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Stress responsive biomarkers for *Lactobacillus plantarum* robustness

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ABSTRACT

Background. *Lactobacillus plantarum* is one of the most versatile Lactic Acid Bacteria (LAB) that is encountered in many environmental niches, including dairy, meat and vegetable, in which it is used as starter culture for fermentation processes. Moreover, it has a demonstrated ability to survive gastric transit, can colonize the intestinal tract of human and other mammals and is commonly used in the formulation of functional probiotic foods for its health-promoting effects in consumers. *L. plantarum*, like other microorganisms, must face adverse environmental conditions during steps of technological processing, storage and consumption that include changes of temperature, pH, solute concentrations, nutrients and oxygen level, which can reduce its growth or survival potential. In order to adapt to environmental stresses and then stabilize their survival potential and their metabolic activity, these microorganisms have developed sophisticated mechanisms to sense changes and trigger a cascade of alterations in gene expression and protein activity.

Wine may be considered one of the most stressful environments (ethanol and acidity) in which several LAB have developed different mechanisms expressing their optimal biological role. *Oenococcus oeni* and *Lactobacillus* species are the main LAB associated with grape must and wine. Wine LAB are responsible for malolactic fermentation (MLF) during which the conversion of malic acid into lactic acid, and the metabolic activities of the associated LAB can lead to the production of compounds that can impact wine aroma and flavor.

Aims. In this study, in order to identify suitable molecular markers capable of improving *L. plantarum* robustness, six *L. plantarum* strains, autochthonous and commercials, have been investigated in response to stress conditions. To assess the effects of these strains on the sensorial quality of wine, volatile organic compounds (VOCs) released during fermentation in the headspace of food matrix have been characterized.

Materials and methods. Gene expression of six *L. plantarum* strains, grown at low pH and in presence of oxidizing factors, such as diamide and hydrogen peroxide, has been monitored by real-time PCR. Moreover, VOCs released by strains inoculated in fresh must (of Apulian grape) in two different moment (co-inoculum and sequential inoculum) have been monitored through PTR-MS in collaboration with Edmund Mach Foundation (FEM) in San Michele all'Adige (Italy).

Results. The results suggest that the expression of the genes analyzed in stress conditions is strains-specific and a combination of *hsp2* and *hsp3* genes as stress responsive markers, may be suitable to identify the most resistant *L. plantarum* strains, to low pH value and oxidative

stress. A total of 50 VOCs were identified. The concentration of most of the volatile compounds was influenced by inoculated strain. The similarities and differences expressed in the volatile profiles of wines reveal the potential of individual strains inoculated as starter cultures to produce wines of a certain character.

Keywords: *Lactobacillus plantarum*, stress response mechanisms, cellular robustness, gene expression, real time PCR, volatile organic compound, PTR MS

SOMMARIO

Introduzione. *Lactobacillus plantarum* è uno dei batteri lattici (LAB) più versatili che si incontra in molte nicchie ambientali, tra cui latticini, carne e verdure, in cui viene utilizzato come coltura starter per i processi di fermentazione. Inoltre, ha dimostrato di essere in grado di sopravvivere al transito gastrico, colonizzare il tratto intestinale di umani e altri mammiferi ed è comunemente usato nella formulazione di alimenti probiotici funzionali per i suoi effetti benefici sulla salute dei consumatori. *L. plantarum*, come altri microrganismi, deve affrontare condizioni ambientali avverse durante le fasi di elaborazione tecnologica, conservazione e consumo che includono cambiamenti di temperatura, pH, concentrazione di soluti, nutrienti e livello di ossigeno, che possono ridurne la crescita o il potenziale di sopravvivenza. Al fine di adattarsi agli stress ambientali e quindi stabilizzare il loro potenziale di sopravvivenza e la loro attività metabolica, questi microrganismi hanno sviluppato sofisticati meccanismi per rilevare i cambiamenti e innescare una cascata di alterazioni nell'espressione genica e nell'attività delle proteine.

Il vino può essere considerato uno degli ambienti più stressanti (etanolo e acidità) in cui diversi LAB hanno sviluppato diversi meccanismi che esprimono il loro ruolo biologico ottimale. Le specie *Oenococcus oeni* e *Lactobacillus* sono i principali batteri lattici associati al mosto d'uva e al vino. I LAB sono responsabili della fermentazione malolattica (MLF) durante la quale la conversione dell'acido malico in acido lattico e le attività metaboliche dei LAB possono portare alla produzione di composti organici volatili (VOC) che possono influenzare l'aroma e il sapore del vino.

Scopo della ricerca. In questo studio, al fine di identificare i marcatori molecolari in grado di migliorare la robustezza di *L. plantarum*, sono stati studiati sei ceppi, autoctoni e commerciali, in risposta a condizioni di stress. Per valutare gli effetti di questi ceppi sulla qualità sensoriale del vino, sono stati caratterizzati i VOC rilasciati durante la fermentazione nello spazio di testa della matrice alimentare.

Materiali e metodi. L'espressione genica dei sei ceppi di *L. plantarum*, cresciuti a basso pH e in presenza di fattori ossidanti, quali la diammide e il perossido di idrogeno, è stata monitorata mediante PCR in tempo reale (rt PCR). Inoltre, i VOC rilasciati dai ceppi inoculati in mosto fresco (di uva pugliese) in due diversi momenti (co-inoculo e inoculo sequenziale) sono stati monitorati tramite PTR-MS in collaborazione con la Fondazione Edmund Mach (FEM) presso San Michele all'Adige (Italia).

Risultati. I risultati suggeriscono che l'espressione dei geni analizzati in condizioni di stress sia ceppo-specifica e una combinazione di geni *hsp2* e *hsp3* come marcatori sensibili allo stress, può essere adatta per identificare i ceppi di *L. plantarum* più resistenti, a basso valore di pH e stress ossidativo. Sono stati identificati 50 composti organici volatili. La concentrazione della maggior parte dei composti volatili è stata influenzata dal ceppo inoculato. Le somiglianze e le differenze espresse nei profili volatili dei vini possono rivelare il potenziale dei singoli ceppi inoculati come colture di partenza per produrre vini di un certo carattere.

Parole chiave: *Lactobacillus plantarum*, meccanismi di risposta allo stress, robustezza cellulare, espressione genica, real time PCR, composti organici volatili, PTR MS

1.INTRODUCTION

1.1 Biotechnology importance of lactic acid bacteria

Lactic acid bacteria (LAB) are characterized as a heterogeneous group of Gram-positive, low-GC (<55mol%) generally nonsporulating and nonmotile, catalase-negative (with the exception of some species of the genus *Pediococcus*), rod (*Lactobacillus, Bifidobacterium*) or cocci (*Streptococcus, Pediococceus, Leuconostoc*) shaped bacteria characterized by ability to produce lactic acid as the major metabolic end-product of carbohydrate fermentation [1]. LAB include both homofermenters, producing mainly lactic acid, and heterofermenters, which, apart from lactic acid, yield a large variety of fermentation products such as acetic acid, ethanol, carbon dioxide, and formic acid [2]. Like all Gram-positive bacteria, their cell envelope is a multilayered structure, which is mainly composed of peptidoglycan with embedded teichoic acids, proteins, and polysaccharides and which is essential for the cellular integrity and shape [3]. LAB are extremely widespread in nature, preferring nutrient-rich habitats. They are part of the normal microflora of the mouth, intestines and human vagina and are also indigenous to food-related habitats, including milk, plants (vegetables and cereal grains), wine and meat. Based on their spoilage-preventing and flavor-contributing characteristics, LAB are employed in numerous industrial applications, ranging from starter cultures, to drive food and beverage fermentations, to bioconversion agents; several specific LAB strains are commonly used in the formulation of functional probiotic foods for their health-promoting effects in consumers [4–6]. LAB fermentation is a safe, economical, and traditional method of food processing, used all over the world, to improve the microbial safety, to offer technological, nutritional and health benefits. Except for a few species (such as *Streptococcus pneumoniae* or *Streptococcus pyogenes*), LAB are nonpathogenic organisms, reason why they have a "Generally Recognized as Safe (GRAS)" and "Qualified Presumption of Safety (QPS)" states [4,7]. During the fermentation process, LAB produce important aroma and flavor compounds through their metabolic activities (e.g. lipolysis and proteolysis), and they contribute to several technological properties in milk based products (yogurt, cheese, butter, kefir and koumiss), vegetables (sauerkraut), meat products (sausages) and wine [8]. LAB play an important role also in bread production especially for their ability to produce antimicrobial compounds, like bacteriocins, and to inhibit the main bread contaminants such as *Aspergillus*, *Fusarium*, and *Penicillium* [9]. Today, the main LAB extensively exploited in food industry belong to *Lactococcus* [10], *Leucocostoc* , *Pediococcus*, *Oenococcus* [11], *Enterococcus* and *Streptococcus* genera [8,12]. The industrialization of food transformations increased the economic importance of LAB, as they play a crucial role in the development of the organoleptic and hygienic quality of fermented

products. LAB, like other microorganisms, must face adverse environmental conditions during steps of technological processing, storage and consumption that include changes of temperature, pH value and osmolarity, or nutrient limitations. Research in this direction would promote better understanding of their responses to stresses.

In **Table 1** examples of application of LAB.

Applications	Products	Strais	References
Dairy products	Cheese	*Lc. lactis; Leuconostoc* spp.	[13] [14]
	Butter and buttermilk	*Lc. lactis* subsp. *lactis, Lc. lactis* subsp. *lactis var. diacetylactis, Lc. lactis* subsp. *cremoris, Leuc. mesenteroides* subsp. *cremoris*	[17] [16]
	Yoghurt	*L. delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus*	[13]
	Fermented, probiotic milk	*L. casei, L. acidophilus, L. rhamnosus, L. johnsonii, B. lactis, B. bifidum, B. breve*	[17]
	Kefir	*L. kefir, L. kefiranofaciens, L. brevis*	[18], [19]
Fermented foods	Meat	*L. sakei, L. curvatus P. acidilactici, P. pentosaceus*	[20], [21], [22]
	Vegetables	*P. acidilactici, P. pentosaceus, L. plantarum, L. fermentum Leuc. mesenteroides, P. cerevisiae, L. brevis,*	[23], [24], [25], [26]
	Cereals	*L. sanfransiscensis, L. farciminis, L. fermentum, L. brevis, L. plantarum, L. amylovorus,*	[27], [28], [29]
Alcoholic beverages	Wine	*O. oeni, L. acetolerans*	[30]

1.2 Lactic acid bacteria in bread and wine

1.2.1 LAB in bread

Lactic acid bacteria (LAB) are the dominant microorganisms in sourdoughs, and the rheology, flavor and nutritional properties of sourdough-based baked products greatly rely on the activity of these bacteria. [31].

The lactic acid produced by the metabolism of LAB (including *L. plantarum*), is the main compound responsible for acidification of the acid mixture and is also implicated in the aroma of bread although with limited effect. Equally, the production of acetic acid favors and improves the organoleptic properties of the final product. However, for a pleasant perception of aroma is required an optimal molar ratio lactate / acetate (2.2-2.7) (quotient fermentation, FQ). Bread quality obtained through the use of this species is comparable to that of bread produced using a more common starter such as *L. sanfranciscensis*, as there are no significant differences regarding the texture, volume, porosity and hardness of the product obtained [32].

Other studies have shown that the rising time and the acidification degree detected during the dough production, have improved thanks to the use of *L. plantarum* [33]. Moreover, a good acidification determines a remarkable reduction of the pH, which has many advantages: i) avoids development of any unwanted fermentation (e.g. *Enterobacteriaceae*), ii) inhibits or slows down in bread the growth of bacteria such as some species of the genus *Bacillus* (responsible for the so-called "Spinning bread"), iii) favors the development of acid-resistant yeasts and iv) improves the structural properties and the shelf-life of the finished product [34,35].

Recent studies have demonstrated the potential application of *L. plantarum* strains to improve the nutritional quality and shelf life of bread made with natural yeast and flour quinoa [36]. Furthermore, the use of *L. plantarum* as a starter culture for gluten-free bread production has been evaluated showing no differences significant compared to the product obtained with conventional starters [37].

LAB have been used for centuries as starter cultures in the food industry and have a long history of use as bio-preservatives, microorganisms able to prevent spoilage and to extend the shelf life of food and feed. In fact, they can produce different kind of bioactive molecules, such as organic acids, fatty acids, hydrogen peroxide and bacteriocins through which they perform their antimicrobial activity. Most of the large number of studies on LAB antimicrobial activity is focused on antibacterial effects [21,38,39], but in recent years the study and application of antifungal LAB has received an increase in interest. LAB can produce low molecular weight antifungal substances, for example phenyllactic acid, p-hydroxyphenyllattattic acid, cyclic

dipeptides as cycle (Gly-L-Leu), cycle (L-Phe-L-Pro), and cycle (L-Phe-trans-4-OH-L-Pro), benzoic acid, methylhydantoin, mevalonolactone and short-chain fatty acids [40–42].

Sourdough starter cultures have been shown to contain lactic acid bacteria that can inhibit the growth of certain molds [43,44] especially different species of *Lactobacillus* genus such as *L. plantarum*, *L. sanfranciscensis, L. rhamnosus* and *L. paracasei* [45].

The study of Schnurer et al., shows how phenyl lactic acid produced by *L. plantarum* strains, used as sourdough starter cultures, be able to prolong shelf life and prevent mold growth on bread baked from the sourdough [41]. However, it is essential that the bacteria do not reduce the leavening power of *Saccharomyces cerevisiae*. In this regard, some studies report that compounds active against filamentous fungi, i.e. molds, do not necessarily inhibit yeasts [46].

Fungal spoilage of foods represents a major cause of concern for food manufacturers and in addition to the great economic losses, another concern is the potential mycotoxin production that may cause public health problems [47]. Contamination by molds can be prevented by irradiating the goods with infrared rays or microwaves, by using modified atmospheres during packaging, or by adding chemical preservatives such as propionic acid [48]. The ancient traditions of using LAB in food, combined with recent knowledge on positive health effects caused by ingestion of probiotic LAB, suggests them as promising alternatives to chemical preservatives [9].

The ability of LAB to inhibit *Aspergillus, Fusarium*, and *Penicillium,* the main contaminants in bread, has been evaluated in several studies. Dal Bello et al., have showed that the production of two cyclic peptides (L-Leu-L-Pro) and (L-Phe-L-Pro) by *L. plantarum* FST 1.7 determines, at low concentrations, the inhibition of mold growth of the genus *Fusarium* (*F. culmorum* and *F. graminearum*) and *Aspergillus* (*A. parasiticus*), responsible for the production of micotoxins [49]. Gerez et al., have tested four LAB strains (*Lactobacillus brevis* CRL 772, *L. brevis* CRL 796, *L. plantarum* CRL 778, and *L. reuteri* CRL 1100) in bread preservation proving their ability to inhibit *Penicillium* sp. growth, through acetic and phenyl-lactic acids, and lengthen shelf life twofold with respect to breads prepared using only *S. cerevisiae* (2 days shelf life) [9].

