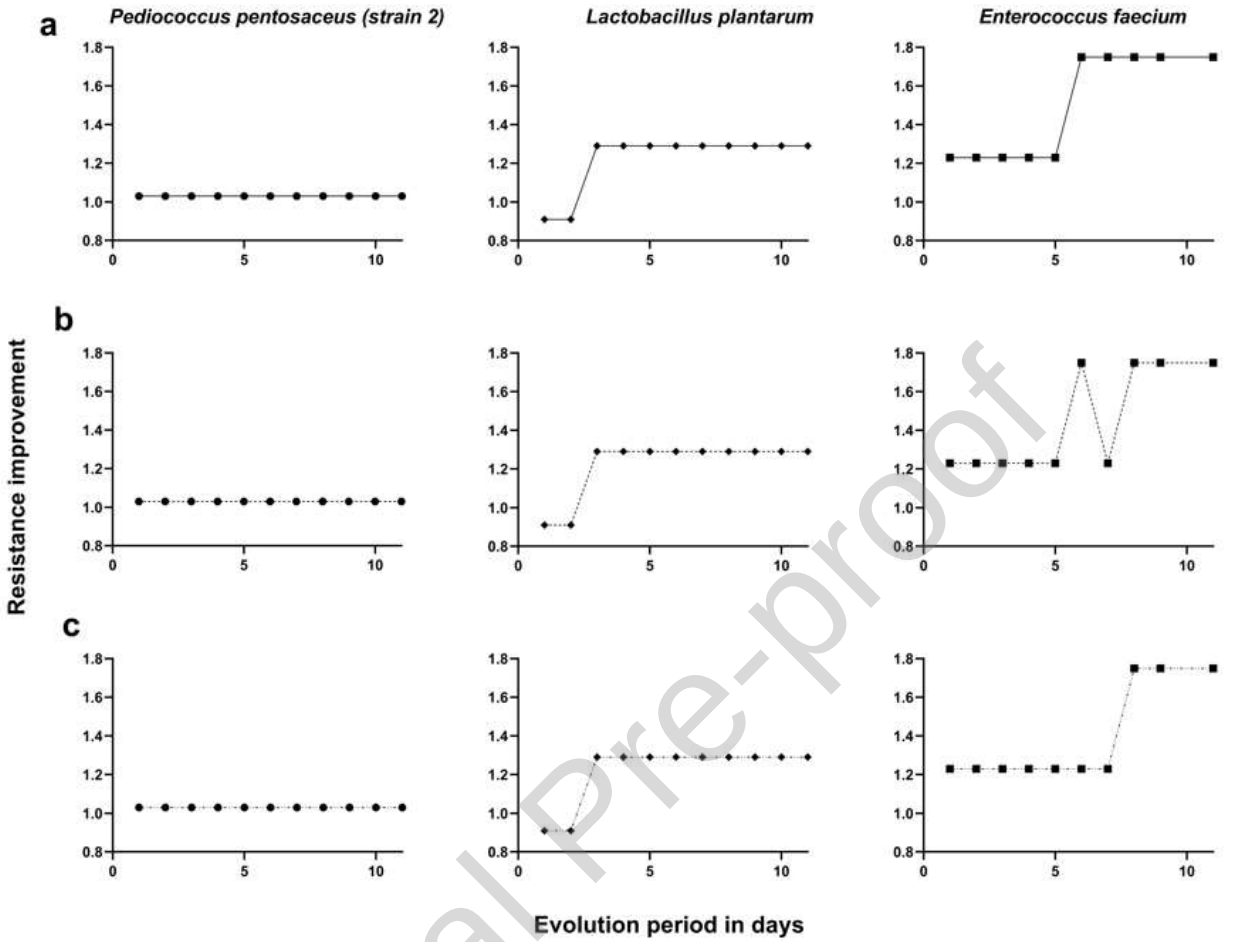


**Figure 3.** Resistance improvement of environmental bacterial isolates in high salinity media, over the evolution period of 11 days. Each plot represents the salinity concentration tolerated by the bacterial isolates, compared with the WT $IC_{50}$ value, through time. Each plot represents a single bacterial strain, and each point represents the resistance improvement of an isolate in one day.

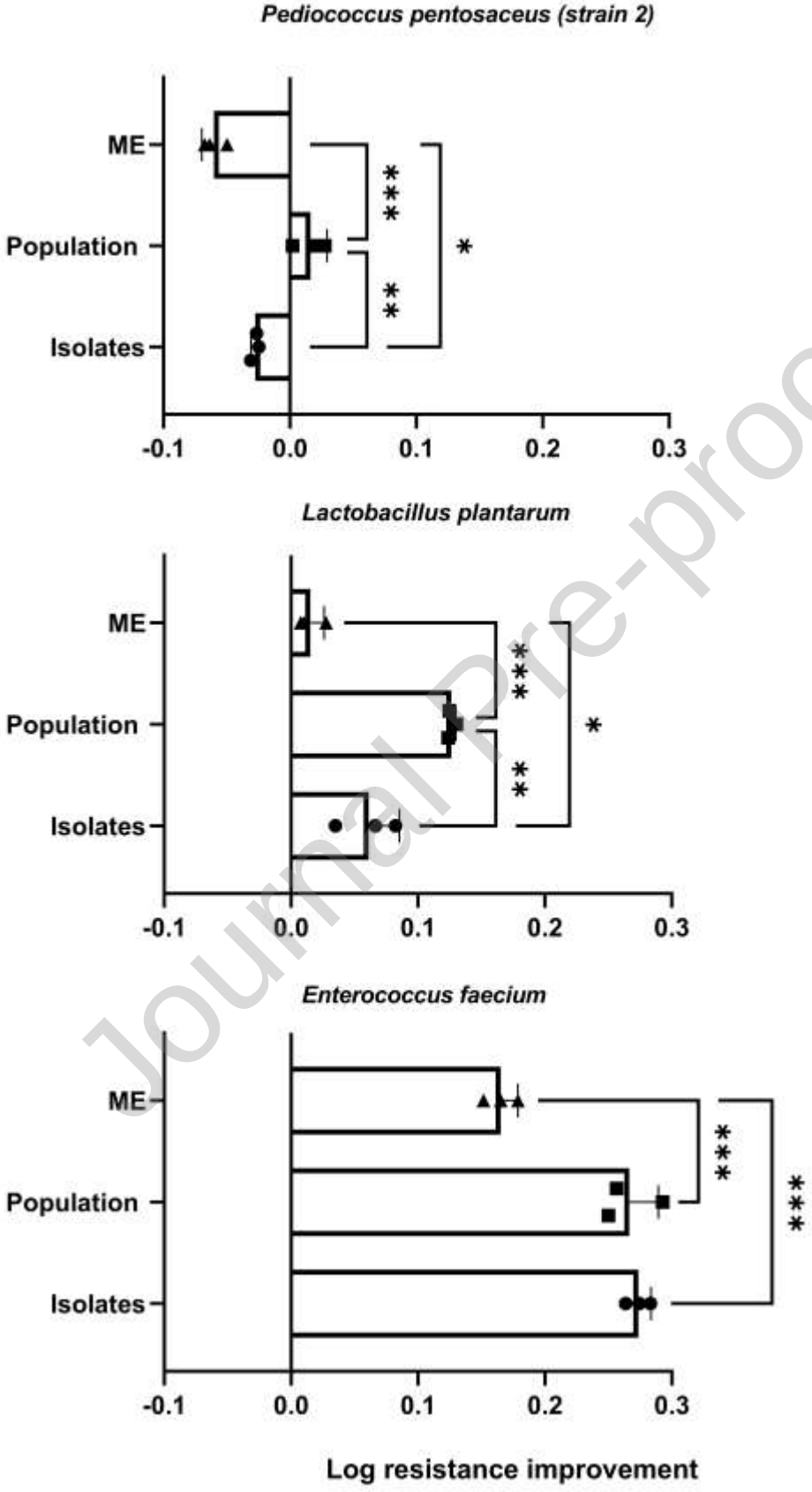

**Figure 4.** Resistance improvement plots for NaCl-evolved individuals, compared with their corresponding bacterial population, for three environmental bacterial isolates. Three biological isolates from each of the evolved populations were used to determine the individual resistance improvement. The ME isolates are also included in the comparison, to appreciate how the medium affected the evolution process. The resistance improvement was calculated by normalizing the values, according to the WT $IC_{50}$ values. All the normalized values were log transformed. The data are presented in triplicates and the error bars are a single SD. The asterisk symbol presents the cases where the differences between two groups were significant ($P<0.05$).