1.2.2 LAB in wine

Until the end of the XIX century, wine was considered the product obtained from alcoholic fermentation conducted by yeasts, especially *S. cerevisiae*, which contribute to the production of major aroma compounds such as esters, higher alcohols, aldehydes and fatty acids, attributing to any subsequent transformations a totally negative value. Pasteur, in his "Etudes sur le vin" of 1866, identified LAB as the most responsible of wine alterations. It took about fifty years for Pasteur's judgment to be partially changed with the recognition that LAB, by converting L-

malic acid into L-lactic acid, could have a useful effect on wine. In fact, the first fundamental contributions on the role of LAB and malolactic fermentation were conducted between 1912 and 1918, but only in the fifties was there a clear perception of the importance of this process from an enological point of view. Subsequently, the imposing amount of scientific research led to the complete knowledge of the biochemistry of malic acid degradation and clarified that LAB, together with this transformation, can carry out reactions that lead to far more complex modifications of the organoleptic characteristics of the finished product. However, if together these transformations have a positive or negative effect on the quality of the wine, it is still the object of conflicting opinions. In any case, the full understanding of the reactions that accompany the malolactic fermentation cannot ignore the knowledge of the eco-physiology of LAB as well as of the physicochemical and biological factors, characteristic of the wine environment, which can influence their metabolic activity.

Throughout the course of winemaking, LAB undergo an evolution as *i)* a number, they increase immediately after the breaking of the berries, during the grape crushing phase until reaching a logarithmic cycle of 10^3-10^4 CFU / mL, to then decrease drastically during the alcoholic fermentation by yeasts, and as *ii)* a variety of species. The bacteria, isolated from the grapes before harvesting, belong to the species *L. plantarum, L. hilgardii, L. casei* and *O. oeni*, which, later becomes the most important species.

Not all species are always represented, certainly there is a natural selection progressively operates during alcoholic fermentation, at the end of which, *O. oeni* is the species mainly found. The evolution of LAB from the vine to the final stages of winemaking is mainly related to the geographical location, the type of cultivar and the winemaking procedures. The genera commonly found in wine are the following*: Lactobacillus, Oenococcus, Pediococcus* and *Leuconostoc* [50]. Nearly all red wines and some white wines (such as Chardonnay and Viognier) undergo malolactic fermentation.

1.3 The Malolactic Fermentation (MLF)

The term "malolactic fermentation" (MLF) describes the enzymatic conversion of L-malic acid to L-lactic acid and CO_2 (**Fig 1**) by one or more species of LAB including bacteria from the genera *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc,* among these, *O. oeni* is best adapted to the harsh wine environment, such as high alcohol, low pH and sulphur dioxide (SO_2) [53,54].
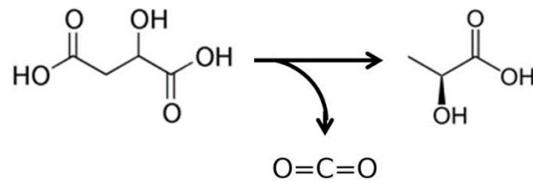
Fig.1 Chemistry of malolactic fermentation

This biological deacidification reaction is well recognized as one of the main metabolic capabilities of LAB in wine, and its conduct is of major commercial importance to the winemaking process. MLF is a secondary fermentation process that normally takes places after the alcoholic fermentation by yeasts, resulting in a wine with a softer mouth feel **(Fig 2).**
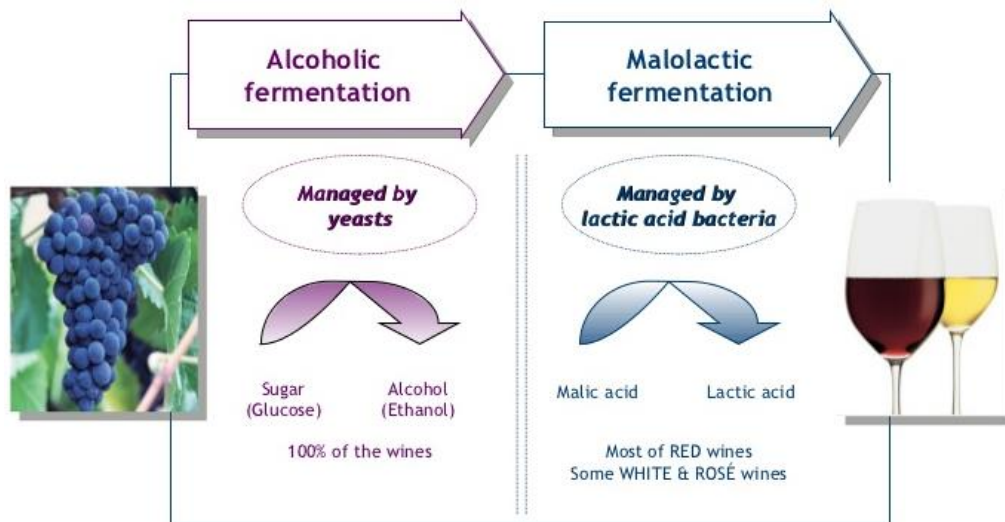

Fig 2 Schematic representation of fermentative processes in wine

The physiological function of the malate fermentation pathway is mainly involved to generate a proton motive force (PMF) as a means to acquire energy to drive essential cellular processes [51]. At the end of MLF, the remaining LAB can still metabolize residual sugars (glucose, fructose, arabinose and trehalose), which could result in spoilage including volatile acidity [52]. For this reason, it is essential to check the potential impact of residual LAB populations.

LAB have three possible enzymatic pathways for the conversion of L-malic acid to L-lactic acid and CO_2 [55]:

1. Direct conversion of malic acid to lactic acid via malate decarboxylase, also known as the malolactic enzyme (MLE).

2. Pathway employing the malic enzyme to convert L-malic acid to pyruvic acid, which is reduced by L-lactate dehydrogenase to lactic acid.

3. Reduction of malate by malate dehydrogenase to oxaloacetate, followed by decarboxylation to pyruvate and reduction to lactic acid.

MLF plays an important role in the winemaking process as it contributes (i) to decrease the acidity; (ii) to enhance the organoleptic characteristic; and (iii) to increase the microbiological stability of wine [53]. However, MLF is not favorable for all wines. This process is a welcome phenomenon in areas with a colder climate, where musts often have an excess of acidity, while it is unwanted in warmer areas where grapes tend be less acidic and a further decrease in acidity by the MLF may be detrimental to sensory and biological properties wine stability [54].

The organoleptic effect resulting from the degradation of malic acid is not only due to the increase in pH, but also to the substitution of the sour taste of malic anion, with the lactic anion which is decidedly sweeter, therefore the wine acquires a more taste soft and velvety. LAB are able to contribute to the formation of aroma through the production of aromatic compounds deriving from the degradation of amino acids. Moreover, there is a decrease in astringency due to the binding reaction between tannins and anthocyanins which reduces the amount of free anthocyanins. The quantity and type of compounds produced are strain-dependent but the composition of the must and the process of winemaking also have a significant influence [55]. One of the most evident flavor changes is the development of a 'buttery' or 'butterscotch' character. Diacetyl has been attributed to be a major contributor to this buttery character in wine. This metabolite can be formed from citric acid in wine, one of the major organic acids present in grapes together with the malate and tartrate. LAB are able to metabolize citric acid in the presence of glucose, leading to the formation not only of diacetyl, but of other compounds including lactate, acetate (volatile acidity), acetoin and 2,3-butanediol [56–58]. Many LAB can also produce a certain amount of polysaccharides. For example, *P. damnosus* produces a β-D glucono composed of a trisaccharide combined with D-glucose. Numerous studies have been carried out on LAB, especially on commercial *O. oeni* strains, demonstrating the potential value of the glucosidase activity of these bacteria as regards the substantial change in the flavor of wines. [59–61].

Yeast are also able to synthesize diacetyl during the alcoholic fermentation, however, the most of it is further metabolized to acetoin and 2,3-butanediol.

The changes in the organoleptic and qualitative profile of the wine due to malolactic fermentation also concern color. Indeed, the deacidification causes a decrease in intensity due to the change between the colored and non-colored forms of the anthocyanins.

1.4 Starter cultures for MLF

Spontaneous fermentation generally has an unpredictable course due to the prohibitive conditions of the wine. The chemical-physical parameters seen previously are often very different from the optimal ones, making the wine an unfavorable environment for bacterial growth. In fact, the low temperatures of the cellars, the low pH and the alcohol content of the wine tend to slow down the development of the bacterial population, with a consequent lengthening of the time required for MLF to degrade all the malic acid. Then, the assignment of this fermentation to the spontaneous microflora, can generate undesirable organoleptic deviations and may require development times unsuitable for production needs.

To have full control of MLF the use of starter cultures is certainly the most effective way since it guarantees the success of the process and, above all, the standardization of wine quality and production [62,63]. In fact, the selection of efficient LAB strains has become one of the main challenges of applied research in oenology [64]. Today, the most tangible result of this interest is not only the availability on the market of bacterial cultures to be used as starters of malolactic fermentation, but also of nutrients (the so-called "malolactic fermentation activators") to be added to the wine to facilitate management of this biological process during winemaking.

One of the advantages of selected cultures is the reduction of the time required to degrade malic acid to lactic acid thanks to the massive inoculation of bacteria (10^6 CFU / mL) in the wine to trigger the fermentation. The inoculated strains are subjected to specific selection processes mainly based on their ability to survive and multiply in wine, thus tolerating stress factors present during the production phases of wines such as pH, alcohol, SO_2 and temperature *O. oeni* is probably the best strain adapted to overcome the harsh environmental conditions of wine, in fact it is the dominant LAB isolated from the MLF and represents the majority of commercial MLF starter. Numerous studies show that lactobacilli are found throughout the wine environment, also during MLF and bottled wine, indicating that some strains have adapted to survive under winemaking conditions. Moreover, lactobacilli associated with wine possess several active enzymes. Because of this, some strains of *Lactobacillus* genera, mainly belong to *L. plantarum* species, have been deeply investigated as alternative starter cultures for MLF [65].

Despite the use of starter cultures and proper oenological technology, the management of MLF in wine can be difficult in some conditions to the point of failure. It is often not easy to identify the causes of their failure; for example, a frequent cause is due to the inadequacy of the bacterial stock with respect to the contingent cellar conditions; or the incorrect use by the manufacturer

of the bacterial preparation in the appropriate manner; or because the same preparation has low cell vitality.

1.5 *Lactobacillus plantarum*

L. plantarum, a facultative heterofermentative LAB, is extremely widespread in the environment. It is a natural inhabitant of the human gastrointestinal tract and widely used as a starter culture in many food industries that deal with bakery products, dairy products, fermented beverages and for preserving meat and fish thanks to its antiputridogenic action [66], and as probiotic for their health-promoting effects in consumers [4,67–69]. The genome of *L. plantarum* strain WCSF1 was the first of the species to be completely sequenced in 2003. The sequence of a second genome (JDM1 strain) with different probiotic functions was recently completed [70].

This species has a particular relevance in oenology: thanks to its ability to conduct MLF, it is usually isolated from red wines during this fermentation process [71], but it is also one of the most concentrated bacteria in the bunches of grapes at harvest [72]. This microorganism is also responsible for a series of wine defects and the production of unwanted compounds such as biogenic amines and ethyl carbamate [73]. *L. plantarum* of enological origin, grows well even at low pH: up to about 3.3 there is an average decrease of only 20% of the growth rate, moreover it tolerates ethanol concentrations well up to 13% and its optimum temperature is about 30 ° C [74].

This species, being an optional heterofermentative agent, possesses all the enzymes of the metabolic pathways of sugars, can also use pentoses, through the way of pentoses phosphates. Moreover, *L. plantarum* has a series of alternative pathways to metabolize organic acids in the absence of sugars. These metabolic pathways are extremely important because they allow it to exploit a series of compounds widely present in wine, including citric acid, glycerol, tartaric acid and above all malic acid through MLF. As an agent of MLF, *L. plantarum* is responsible for decreasing the acidity of wines and improving their aroma and taste [75].

L. plantarum is able to derive energy from some amino acids in the absence of other nutrients, through the decarboxylation of the amino acids present in the wine. From the degradation of amino acids some LAB, including *L. plantarum*, can lead to the production of biogenic amines (BA), low molecular weight organic bases that cause headaches, allergies, respiratory and heart problems at high concentrations. In literature, however, there are strong indications that *L. plantarum* is, in general, unable to produce BA but able to degrade its [76].

Therefore, *L. plantarum* appears to be a more efficient alternative to *O. oeni* as a starter culture in the winemaking process [75,77].

In fact, selected strains of *L. plantarum* have shown to have a higher malolactic activity even at low pH values, capable of metabolizing very little sugar and, of considerable importance, unable to produce acetic acid. The starting efficiency of malolactic fermentation depends on the ability of the strain to survive and proliferate in wine. Resistance to the difficult conditions of wine is strictly strain-dependent. Therefore, the isolation, identification and typing of *L. plantarum* strains naturally present in wine immediately after spontaneous malolactic fermentation are important steps for the development of new malolactic starters, able to start and complete malolactic fermentation without producing undesired intermediate compounds. Recent studies have shown that strains of *L. plantarum* possess a resistance mechanism capable of tolerating not only the low pH but also high concentrations of ethanol [74,78,79].

1.6 Stress physiology of LAB

LAB are widely applied in food industry, agricultural production, animal husbandry, pharmaceutical engineering, in which they encounter a wide range of stresses in their constantly changing environments both abiotic (during food production, manipulation of starter or probiotic cultures), and biotic (in the host or in complex ecosystems) [8,80]. Variations in temperature, pH, solute concentrations, nutrients, and oxygen level can inhibit cell growth and lead to cell death. So that the LAB can perform their activity it is necessary to survive, maintaining high viable counts, the technological stress encountered during food manufacture, as well as the hostile environments found within the product to which they are added and the host intestinal flora [81,82]. Then, cellular robustness plays a key role, especially in view of the development of new applications such as pharmaceutical preparations, live vaccines and probiotic foods. Several studies show that LAB are particularly robust: Mills et al. have reported that *Lactobacillus spp*. survived the low pH of the stomach (pH 2.0 to 4.0), while *O. oeni* strains were able to proliferate in the presence of 13% ethanol at pH 3.2 and 18°C [83].

Booth has defined the stress as "any change in the genome, proteome or environment that imposes either reduced growth or survival potential. Such changes lead to attempts by a cell to restore a pattern of metabolism that either fits it for survival or for faster growth" [84]. Bacteria have developed defense mechanisms against stress that allows them to survive in a hostile environment with different ways to sense changes and trigger a cascade of alterations in gene expression and protein activity [6,85]. Indeed, a stressful environment usually affect the microbial cell physiology and in response to environmental stresses, microorganisms have

developed signal transduction systems that control the coordinated expression of genes involved in different cellular processes such as cell division, DNA metabolism, membrane composition, transport protein [85,86] **(Fig. 3).**
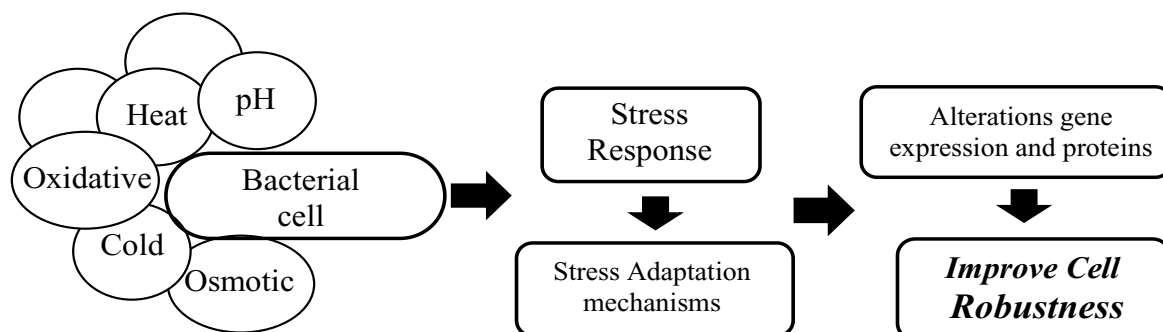
Fig. 3 Examples of stress response mechanism in lactic acid bacteria

Many studies conducted on lactobacilli and bifidobacteria have shown that exposure to acidic environments leads to changes in cell membrane lipid composition [87–89]. Broadbent et al. have found a dramatic increase in the ratio of saturated to unsaturated fatty acids and cyclopropane fatty acids (CPFA) content in the membranes of acid-adapted *L. casei* ATCC 334 [90]. These changes modulate membrane features including fluidity, hydrophobicity and proton-permeability [91,92]. In *O.oeni*, the main bacterial starter used for secondary fermentation in wine, under stress conditions, changes in the composition of fatty acids of cell membranes usually occurred, as well as the synthesis of stress proteins [93]. During industrial processing (starter handling and storage) and in the passage through the gastrointestinal tract (acidity and bile salt) bacteria find several environmental stress conditions, which can cause structural and physiological injury to the cells, resulting in loss of viability. For that, most of bacteria react by blocking the replication processes and activating adaptation mechanisms [94]. The current knowledge on the environmental stress responses in LAB varies between species and depending on the type of stress [95]. Within the stress responsive mechanisms, knowledge is accumulating on stress such as acid, heat, cold, osmotic and oxidative stress.