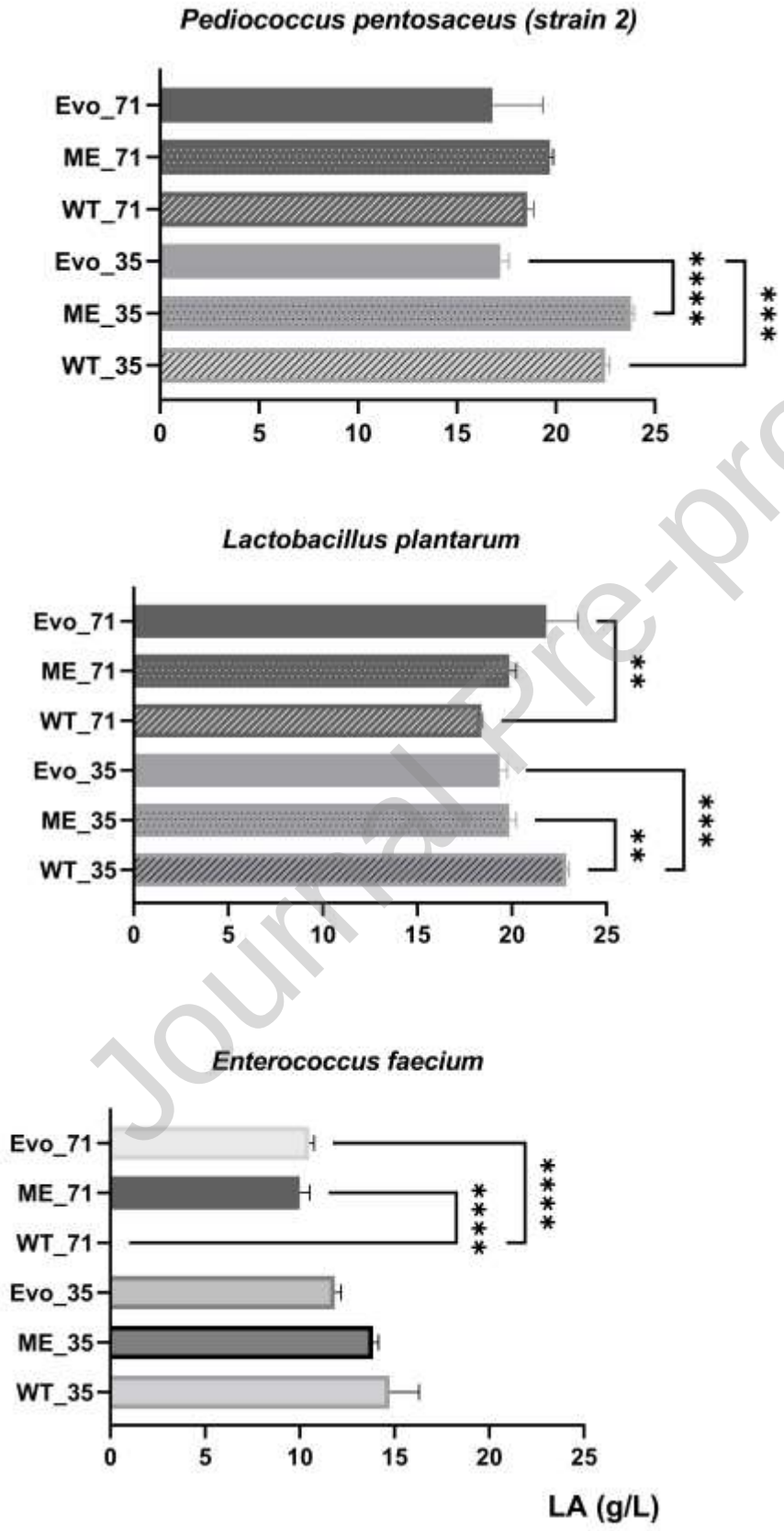

**Figure 5**.Validation experiment of ALE in seaweed hydrolysate. The evolved, ME, and WT strains were grown in seaweed hydrolysate (35 g/L NaCl), and in seaweed hydrolysate with addition of NaCl (71 g/L NaCl). The data are presented in triplicates and the error bars are a single SD. The asterisk symbol presents the cases where the differences between two groups were significant (P<0.05).