1.7 Stress response mechanisms in LAB

The ability of LAB to tolerate industrial stress is essential, considering their economic importance for food fermentation and their health-related implications as probiotics [51]. To

survive these harsh conditions, LAB have evolved both physiological and genetic mechanisms [96] including:

• the production (i.e. up-regulation or de novo synthesis) of proteins involved in damage restoring, in the preservation of cell homeostasis, and/or in the eradication of the stressing cause (i.e. achieved by modulation of stress regulons);

• the modifications of cellular structures resulting in a temporary enhanced resistance or tolerance to the stress (i.e. changes in cell morphology, alterations in membrane fatty acids composition and content);

• the cell entrance into a quiescent physiological condition;

• the evasion of host defenses, in case of host-microbe interaction [97].

Below the main stress factors encountered by LAB in their industrial applications and their adaptation mechanisms are discussed.

1.8 Mechanisms of acid resistance

LAB are generally neutrophils (optimal pH for growth between 5 and 9) except for some species of the genera *Lactobacillus, Leuconostoc* and *Oenococcus*. Mild to medium acidic environments are typical of fermented foods such as sauerkraut, green olives, pickles, sausages, baked goods, cheeses and fermented milk drinks. Moreover, upon ingestion, probiotics have to face the strong acidity of the initial part of the GI tract, which is also combined with the action of digestive enzymes. In the stomach, the pH varies between 1.5 and 5, depending on digestion phase and fasting periods. While most LAB generally well tolerate low pH, other beneficial microorganisms, such as bifidobacteria, exhibit a poor acid resistance and this often constrains their application as functional ingredients of probiotic food [6]. The resistance to acids is fundamental for LAB growth, for their application in fermentation processes and production and functionality of a probiotic culture. LAB, as well as other bacteria, developed sophisticated adaptation mechanisms, which are able to increase their cell robustness [80]. Acid stress can be described as the combined biological effect of low pH and weak (organic) acids, such as acetate, propionate and lactate present in the environment (food) as a result of fermentation, or alternatively, when added as preservatives. Weak acids in their protonated form can diffuse into the cell and dissociate, thereby lowering the intracellular pH (pHi) resulting in the inhibition of various essential metabolic and anabolic processes, and influencing the transmembrane ΔpH, impairing not only the pH homeostasis but also the transport systems that depend on the proton-motive force [98]. In LAB, acid tolerance (AT) increases in at least two distinct physiological states: (i) during logarithmic growth an adaptive response referred to as L-ATR (lactic acid

tolerance response), can be induced by incubation at a non-lethal acidic pH; and (ii) after entry into the stationary phase, AT increases as a result of the induction of a general stress response [81].

LAB can respond to acid stress by i) up-regulating general stress proteins and chaperones such as cytoplasmic (DnaK and GroEL) and periplasmic (HdeA and HdeB) chaperones, Clp protease complex, able to remove damaged proteins (due to low pH) participate in protein homoeostasis ii) the production of a new set of shock proteins, known as stress proteins [101] and/or iii) carrying out specific adaptation and protective mechanisms such as the Arginine Dihydrolase (ADS) pathway, an increased activity and amount of the F_0F_1-ATPase, the amino acid decarboxylation-antiporter reactions, the pre-adaptation and cross-protection systems.

Pre-adaptation and cross-protection. These are effective methods to strengthen the resistance of LAB against acidic environments. The pre-exposure of LAB to a sublethal stress can increase their resistance to the same type and/or other kinds of lethal stress. According to van de Guchte et al., bacteria cells develop stress-sensing systems, which allow them to better resist harsh conditions and sudden environmental changes [81]. At the basis of cross-protection different stimuli such as heat, oxygen, cold and low pH can are involved. For example, heat pre-treatment provokes an acid resistance response in *L. plantarum* promoting its growth under low pH [102] while Chu-Ky et al. have shown how acid adaptation enhanced *L. fermentum* resistance in acidic conditions [103]. After 180 min of acid stress exposure, in a gastro intestinal juice simulator, the vitality of the acid-adapted bacterium was much higher than that of unstressed control. These systems are used by bacteria not only to counteract the acid conditions they encounter during life cycle but are also used as a response to stresses such as heat, cold and osmosis.

Addition of protective agents. The supplement of acid stress protectants in LAB, such as amino acids, fatty acids or fermentable sugars, can reduce damage caused by the acidic environment. For example, arginine, aspartate and tween-80 can improve acid resistance of *L. casei* Zhang [70,104] and increases the survival capacity of *L. rhamnosus* (1000 times higher than the control) [91]. High concentrations of maltose or glucose improved the viability of *L. plantarum*, while the addition of tryptone and yeast extract, increased the tolerance of *L. acidophilus* [105]; finally, glutathione carried out a protective action against LAB in acidic conditions [106].

Like in other biological fields, there are continuous advancements that culminate in the discovery of new acid-resistance mechanisms and biomarker candidates. For instance, two recent works based on proteomic analysis, in *Lactobacillus pentosus* [107], and transposon mutagenesis, in *Lactobacillus paracasei* [108], have proposed novel genes involved in acid

14

resistance. Interestingly, some of these biomarkers influence cell auto-aggregation, which underlies the probiotic ability to adhere to host mucosa [107,108].

1.9 Oxidative stress

Aerobic bacteria produce energy primarily through oxidative phosphorylation. Although many LAB are typically microaerophiles and can produce catalase (if grown in a medium containing heme) or pseudo catalase (non-heme catalase), several species, such as *Lc. lactis* and *E. faecalis*, grow well anaerobically. These lack effective oxygen scavenging cellular mechanisms such as catalases and are thus unable to synthesize ATP by respiratory means, but they have an exclusively fermentative metabolism. They can be susceptible to aerobic conditions during food processing (e.g. after spray drying or during fermentation), because it is naturally present in food or permeates in them through packaging. For example, dairy foods, such as yoghurt, contain high levels of oxygen, which is incorporated during the various processing phases (homogenization, mixing and agitation) or permeated through the packaging material during the shelf life storage [81]. To survive these harsh conditions, bacteria have developed adaptation responses that lead to reprogramming gene expression. As reported by several studies, in *Oenococcus,* the *trxA* gene, encoding a thioredoxin, was found to be induced not only by the presence of H_2O_2 but also by heat-shock while the *trxB* gene encoding the thioredoxin pathway enzyme, thioredoxin reductase has been identified in *L. bulgaricus* [81]. In *Lc. lactis*, FlpA and FlpB regulate the uptake of Zn (II). The inactivation of *flpA* and *flpB* genes, resulting in exhaustion of the intracellular pool of Zn (II), increases microorganism sensitivity to H_2O_2. This finding suggests that *Lc. lactis* uses Zn (II) as a defense mechanism against oxidative stress [109].

Oxidative stress is caused by an imbalance between intracellular oxidant concentration, cellular antioxidant protection and oxidative change of macromolecules (such as membrane lipids, proteins) [110]. Exposure to oxygen causes an accumulation of toxic oxygenic metabolites, which, in turs, lead to a partial or complete growth inhibition. Understand how oxygen can perform a toxic action on the anaerobic microorganisms, requires a brief description of Reactive Oxygen Species (ROS). ROS are generated from endogenous sources as well as from the environment; they can readily diffuse across cellular membranes and may cause oxidative damage in biomolecules such as membrane lipids, proteins and nucleic acids impairing their biological functions or constituting one of the major causes of aging and cell death [111,112]. For example, [113] have shown that oxidative stress causes a change in the fatty acid

composition in the cell membrane of *L. helveticus*. Examples of the main toxic forms of oxygen are reported below:

i) superoxide anion (O_2*^-), its toxicity is due to its instability, it is highly reactive and can oxidize all the organic compounds of the cell; the optional aerobic and anaerobic bacteria produce the enzyme superoxide dismutase (SOD) to neutralize it, while bacteria that lack this enzyme, such as *L. plantarum*, using high level (20–30 mM) intracellular accumulation of Mn^{2+} ions as a scavenger for oxygen radicals [114];

ii) hydrogen peroxide (H_2O_2) a powerful bactericide and oxidant, able to generate other cytotoxic oxidizing chemical species such as hydroxyl radicals in the presence of transition metals; contains the peroxide anion, which is also toxic, it is neutralized by enzymes such as catalase or peroxidase; iii) hydroxyl radical (HO*), is highly reactive with biological molecules, it forms in the cell cytoplasm following the action of ionizing radiation [115].

Usually, bacteria use antioxidant enzymes, in SOD and catalase (CAT), to protect themselves from ROS by eliminating superoxide and H_2O_2, respectively. Recently, An et al. reported that the cooperation between SOD and CAT could significantly enhance oxidative resistance in *L. rhamnosus* [116].

LAB produce ATP by substrate level phosphorylation, therefore the regeneration of NAD^+ from NADH assumes critical importance. The organic substrate undergoes a series of oxidative and reductive reactions mediated by pyridine nucleotides such as NADH. The simplest way to oxidize NADH is by the reduction of molecular oxygen (O_2) via the activity of NADH oxidase. Many studies on the aerotolerance of LAB suggest that the ratio and specific activities of the NADH oxidase and NADH peroxidase determine the elimination of oxygen from the cell [117,118]. The NADH oxidase system appears to be commonly spread within LAB. Two types of NADH oxidases have been identified in LAB, the NADH- H_2O_2 oxidase and NADH- H_2O oxidase.

While the NADH- H_2O_2 oxidase catalyzes the reduction of O_2 to H_2O_2 (1)

$$NADH + H+ + O_2 \longrightarrow NAD+ + H_2O_2 \quad (1)$$
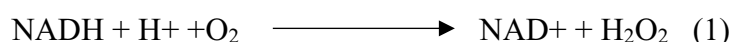
the NADH- H_2O oxidase carries out the four-electron reduction of oxygen to water (2)

$$2NADH + 2H+ + O_2 \longrightarrow NAD+ + 2 H_2O \quad (2)$$
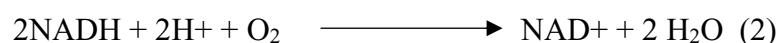
The activities of NADH oxidase can also result in the incomplete reduction of oxygen, generating reactive oxygen species such as the superoxide anion (O^{2-})

$$NADH + 2O_2 \longrightarrow NAD+ + H+ + 2O_2$$
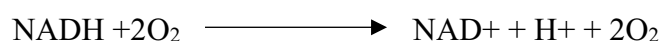
Accordingly, some lactic acid bacteria possess NADH peroxidase that reduce H_2O_2 to H_2O as shown below:

$$NADH + H+ + H_2O_2 \longrightarrow NAD+ + 2\ H_2O \quad [118]$$

Membrane lipid composition was found to be modulated by oxidative environment in both probiotic lactobacilli [113] and bifidobacteria [119], suggesting that these changes may increase resistance to ROS.

1.10 LAB Robustness

In general, the term robustness refers to "the persistence of a system's characteristic behavior under perturbations or conditions of uncertainty". In biology, the concept of robustness closely relates to "stability," "homeostasis," and "canalization," but it covers a broader class of phenomena [120,121]. For instance, homeostasis refers to maintaining a steady state, but robustness can also apply to dynamic processes in development. For which is fundamental to specify i) the characteristic behavior that remains unchanged and ii) for which type of disturbances or uncertainties this invariance property holds [122]. Furthermore, cellular robustness can be measured as limits of intracellular parameters such as gene expression levels [123].

As described in the previous chapters, LAB are widely used in the food industry. Due to this extensive industrial use there is a strong interest in unraveling the molecular mechanisms involved in industrial robustness [124]. Indeed an essential characteristic to enable advanced industrial application of these starter and probiotic cultures is their robustness towards the stresses encountered in the industrial pipeline, which include oxidative, temperature, osmotic and/or solvent stress during industrial fermentation, as well as industrial processing stresses, such as freeze-drying.

At present, several strategies to intervene on process techniques and/or to develop strains that are more robust have been proven successful:

-protective strategies: encapsulation, carrier media and addition of protective agents;

-enhancing strategies: addition of prebiotics and methods taking advantage of stress adaptation and cross-protection;

-genetic approaches: genetic engineering and selection of resistant strains by adaptive evolution [125].

Today we are witnessing a growing commitment to engineer microbes through more "natural", classical strain-improvement methods, including those based on adaptive evolution [126], which allows to obtain microbial strains with tailored characteristics through a selective pressure. Currently, this strategy provides a valuable tool for diverse biotechnological applications [127], including the isolation of probiotics with improved robustness to specific environmental stress. Indeed, by progressively exposing microbial cultures to a stress, over numerous generations, it is possible to select spontaneous mutants that are more tolerant to that kind of stress. For instance, after gradual adaptation of *L. acidophilus* to high temperatures, a thermotolerant variant was selected which showed greater stability under both heat and acid conditions [128]. Likewise, an acid-resistant mutant of *L. casei*, obtained by a 70 days-adaptive evolution, exhibited higher pHi, increased biomass production and survival during acid stress [70], as well as a distinctive proteomic pattern compared to wild type [129]. Moreover, acid-tolerant lactobacilli could be obtained by a mixed approach that joined adaptive evolution and genome shuffling, thereby speeding up the evolution towards the desired phenotypes [130]. Even among bifidobacteria, acid-resistant [131–133] and heat tolerant [134] derivative strains could be obtained by prolonged exposure to low pH conditions and sequential heat shocks, respectively. Interestingly, the increased acid resistance was also associated to higher fermentative capacity and enhanced tolerance to other types of stress, which corroborates the principles of cross-protection [125].

The use of methods, such as cross protection and the controlled pre-exposure of bacteria to sublethal stresses, seems to be a valuable strategy to increase their stress tolerance, and thus obtaining robust cells, while optimizing vitality and functionality. However when this approach is transferred from laboratory to an industry scale, some difficulties arise, including a scarce stability of the stress-induced resistant phenotypes, variable results and negligible achievements in terms of robustness improvement [135,136].

Therefore, more studies are needed to understand strain-specific differences in stress response performance, as well as to develop methods with enhanced feasibility at the industrial scale [137].

1.11. On-line monitoring of volatile organic compounds

1.11.1 PTR-MS instrument

There are numerous research fields in which the rapid and sensitive monitoring and quantification of Volatile Organic Compounds (VOCs) are fundamental as environmental sciences, medicine and food sciences. VOCs play an important role in the perception of aroma

and flavor and, thus, in food appreciation. Their detection helps to control product quality and to monitor industrial processes. The Proton Transfer Reaction Mass Spectrometer (PTR-MS) represents an analytical technique developed by Werner Lindinger of the University of Innsbruck [138] which allows to monitor processes in real time and take on-line measurements of many VOCs released, without the need for a pre-treatment of sample [139].

The chemical analysis technique with PTR-MS is based on the transfer of the H^+ proton from the H_3O^+ ion to all those compounds that have a proton affinity greater than that of water. After injecting the volatile mixture to be measured in the drift tube, all volatile compounds with proton affinity higher than the water will react with the hydron ions according to the following reaction:

$$H_3O^+ + R \longrightarrow RH^+ + H_2O$$
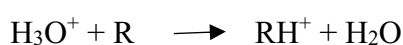
where R indicates the neutral volatile molecule.

Most VOCs (including terpenes, benzene, aldehydes, alcohols, etc.) have a proton affinity greater than that of water and therefore can receive the H^+ proton from the H_3O ion. The **Fig. 4** shows a common version of PTR-MS apparatus.
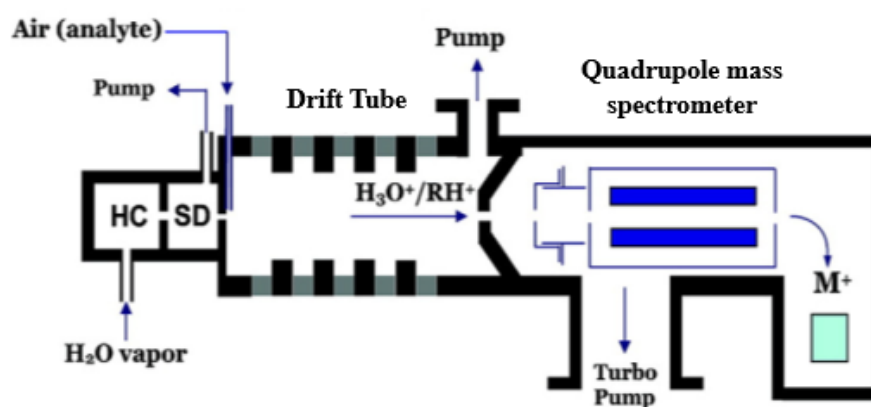
Fig. 4 Simplified representation of a PTR-MS workflow utilizing a quadrupole mass filter (HC = hollow-cathode discharge source, SD = source drift region)

The proton transfer reaction has the advantage of inducing a low level of fragmentation of the compound that receives the H^+ proton (compared to electronic impact and charge transfer) thus facilitating both the interpretation and processing of mass spectra.[140–142], but provided only the nominal mass of the ions measured and thus little chemical information. The recent combination of PTR-MS with time-of-flight mass analyzer (PTR-ToF-MS) has been proposed to overcome these limitations and improve the analytical capability of this technique, building

a device which can register entire mass spectra of complex VOC mixtures with high mass resolution in real time [143–145]. The high mass resolution provided by the ToF analyzer allows to separate and tentatively identify isobaric compounds but not to distinguish isomeric compounds. Direct headspace air injection into the PTR-ToF-MS is difficult in the case of samples with high ethanol concentration in a headspace. To remedy these problems the PTR-ToF-MS was also coupled with a fast gas chromatography (FastGC) (IONICON ANALYTIK GmbH, Innsbruck, Austria) as it is described by Romano et al. 2014 [146].

1.11.2 PTR-MS applications

Due to the importance of VOCs in food science and technology, fast and high-sensitivity methods have been developed for their identification and quantification. Numerous papers show the application of PTR MS on a wide variety of food matrices such as olive oil (Aprea et al., 2006), berryfruit (Boschetti et al., 1999), Grana Padano, Parmigiano Reggiano, and Grana Trentino (Boscaini et al., 2003), milk (Fabris et al., 2010), roasted coffees (Yenr et al., 2014), apple (farneti et al., 2015), fermented food matrix (Makhoul et al., 2014).

Wine quality is strongly influenced by aroma and flavor given to the beverage from its VOCs, which can be released from non-volatile precursors of grapes and oak wood or can originate during malolactic fermentation by LAB (Gonzalez et al., 2011).

FastGC allowed to eliminate the effect of ethanol and perform PTR-MS analysis without the need to drastically change the ionization conditions. At the same time the chromatographic separation provided an additional dimension to the data without affecting the analytical throughput.

2. AIMS OF THE RESEARCH

The availability of complete genome sequences of industrial *L. plantarum* strains may be useful to develop functional genomics technologies that integrate molecular biology and classical physiology in order to understand stress adaptation mechanisms at strain level and, further, identify cellular biomarkers. These technologies can discover cellular components that may function as biomarkers for stress adaptive behavior and enhanced robustness in commercial *L. plantarum* strains dealing with food or storage stress conditions.

In this study, in order to identify suitable molecular markers capable of improving *L. plantarum* robustness, six *L. plantarum* strains, autochthonous and commercials, have been investigated in response to stress conditions. To assess the effects of these strains on the sensorial quality of wine, volatile organic compounds (VOCs) released during fermentation in the headspace of food matrix have been characterized.

The knowledge of regulators and a better understanding of LAB stress responses could provide relevant insight into improving performance of current industrial starter strains. The use of selected strains with particular characteristics of resistance to stress, will determine certainly an increase in the quality and conservation of the products.

3. MATERIALS AND METHODS

3.1 Whole genome sequences of *L. plantarum* strains and identification of conserved stress responsive markers

3.1.1 Microbial strains and growth conditions

Six *L. plantarum* strains isolated from wine, already characterized for their pro-technological potentials have been included in our trials. Four commercially available *L. plantarum* strains identified as *A, B, C,* and *D* were kindly provided by Lallemand Inc. (Toulouse, France), while the two *L. plantarum* strains identified as *E* and *F* are available in the strains collection of the Industrial Microbiology laboratory, University of Foggia (Italy).

Lactobacilli have been cultivated in MRS (De Man Rogosa Sharpe) broth (pH 6.2), a non-selective medium used for the growth of lactic acid bacteria, available as lyophilized powder (Oxoid, UK). It was prepared by resuspending 52 g in 1 L of distilled H_2O.

Solid MRS was prepared by adding 15 g/L agar. All media were autoclaved at 121 °C for 15 minutes. *L. plantarum* strains were stored in glycerol stock at -80 ºC in tubes of 2 mL and routinely grown in MRS broth (pH 6.2) anaerobically at 30 ºC when required.

3.1.2 Genomic DNA extraction

Overnight strains cultures, grown at optimal conditions, 30 °C in MRS broth (pH 6.2), were diluted and incubated at the same temperature until exponential middle phase (OD_{600} about 1). Genomic DNA was extracted from bacterial cultures by using the Ultraclean Microbial DNA Isolation kit (MoBio), according to the manufacturer's instructions. Briefly, DNA extraction is divided into two different phases: a first phase of lysis and a second phase of purification. In the first step the microbial culture is centrifuged at 10,000 rpm for 1 min; the supernatant discarded, while the obtained pellet is resuspended in the Micro Bead solution consisting of Guanidine Thiocyanate Solution able to denature intracellular proteins, to stabilize and disperse homogeneously the microbial cells prior to lysis.

Then, the solution is added to a Micro Bead Tube containing beads, followed by lysis solution consisting mainly by SDS an anionic detergent useful to breaks down fatty acids and lipids associated with the cell membrane. The tubes are heated at 65°C, to increase yield, for 10 minutes and vortexed at maximum speed for 10 min.

Finally, the tubes are centrifuged and a Solution MD2 is added to the supernatant, which is incubated at 4°C for 5 min.

The Solution MD2 contains a reagent to precipitate non-DNA organic and inorganic material including cell debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

In the second purification step, the mixture is centrifuged at 10.000 rpm for 2 min.

The supernatant, then, is transferred to a new tube followed by a highly concentrated Salt Solution, necessary to bind DNA to the spin filter membrane, and vortexed for 5 seconds before being transferred into silica Spin Filter.

The filter is washed with a ethanol based wash solution, which removes residues of salt, and other contaminants while allowing the DNA to stay bound to the silica membrane, and is centrifuged for 1 min at 10,000 rpm. The filtrate is eliminated, the column is reinserted into the tube and centrifuged to remove residual ethanol wash solution.

Then, the Spin Filter is placed into a new Tube and the Elution Buffer is added to the center of the white filter membrane.

After leaving at room temperature for 1 min., a centrifugation at 10,000 rpm for 1 min. is performed. The Spin Filter is removed and the eluent (DNA) is stored at -20 ° C.

3.1.3 Quantification and electrophoresis on agarose gel

The DNA quantification was performed by NanoDrop instrument. It is a spectrophotometer that allow quantifying samples of different nature (e.g., DNA, RNA) using micro volumes without the use of classic cuvettes. This tool consisting of an instrumental part and a software to be installed on a computer.

A critical point is represented by the upper and lower optical surface of the retention system spectrophotometric, which should be fully cleaned prior used. After that, $2\mu l$ of nucleic acids samples are loaded in the microspots.

Furthermore, a software measures the absorbance at different wavelengths and automatically calculates the concentration ratio e sample purity.

The **Fig. 5** shows a typical nucleic acid spectrum.

The ratio of the two absorbances $\lambda = 260$ / $\lambda = 280$ should be between 1.6 and 1.8 for DNA, between 1.8 and 2.0 for RNA.

The purity ratio between the absorbance at $\lambda = 260$ / $\lambda = 230$ is a second measure of DNA control, that must be between 1.8 and 2.2. Purity ratios that are significantly below the expected values may indicate the technique of extraction and purification of the acid nucleic use may require further optimization.
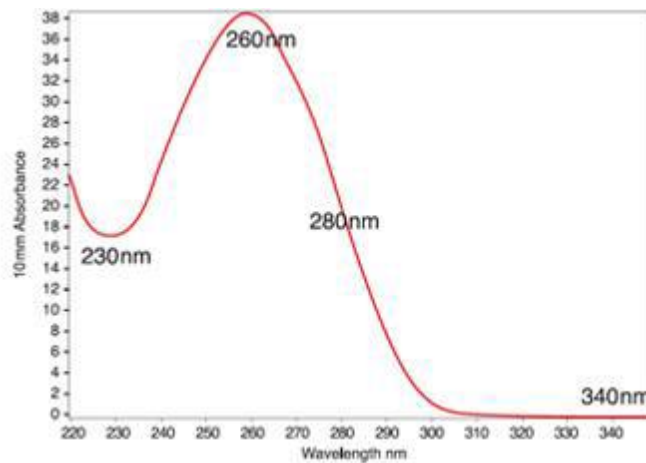
Fig.5. Typical nucleic acid spectrum

Electrophoresis. This technique is used routinely to quantify and verify the quantity and quality of DNA and RNA extracted and to control the size of amplified fragments by PCR. It exploits the charge that the molecules have once placed in a saline solution. In response to the passage of electric current, the molecules will tend to migrate towards the electrode with charge opposite to its own, separating according to their weight molecular as the larger molecules migrate more slowly than most small.

The DNA, which has a negative charge, will then tend to migrate to the anode having charge positive. Using known molecular weight markers is also possible to estimate the size and concentrations of our samples. The electrophoresis comes normally carried on agarose gel at 1 to 2% in 1x TAE buffer (1g of agarose in 100 ml TAE 1x). The gel solid is immersed in the TAE 1x buffer of the pan where the stroke takes place. It comes conducted with a voltage that depends on the size of the gel.

Preparation of a 100 ml gel at 1%:

- 1gr of agarose is weighed, placed in a bead and added 100 ml of TAE 1x and 0,85 μl of ethidium bromide.

- Melt the agarose (in the microwave oven) and pour the liquid into a support where previously one or more combs have been inserted. Furthermore, samples are diluted and labelled with a blue dye, the Loading Dye, which will form two separate bands in the gel.

The experimental conditions were as follows:

1% agarose gel, 30min running at 100V, 5μl of DNA loaded.

3.1.4 Genome sequencing

For genome sequencing and assembly 2 μg of Genomic DNA was subjected to library preparation using the "TruSeq DNA Sample Prep Kit FC-121-1001" according to the

manufacturer's instructions. Whole genome sequencing of *L. plantarum* strains was performed using the Illumina Miseq platform **(Fig.6)** Before assembly, the raw reads were subjected to a filtering, using PRINSEQ v0.20.3 software [147]. This filtering step was performed in order to remove: the 3' ends showing a quality score below 25 (Q<25); the reads shorter than 75 bp; the reads with an average quality score below 25 (Q<25); the reads containing a percentage of unknown based ("N" characters) equal or greater than 10% and the duplicated reads

Fig.6 Workflow of gene sequencing

3.1.5 Genome annotation

Genome annotation was carried out in collaboration with the bioinformatics group of Lallemand Inc. Briefly, genome annotation ("Open Reading Frames" (ORF) calling, gene function prediction) of the assembled genome is performed using the RAST (Rapid Annotation using Subsystem Technology) server [148]. Start codons of the predicted ORFs are verified manually, aligning the *L. plantarum* strains analyzed with homologous ORFs from the following reference *L. plantarum* strains, WCFS1, ZJ316, STIII, JDM1 and 16, already available in gene bank. ORF functional annotations were refined by aligning ORF nucleotide sequences to the Cluster of Orthologous Groups (COG) database [149] using BlastP and by using the functionality of InterProScan v5.0 in Blast2GO [150] searching for matches against the PRINTS (v42.0), Pfam (v27.0) and TIGRFAMs (v13.0) databases. The TMHMM (v2.0) and Phobius (v1.01) prediction search tools were used respectively to predict transmembrane domains and the presence of signal peptides.

3.2 Quantitative analysis and identification of suitable stress responsive markers

3.2.1 Bacterial strains and growth conditions

The effects of stress conditions were investigated on the six *L. plantarum* strains.

Acidic stress conditions. Overnight bacterial cultures, grown in optimal conditions, 30°C in MRS broth (pH 6.2), have been diluted and incubated at the same temperature until OD_{600} = 1.5 (exponential middle phase). At this value, each culture has been divided into two aliquots and centrifuged to 3000 rpm for 7 min at 21°C. The obtained pellets were resuspended in MRS pH 6.2 (control cells) and MRS pH 3.5 respectively.

Oxidative stress conditions. Sensitivity of the cells to oxidative stress was assayed using dithiothreitol (DTT)* and diamide (SIGMA-ALDRICH) [151] and H_2O_2 (30%, FLUKA) as oxidative stressors. Overnight cultures, grown at 30°C in MRS broth, have been diluted and incubated at the same temperature until OD_{600} = 1.0; then, oxidative stressors has been added, DTT and diamide at final concentration 5Mm, H_2O_2 at final concentration 1mM, and growth continued until OD_{600} = 1.5.

*DTT (HSCH_2(CH(OH))_2CH_2SH), a reducing agent, was used to measure the oxidative potential of diamide [151].

3.2.2 RNA isolation and cDNA synthesis

During the experimental test bacterial cultures have been exposed to different stress conditions created by the acidification of medium and addition of oxidative factors.

Acidic stress conditions. RNA was extracted from control cells, grown at 30 °C at OD_{600} =1.5, and from cultivated cells in acidic conditions (pH 3.5) at 30 °C for 15', 60' and 120'.