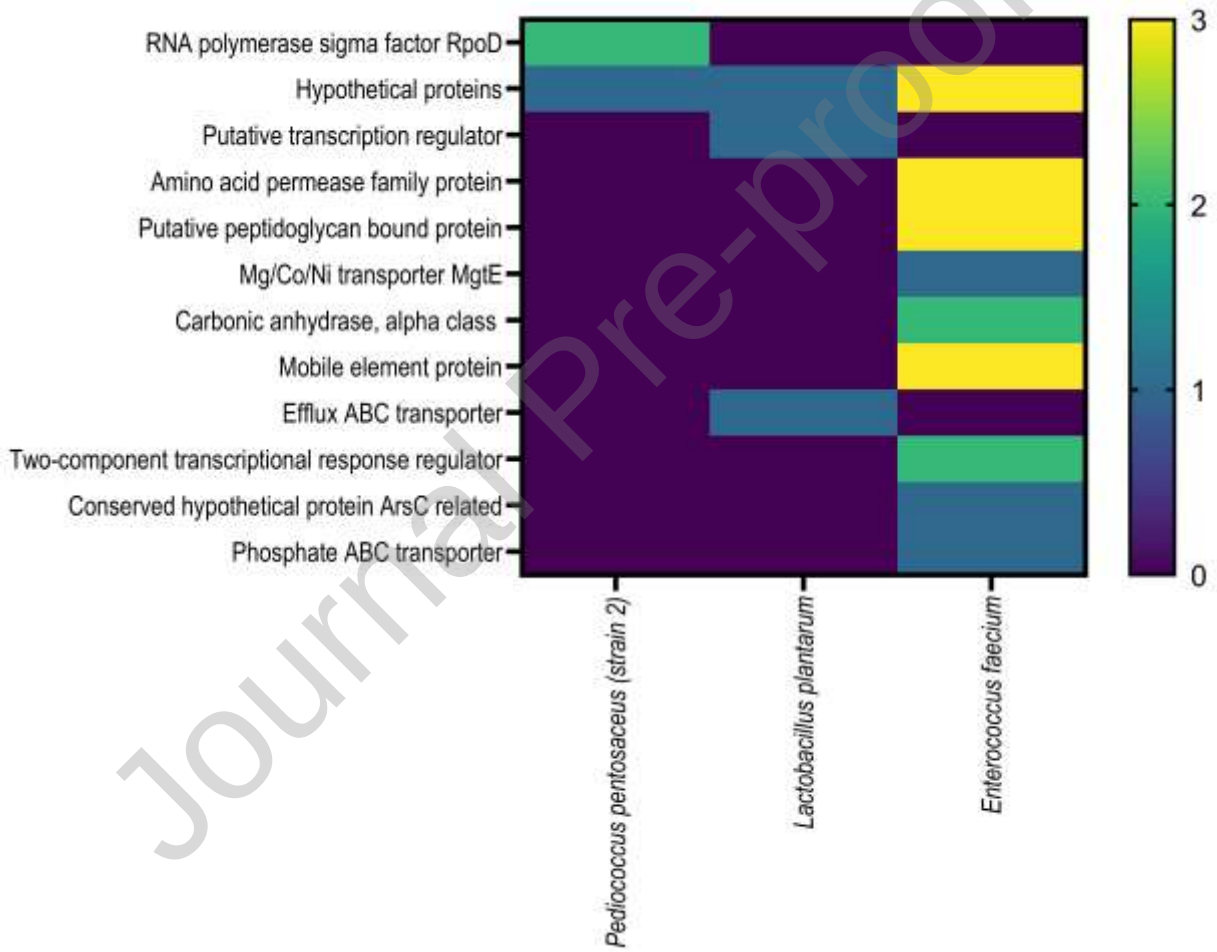




**Figure 6.** SNPs mutations detected after an ALE experiment for adaptation in high salinity media. The colors show how many times a genetic mutation appeared among the three lineages of a bacterial species.

**Table 1.** NaCl concentrations (g/L) where the growth is inhibited by 50%, for NaCl-evolved bacterial population, NaCl-evolved bacterial isolates, media evolved strains (ME), and Wild type strains (WT). The data are provided as means of triplicates and SD.

| | **Bacterial population** | **Individual bacterial isolates** | **ME** | **WT** |
|---|---|---|---|---|
| ***Pediococcus pentosaceus* (Strain 2)** | 71.87 ± 0.18 | 63.87 ±0.16 | 60.23 ± 0.11 | 69.41 ± 0.20 |
| ***Lactobacillus plantarum*** | 73.59 ± 0.05 | 63.66 ± 0.27 | 56.96 ± 0.12 | 53.83 ± 0.13 |
| ***Enterococcus faecium*** | 73.09 ± 0.33 | 76.27 ± 0.14 | 59.41 ± 0.15 | 53.25 ± 0.24 |

**Table 2.** Relative lactic acid production (%) for different seaweed species, bacterial starter cultures, and fermentation methodologies.

| **Seaweed** | **Microorganism** | **Methodology** | **Relative LA production (%)** | **Reference** |
|---|---|---|---|---|
| *Gracilaria sp.* | Mix of *Lactobacillus acidophilus* & *Lactobacillus plantarum* | Optimization of fermentation parameters including: inoculum, agitation, & temperature | 65.38 | Lin *et al.* 2020 [1] |
| *Ulva sp.* | *Lactobacillus plantarum* | Poly(vinyl-alcohol)-immobilized bacteria & continuous fermentation | 92.93 | Nagarajan *et al.* 2020 [2] |
| *Ulva sp.* | *Lactobacillus* | Combination of specific | 77.74 | |

| | | | | |
|---|---|---|---|---|
| | *rhamnosus* | macroalgae & LAB species | | Nagarajan *et al.* 2022 [3] |
| *Gracilaria sp.* | *Weisella paramesenteroides* | | 72.96 | |
| *Sargassum cristaefolium* | *Lactobacillus plantarum* | | 78.45 | |
| Mix of *Saccharina latissima* hydrolysates | *Pediococcus pentosaceus* | Adaptive laboratory evolution (ALE) strategy for adaptation in 71.00 g/L NaCl | 57.98 | This study |
| | *Lactobacillus plantarum* | | 75.29 | |
| | *Enterococcus faecium* | | 36.24 | |

**Declaration of interests**

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

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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## Highlights

- Seaweed isolated lactic acid bacteria resisted 71 g/L NaCl.
- Equal lactic acid concentration in natural seaweed- and hypersaline media.
- Increased salt affected cell metabolism, cell wall and strain-specific proteins.