Oxidative stress conditions. RNA was extracted from control cells, grown at 30 °C at OD_{600} =1.5, and from cultivated cells in the presence of oxidative stressor for

- 30', 60', 120' and 160' for commercial strains in presence of diamide;
- 30', 60' and 90' for autochthonous strains in presence of diamide;
- 30', 60' and 90' for all strains in presence of H_2O_2.

Total RNA from all the strains analyzed was extracted from all cultures exposed to different low pH value and oxidative stress conditions using the UltraClean Microbial Isolation Kit (Mo Bio Laboratories, USA) according to the manufacturer's instructions.

The RNA quantification was performed by NanoDrop instrument.

cDNAs were synthesized using the QuantiTect Reverse Transcription Kit (QUIAGEN), which includes a DNase treatment in order to eliminate the residual contaminant DNA fragment, and reverse transcription. Absence of chromosomal DNA contamination was confirmed by real-time PCR.

3.2.3 Quantitative Reverse Transcriptase PCR (RT-qPCR) and transcriptional profiling

RT-qPCR is a technique used to establish the expression level of the interest gene and allows to a DNA quantification absolute or relative (in the case of studies on gene expression). This technique uses fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR reaction. The quantification was carried out using SYBR Green, a DNA intercalator that binds to the double helix and emits fluorescence if appropriately excited. RT-qPCR was carried out on Applied Biosystem 7500 Real-Time PCR System. After twenty-fold dilution of cDNA, 5 µL were added to a 15 µL of a real-time PCR mixture (9 µl of Power SYBR Green PCR Master Mix (Applied Biosystems), 0,5 µl of each primer 100nM and 5 µl of RNase-free water). In each run a DEPC-water was used as negative control. Thermal cycling conditions were designated as follows: initial denaturation at 95°C for 5 min followed by 40 cycles at 95°C for 10 s, 60°C for 30 s, 95°C for 15 s.

A melting curve analysis was performed in order to verify the specificity of RT-qPCR. The efficiency of amplification was determined by running a standard curve with serial dilutions (ratio 1:10) of cDNAs. RT-qPCRs were performed in duplicate for each sample of cDNA.

For each measurement, a threshold cycle value (CT) was determined. This is defined as the number of cycles necessary to reach a point in which the fluorescent signal is first recorded as statistically significant above background.

In this study, the threshold value was determined with a baseline settled automatically [152,153]. However, the RT-qPCR requires the use of internal controls (usually known as housekeeping genes) for data normalization. The expression of these genes is often considered to fluctuate very little in comparison to other genes or the genes that are analyzed under determinate conditions, although in given conditions, their expression can vary considerably [154–156]. The constitutive *ldhD* and *tuf* genes were chosen as internal controls for these experiments [79,157,158].

The expression of stress responsive genes analyzed was investigated by the $2^{-\Delta\Delta Ct}$ method [159] using specific primer pairs reported and results were normalized by using the constitutive *ldhD* and *tuf* genes [79,157,160]. The expression of genes reported in **Tab.2** was monitored by RT-qPCR after inducing stress conditions.

Strains viability was also monitored by plate count analysis (data not shown).

Target gene	Function	Forward primer (5' → 3')	Reverse primer (5' → 3')
ldh	housekeeping gene	ACGCCCAAGCTGATGTTATC	AGTGTCCCACGAGCAAAGTT
tuf	housekeeping gene	TTACTATCAACACTGCCCACG	ACAACTAAGATCGCACCGTC
hsp1	heat shock protein	AGGTTGATGTCCCTGGTATTC	TTAAGACACCGTCAGCTTGG
hsp2	heat shock protein	CGGTGAAGTATGCTGACGAA	TTACCTTGCTATCCGCAAC
hsp3	heat shock protein	CGCGAGTGAACGTCAAACTG	ATCCGCAGCTGCCTTCTTT
dnaK	DnaK molecular chaperon	TCAACCGTGTCACCCAAGTA	TCCTTCAGTTGTGGCATTCA
groEL	GroEL molecular chaperon	ACCGGATTGAAGATGCTTTG	AACCAGCATTTTCAGCGATT
ctsR	CtsR, heat shock transcriptional regulator	AATTTGGTCGATGATGCTGAT	TAAGTCCCGGTCCGTTAATCC
hsp33	Hsp33 molecular chaperon	GTCGCGGGTAATGAGATGTT	GACCGTTCCCGTTGATTTTA
clpE	ClpE molecular chaperon	TTTACCAACCCCAGCTTCAC	GGCAAAATCGATCCAGTGAT
clpP	ClpP protease	TAGATTGCTAAGCCGGCAGT	ATGTTATCCGGTCCCATTGA
clpB	ClpB molecular chaperon	AGTTACCGGCGTCCATACTG	GACTCAAAGCCGTCCTCAAG
clpC	ClpC molecular chaperon	ATCCTTTCCTCGCGAATTTT	TGGCGTTCCTTCAGTCTTCT
ftsH	FtsH (protease and chaperone activity)	AAAACTGCAGAATCGACGCAATGGAC	GCTCTAGACGCTCATAACCGAATTAACG
trxA2	Encoding a thioredoxin	ATGGTCGCAGCAACTACTG	TTATAGATATTGAGCTAAAGTTTG
trxB1	Encoding thioredoxin reductase	ATGGCAAAGAGTTACGACG	TTCAGAACCAGTCCCAATGAC

Tab.2 Genome sequences of genes used in this study

3.2.4 Statistical analysis

Data represents the mean ± standard deviation (SD) of two biological experiments and three technical replicates. A one-way analysis of variance (ANOVA) was performed to test whether there was a significant evolution in gene expression levels during stress using the statistical software PAST version 2.17C [161]. P values <0.05 were considered as statistically significant.

3.3 Monitoring the effect of *L. plantarum* strains on the evolution of VOCs during winemaking process using PTR-MS

3.3.1 Microbial Strains and Growth Conditions

A commercial strain *Saccharomyces cerevisiae* DV10 (Lallemand Inc. produces) and six *L. plantarum* strains already characterized for their pro-technological potentials and available in the strains collection of the Industrial Microbiology laboratory, University of Foggia (Italy) were tested for VOCs measurement during fermentation processes. Bacterial strains identified as F, G, H, I and L have been isolated from wine while the strain M has been isolated from human saliva and is usually used as a microbial model in the study of host-microbe interactions, due to its ability to survive gastrointestinal (GI) transit. *L. plantarum* strains have been cultivated in MRS broth (pH 6.2) while the yeast has been grown in YPD broth for 24h at 30°C. Before the measurement of bacteria VOCs during AF and MLF, the growth curves of the tested strains have been built to monitoring VOCs during bacterial grown.

Overnight pre-grown strains were inoculated in MRS broth for 24h. After an overnight pre-culture at 30 °C in MRS broth the strains were inoculated into 3 mL of medium inside a 20 mL vial and closed with a screw cap with a silicone/PTFE septum for 32 hours and with 8 cycles of measurements.

3.3.2 VOCs analysis by PTR-ToF-MS

In order to characterize the VOCs released during fermentation in the headspace of wine, and then to assess sensorial quality, a commercial PTR-ToF-MS (Proton Transfer Reaction- Time Of Flight- Mass Spectrometer) 8000 apparatus from IONICON ANALYTIK GmbH (Innsbruck, Austria), has been used in its standard configuration (V mode). The ionization conditions in the drift tube involved 110 °C drift tube temperature, 2.30 mbar drift pressure and 550 V drift voltage. This led to an *E/N* ratio of about 140 Townsend (1 $Td = 10-17$ cm2 V−1 s−1) where *E* corresponds to the electric field strength and *N* to the gas number density. The inlet line consisted of a PEEK capillary tube (internal diameter 0.04 inches) heated at 110 °C. The inlet flow was set at 40 sccm.

Direct headspace air injection into the PTR-ToF-MS is difficult in the case of samples with high ethanol concentration in a headspace. To remedy these problems the PTR-ToF-MS was also coupled with a fastGC add-on (IONICON ANALYTIK GmbH, Innsbruck, Austria) as it is described by Romano et al. 2014. The polar capillary column [MXT®-WAX (Siltek® - treated stainless steel), 6 m, 0.25 mm ID, 0.25 μm df] was maintained under pure N2 with a constant flow rate of 4 sccm. Sample headspace air was injected with the flow rate of 150 sccm

into a fastGC sampling loop for 4 s, guaranteeing its total filling. The chromatographic measurement was registered for 130 s with the thermal ramp from 40 to 220 °C which resulted the thermal gradient of 2.25 °C/s. Between two measurements an interval of 100 s was set to prevent memory effects. The acquisition time was decreased in order to obtain a higher (retention) time resolution in the chromatograms. Each spectrum consisted of 1000 acquisitions lasting 35 µs each, resulting in a time resolution of 210 ms.

3.3.3 Vinification assay

Fermentations have been performed in bottles containing 0,400 L of Apulian grape red must (previously pasteurized) a 20°C.

All samples have been inoculated with the yeast. Bacteria have been inoculated either with yeast (co-inoculum) and after the completion of alcoholic fermentation (sequential inoculum) to obtain an initial cell density of 1×10^7 CFU/mL. The two different moment of inoculation have been chosen to verify the behavior of the selected bacteria introduced in a winemaking phase where there are not still hostile situations (high alcoholic content or low temperature) characteristic of alcoholic fermentation. In this way it is possible to obtain an adaptation of the bacteria directly in the must-wine environment in favor of their development. Yeasts/bacteria co-inoculation represents a novel strategy in industrial wine fermentations which could lead to enhance the quality and safety of product/beverage.

The experimental tests were conducted for 20 days.

Fermentations were carried in three biological replicates. 3 mL of samples were put in 20 mL vials with silicone/PTFE septum. All the tubes were sampled in triplicate. Samples were analyzed in a random order to minimize possible memory effects.

The experimental design consisted of 36 samples (6 different strains × 6 replicates), 5 vials with must inoculated only with yeast, 6 vials with must only and 1 vial with blank.

Measurements were performed in an automated way by using a multipurpose GC automatic sampler (Autosampler, Gerstel GmbH, Mulheim am Ruhr, Germany) as described in Makhoul et al. 2014.[139] Each sample was measured once a day during alcoholic fermentation and twice a week during malolactic fermentation, which was sufficient for the accumulation of VOCs in the headspace of the vial according to preliminary tests. A gas calibration unit (GCU, IONICON ANALYTIK GmbH, Innsbruck, Austria) was employed to generate zero air for flushing sample headspace in order to prevent the microbiological and chemical contamination of a sample headspace. Due to high ethanol concentration in the sample headspace, in order to prevent primary ion depletion and ethanol cluster formation [146] an Argon dilution system was applied

after headspace sampling. The dilution ratio was 1 part of headspace to 3 parts of Argon. Sample headspaces were flushed with a flux of 40 sccm of Ar.

PTR-MS analysis is very rapid and can reach detection limits as low as 0.1 parts per trillion by volume. The coupling of PTR-MS with Time-of-Flight (ToF) mass analyzers further improved time and mass resolution and allows the monitoring of VOCs during food fermentations. The automation used for the experiments will be the same as the one described in previous works by Makhoul et al. and Campbell-Sills et al. [139,162] .

3.3.4 Data processing and statistical analysis

Data processing of PTR-ToF-MS spectra with or without fastGC included dead time correction, calibration of mass spectral data and peak extraction steps performed according to a procedure described by Cappellin et al.[163]. The baseline of the mass spectra was removed after averaging the whole measurement and peak detection and peak area extraction was performed by using a modified Gaussian to fit the data [164]. Peak intensity in ppbv (part per billion by volume) was estimated using the formula described by Lindinger et al. using a constant value for the reaction rate constant coefficient ($k = 2 \times 10{-9}$ cm3/s) for H_3O^+ as primary ion [138]. This introduces a systematic error for the absolute concentration for each compound that is in most cases below 30% and could be accounted for if the actual rate constant coefficient is available [144].

All data detected and recorded by the PTR-ToF-MS were processed and analyzed using MATLAB (MathWorks, Natick, MA) and R (R Foundation for Statistical Computing, Vienna, Austria).

4. RESULTS AND DISCUSSION

4.1 Strains, DNA extraction and quantification

The six *L. plantarum* strains were routinely grown in MRS broth (pH 6.2) at 30 ºC and DNA was extracted at middle exponential phase (OD_{600} aroud 1) by using the Ultraclean Microbial DNA Isolation Kit (MoBio, according to the manufacturer's instructions. DNA quantification was performed using a NanoDrop system, and a suitable yield for genome sequence was observed both in terms of quality and quantity.

Calculations of the relationships between readings at $\lambda = 260$ / $\lambda = 280$ and $\lambda = 260$ / $\lambda = 230$ gave values ranging between 1.8 and 2.1, a range in which a good extract of pure DNA is estimated.

Gel electrophoresis is a powerful means for revealing the condition (including the presence or absence) of DNA in a sample. Impurities, such as detergents or proteins, can be revealed by smearing of DNA bands. RNA, which interferes with 260 nm readings, is often visible at the bottom of a gel.

4.2 Genome sequencing and assembly

DNA library construction and genome sequence of reference strains were performed by Macrogen Inc. (South Korea) using a Miseq platform.

Genome analysis, assembly and functional annotation were performed in collaboration with Lallemand Inc. (Toulosue, France) using bioinformatics tools such as the RAST (Rapid Annotation using Subsystem Technology) and the COG (Cluster of Orthologous Groups) database.

Lactobacillus plantarum E and *L. plantarum F* strains whole genome sequence has been completed and the main parameters are shown in the following table (**Tab.3**).

Sample ID	Total read bases (bp)	Total reads	GC (%)	AT (%)	Q20 (%)	Q30 (%)
E	4,254,852,250	42,127,250	43.516	56.48	95.049	91.934
F	3,234,427,076	32,024,476	43.280	56.72	95.172	92.120

Tab 3. Completed genome sequence of *L. plantarum* strain E and F

Sample ID: Sample name.

Total read bases: Total number of bases sequenced.

Total reads: Total number of reads. In Illumina paired-end sequencing, read1 and read2 are added.

GC (%): GC content.

AT (%): AT content.

Q20 (%): Ratio of reads that have phred quality score of over 20.

Q30 (%): Ratio of reads that have phred quality score of over 30.

4.3 Stress tolerance

Real-time RT-PCR assays were performed to measure the relative quantity of genes transcripts encoding for different metabolic and cell maintenance functions. These genes have been selected because they are highly expressed in *L. plantarum* under different stress conditions [79].

Genes are considered to be up- or down-regulated if their relative expression levels are at least two-fold higher or lower respectively, compared to the control sample [155].

4.3.1 Acidic stress

Tolerance to the typical pH of the wine represents an important feature for the oenological LAB, as it allows a better development of MLF led by these microorganisms. Furthermore, the viability of LAB in an acidic environment is a key factor for those with potential probiotic properties, as to perform their beneficial effect in the gut they must survive to the harsh conditions of the stomach. For this purpose, the ability to tolerate low pH values (pH 3.5) by *L. plantarum* strains analyzed was investigated.

Relative expression levels of 12 different stress responsive markers previously characterized and usually induced by stress commonly encountered in food (such as low pH value, high ethanol concentration, sulphite, etc.) were evaluated on *L. plantarum* strains. Exponential-phase cultures have been exposed to shift to pH 3.5. Total RNAs have been isolated before and at 15', 60' and 120'after stress treatment. A quantitative real time PCR approach and the *ldhD* and *tuf* genes as control genes were used.

The expression of stress responsive markers was investigated at low pH and the following conclusions were reported:

• No significate differences in viability (as CFU/ml) between control and stress induced strains were detectable (data not shown)

• The expression of the stress genes analyzed at low pH was observed to be strains-specific (differences between strains).

• Among all the stress genes analyzed, an induction higher than 2 –fold (arbitrary choice for gene induction) for *hsp 2* gene was observed in all the strains analyzed, although differences between strains were clearly visible.

• Within all the commercial strains analyzed, the expression of *hsp 2* gene was higher in *L. plantarum* strain *C.* (see **Fig.7**)

Fig 7. Example of relative gene expression (RGE) in a) *L. plantarum* A, b) *L. plantarum* B, c) *L. plantarum* C, and d) *L. plantarum* D, e) *L. plantarum* E and f) *L. plantarum* F under low pH stress, of genes analyzed determined by quantitative real-time RT-PCR. The relative levels of expression were calculated normalizing the levels of *ldhD* gene, and the respective gene expression level under unstressed condition. The results are represented with their standard deviations indicated by vertical bars.

a)

GroEL

CtsR

hsp33

ClpE

ClpB

ClpC

ClpP

b)

ClpB — RGE vs pH 3.5 (0 min, 15 min, 60 min, 120 min), legend B

ClpC — RGE vs pH 3.5 (0 min, 15 min, 60 min, 120 min), legend B

ClpP — RGE vs pH 3.5 (0 min, 15 min, 60 min, 120 min), legend B

c)

hsp1 — RGE vs pH 3.5 (0 min, 15 min, 60 min, 120 min), legend C

hsp2 — RGE vs pH 3.5 (0 min, 15 min, 60 min, 120 min), legend C

hsp3 — RGE vs pH 3.5 (0 min, 15 min, 60 min, 120 min), legend C

DnaK — RGE vs pH 3.5 (0 min, 15 min, 60 min, 120 min), legend C

d)

hsp1

hsp2

hsp3

DnaK

GroEL

CtsR

hsp33

ClpE

ClpB — pH 3.5

ClpC — pH 3.5

ClpP — pH 3.5

e)

hsp1 — pH 3.5

hsp2 — pH 3.5

hsp3 — pH 3.5

DnaK — pH 3.5
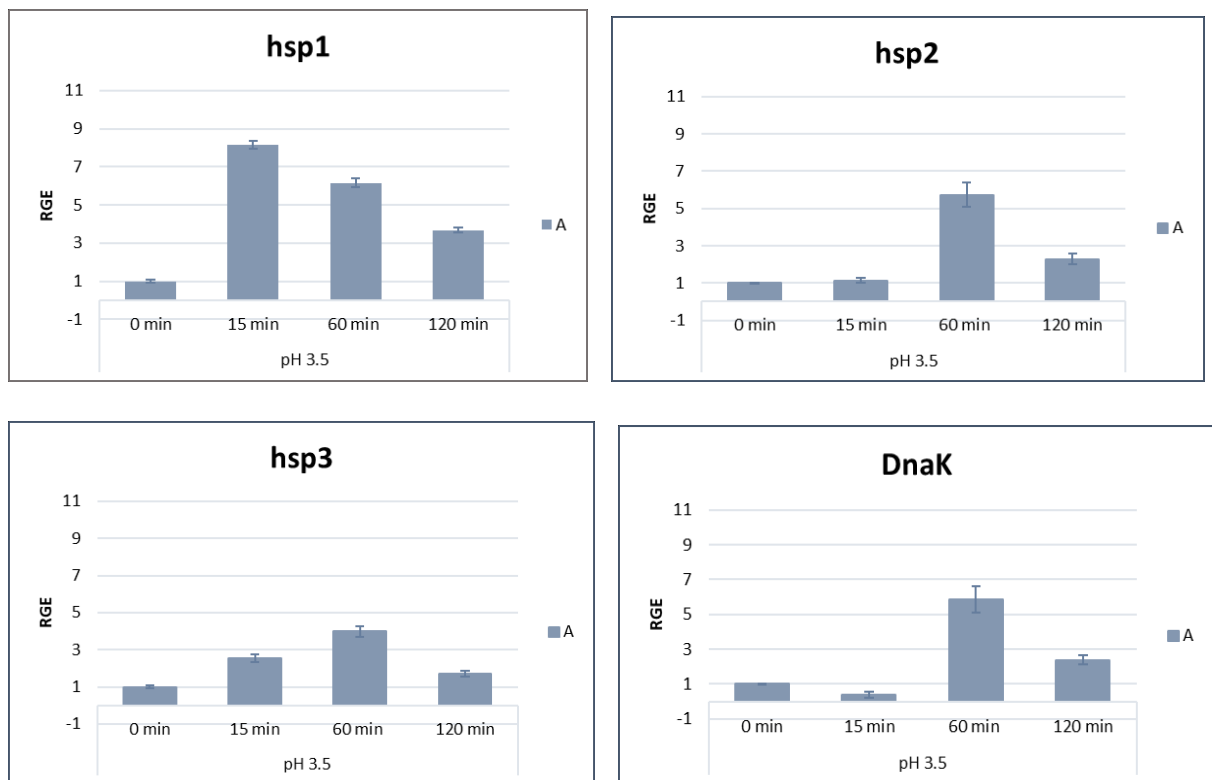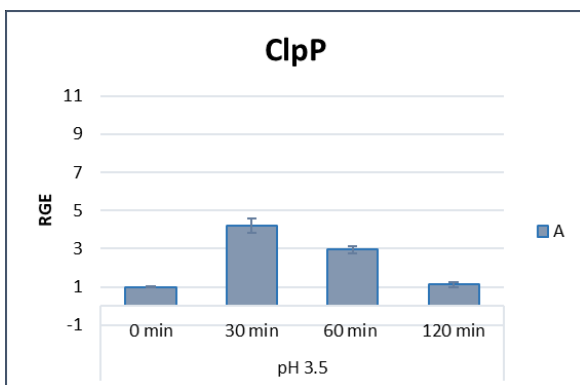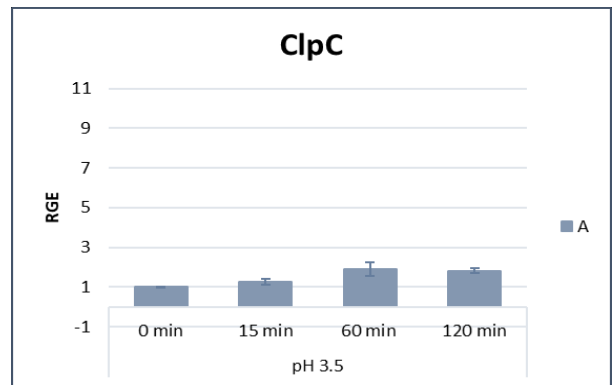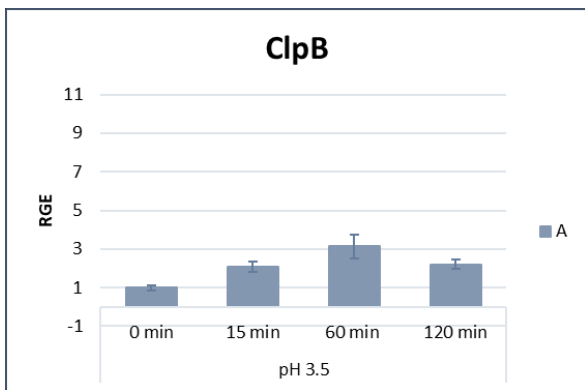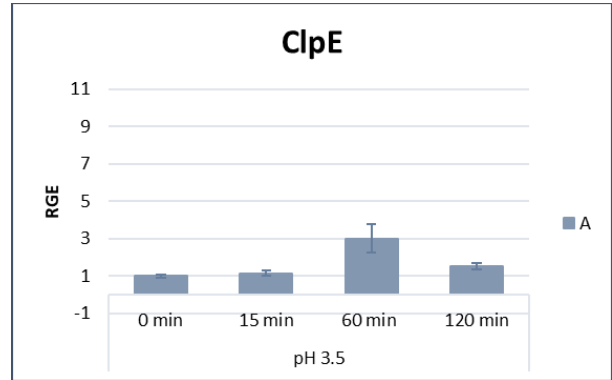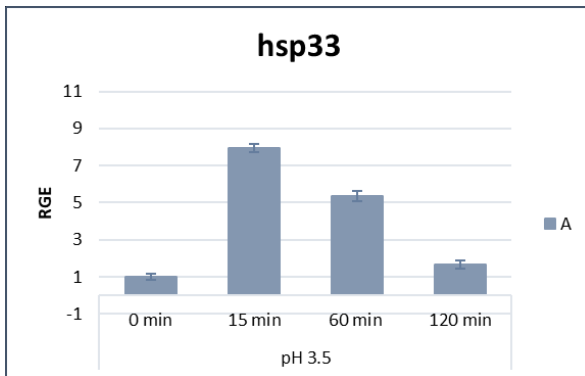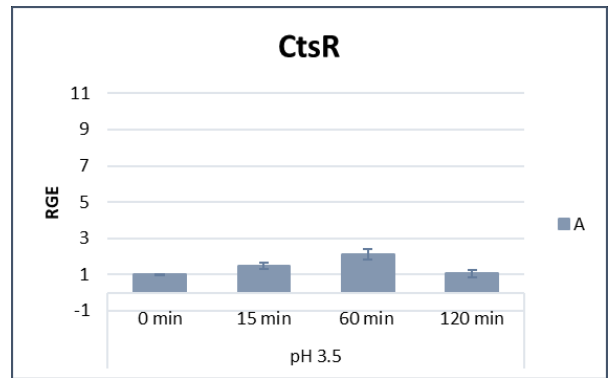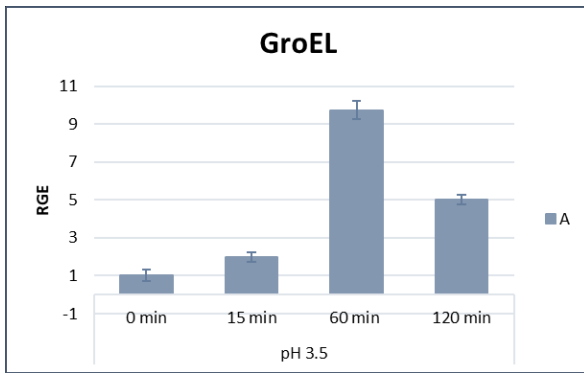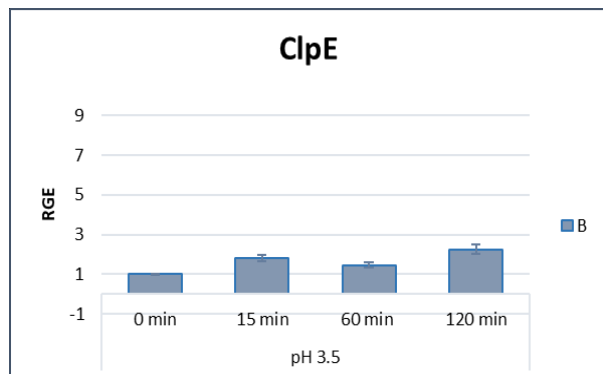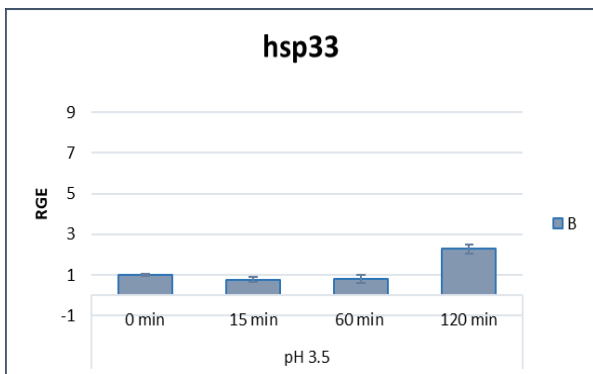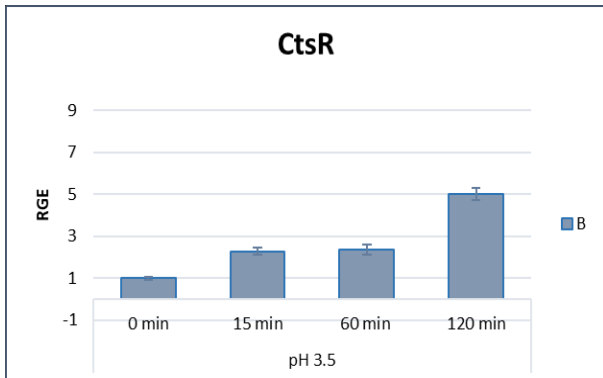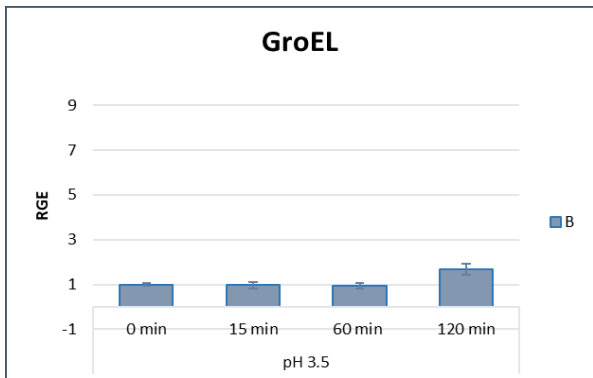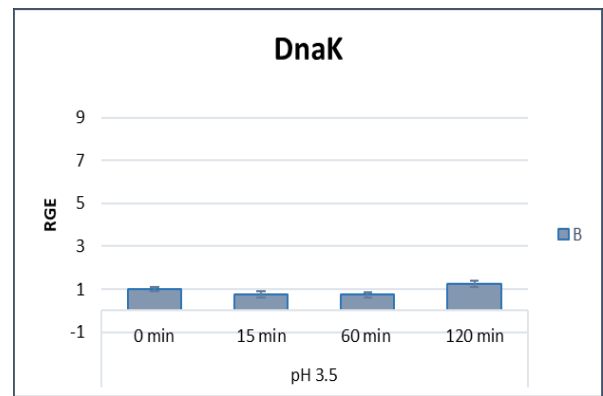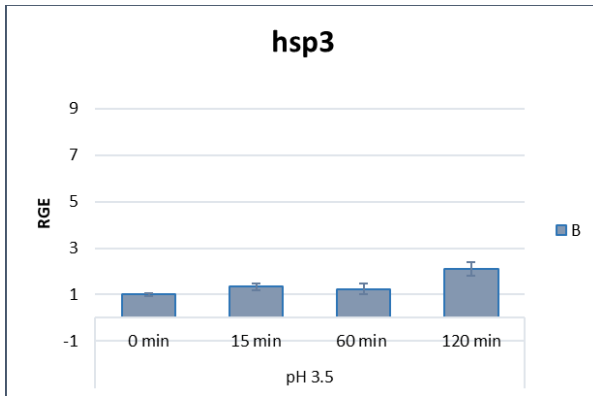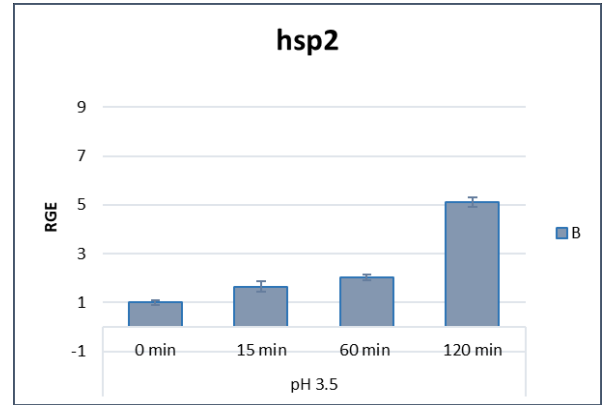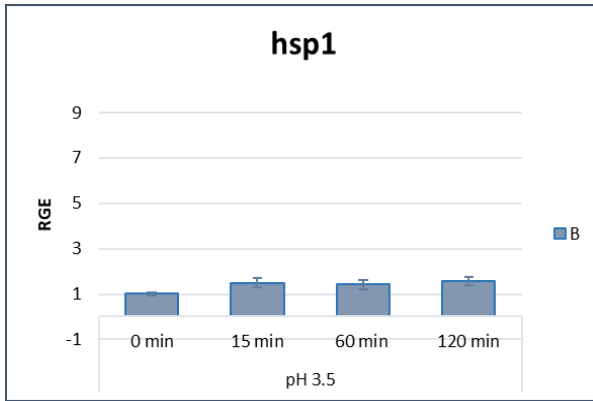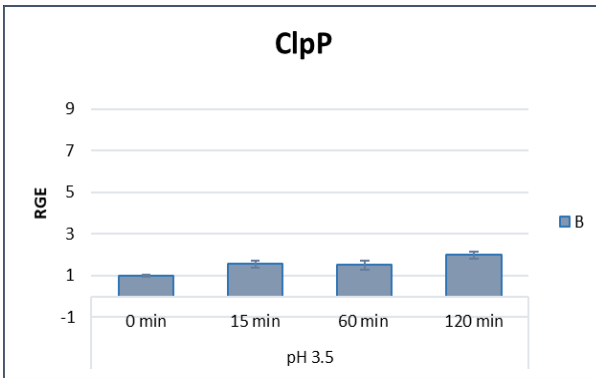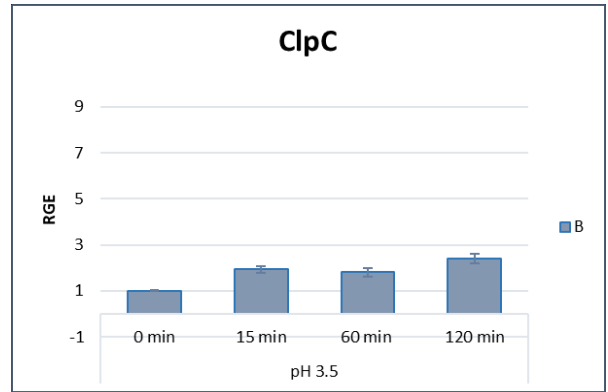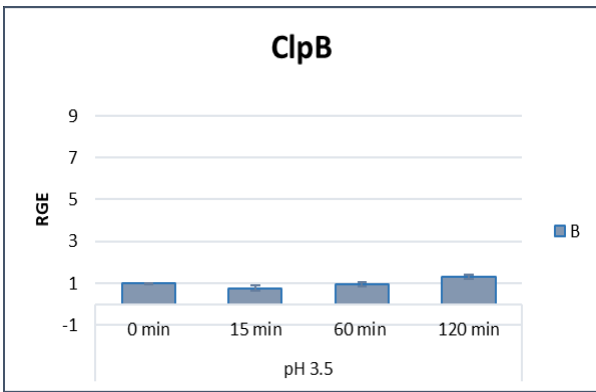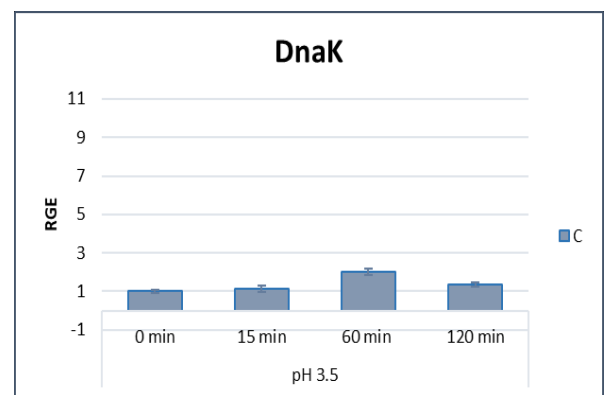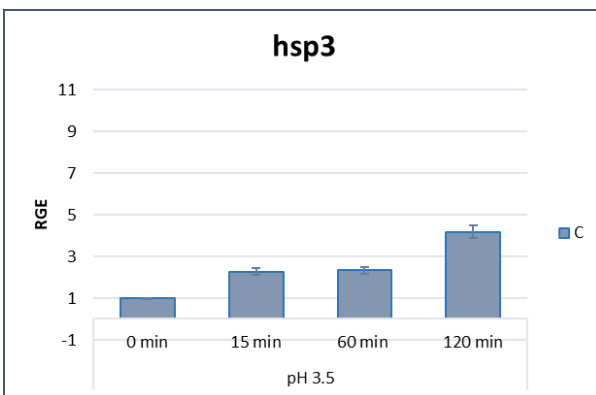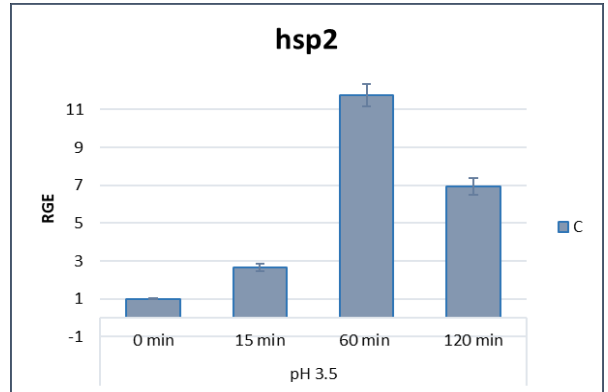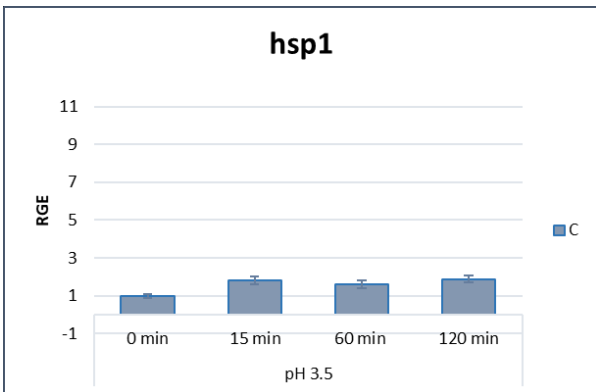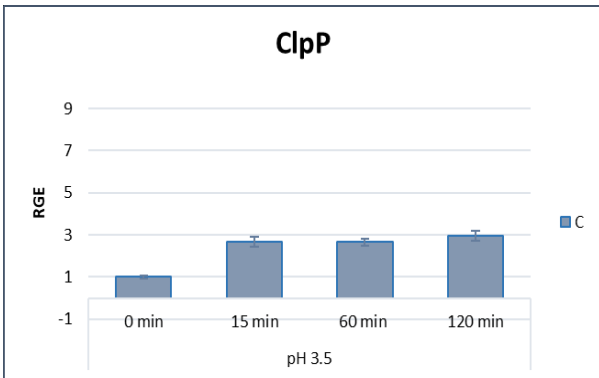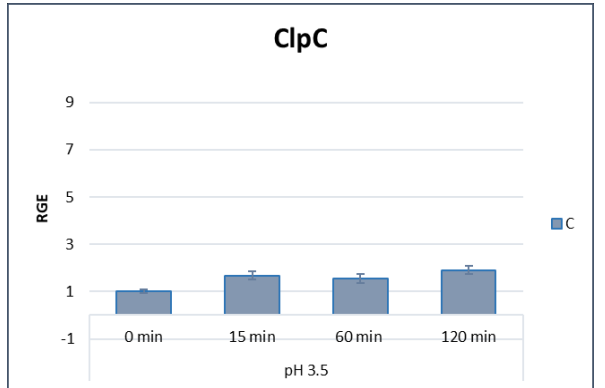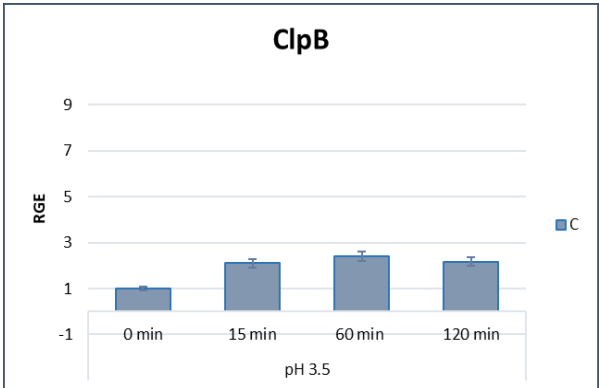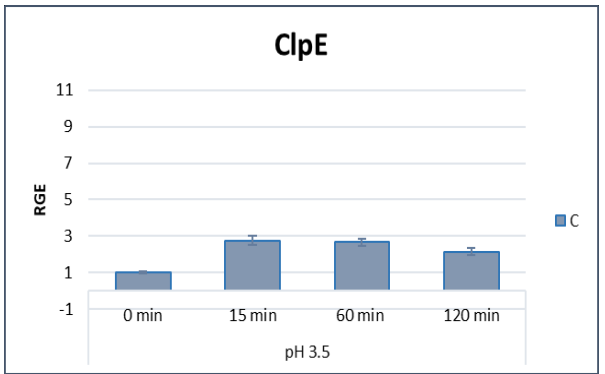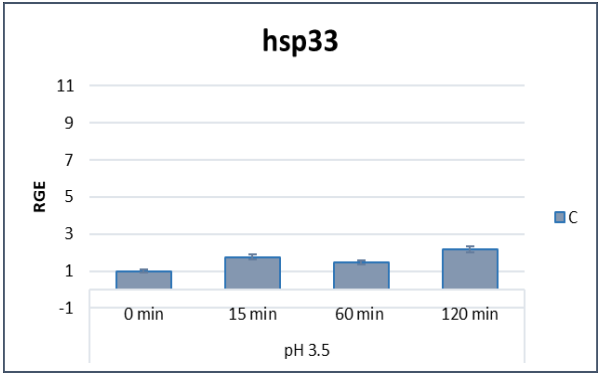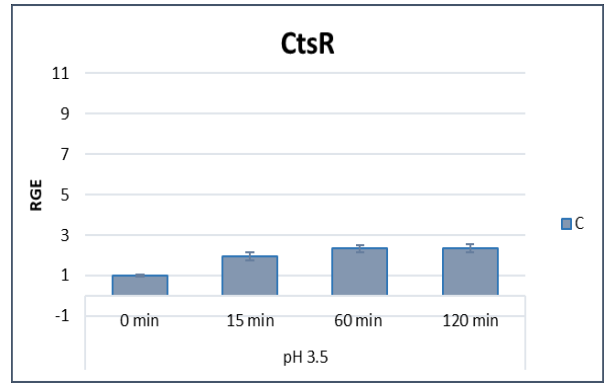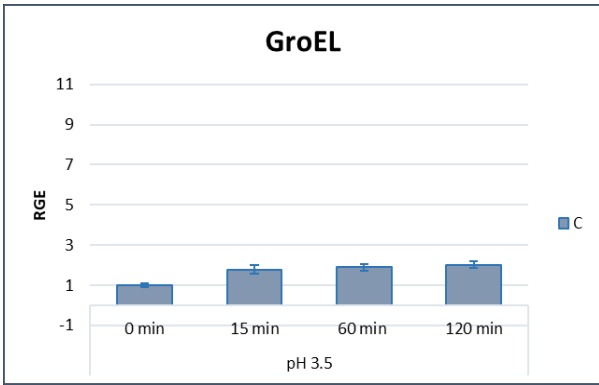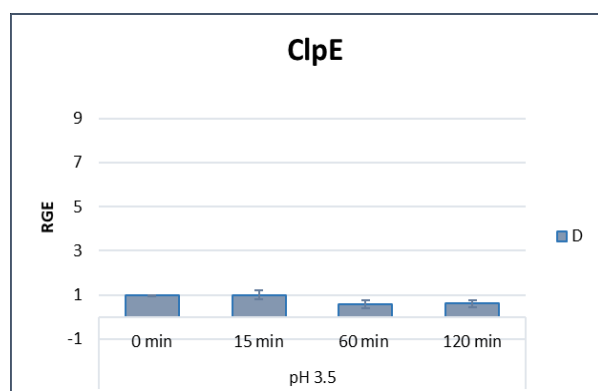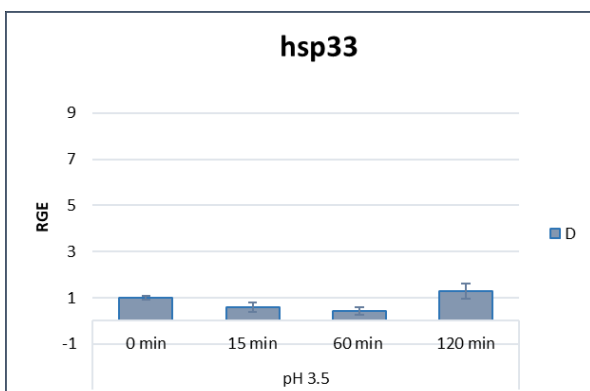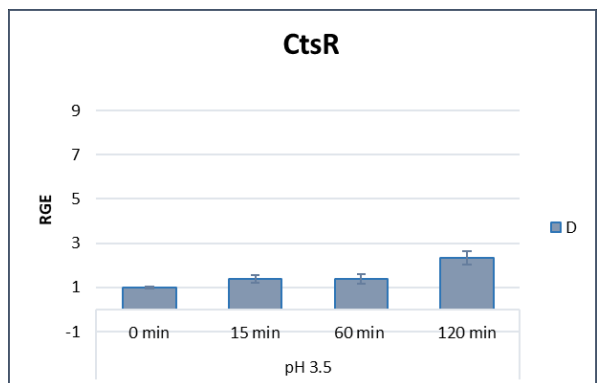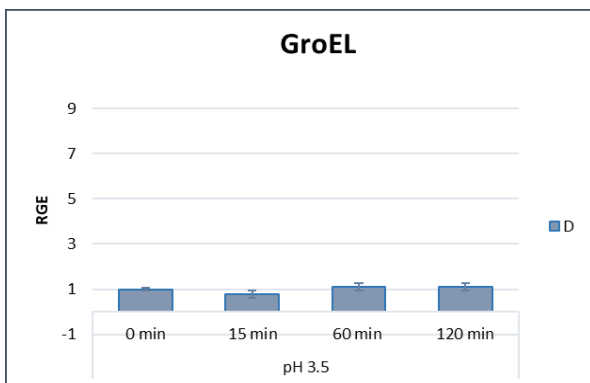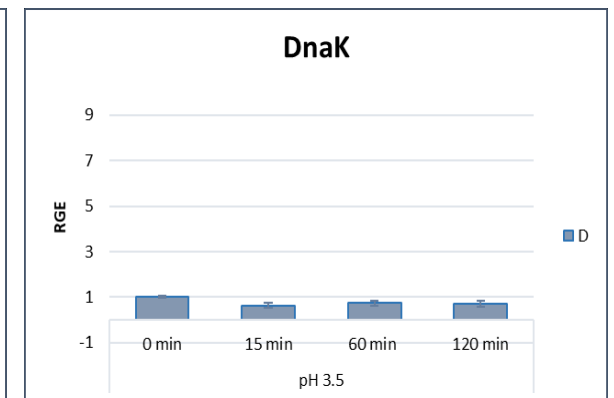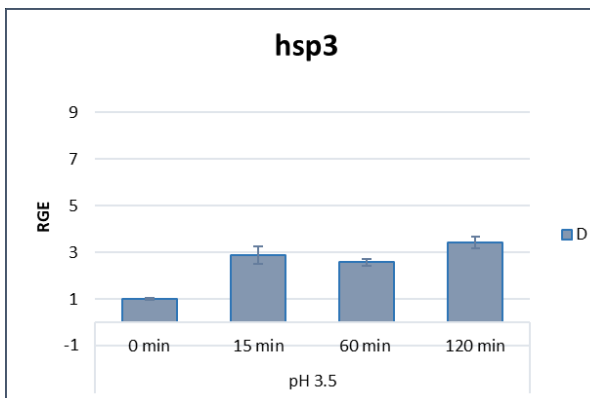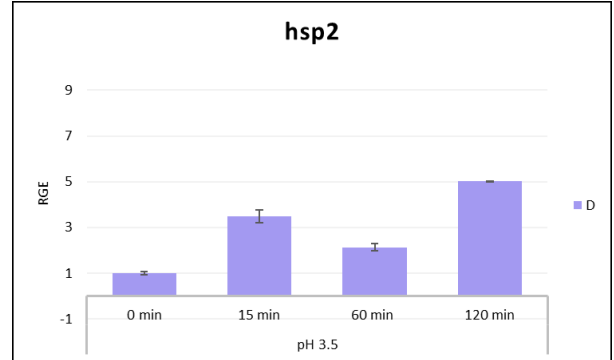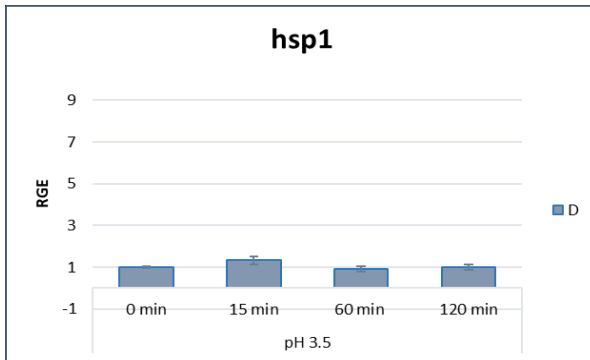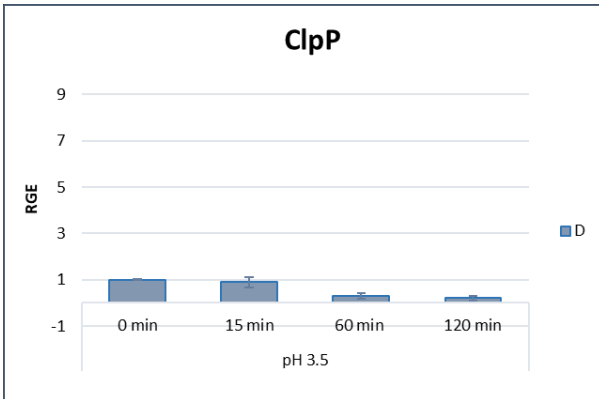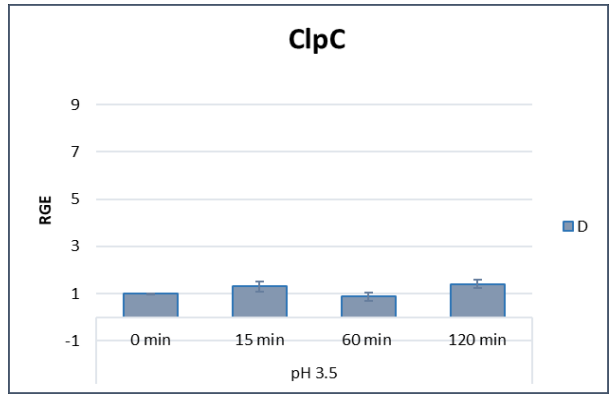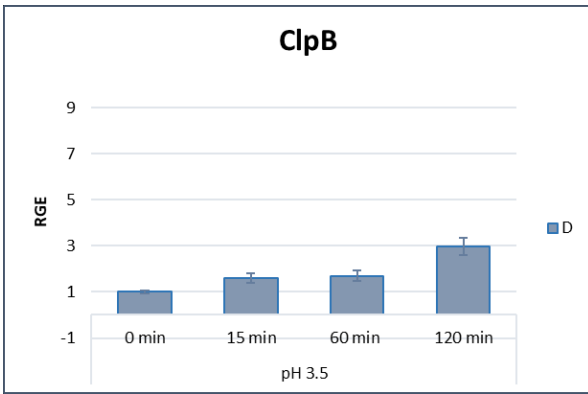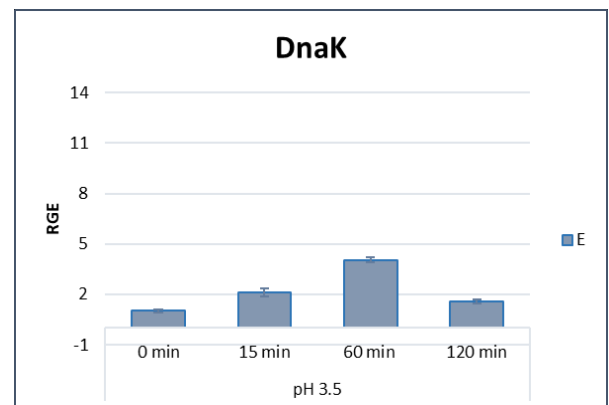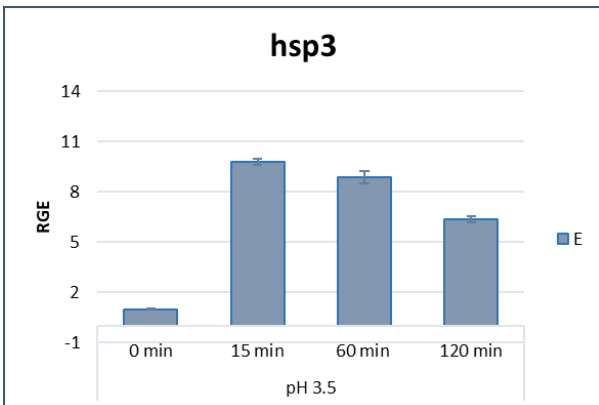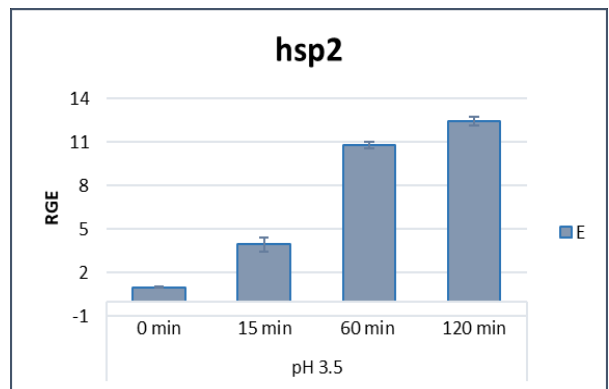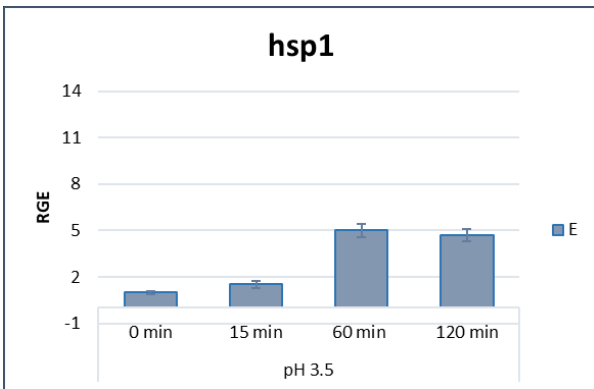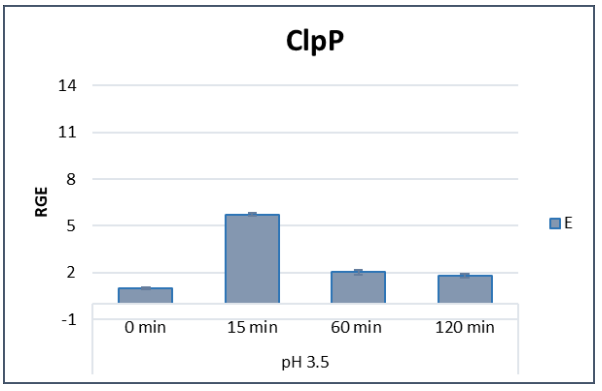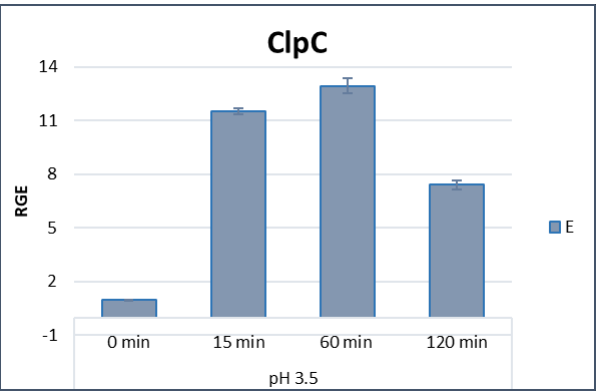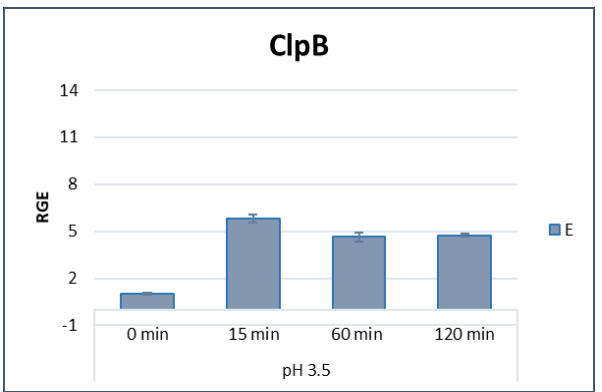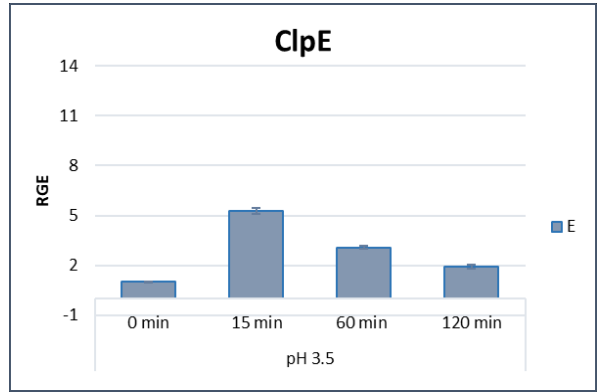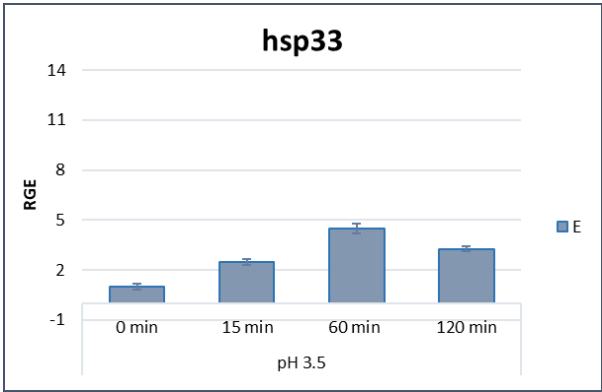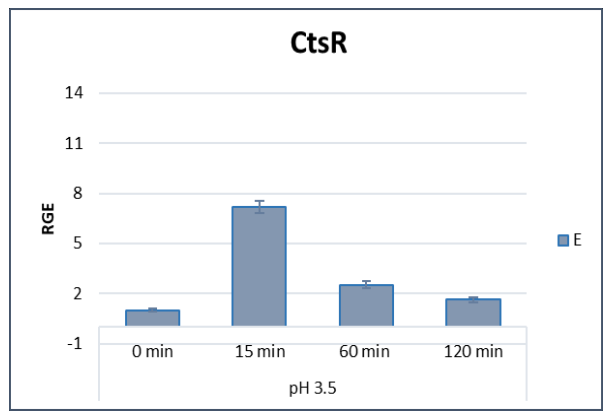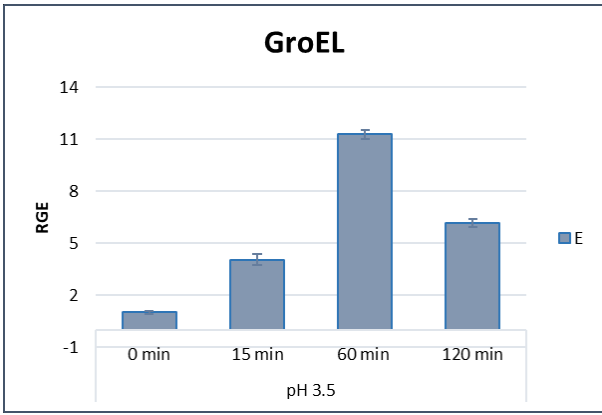
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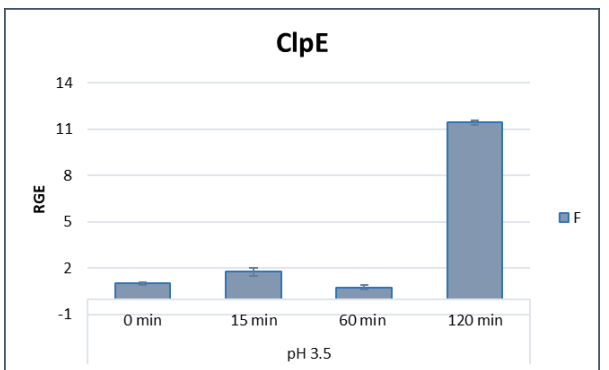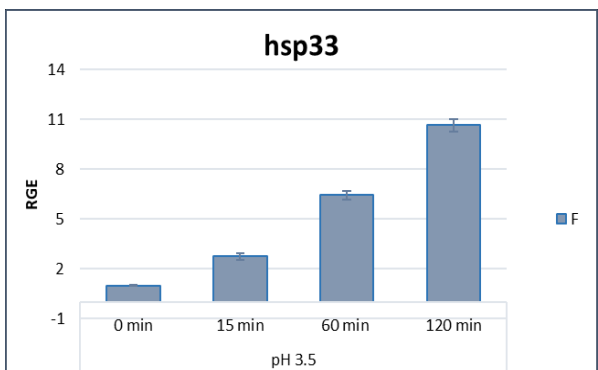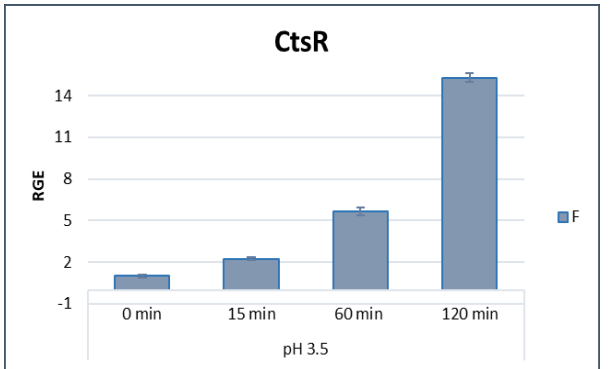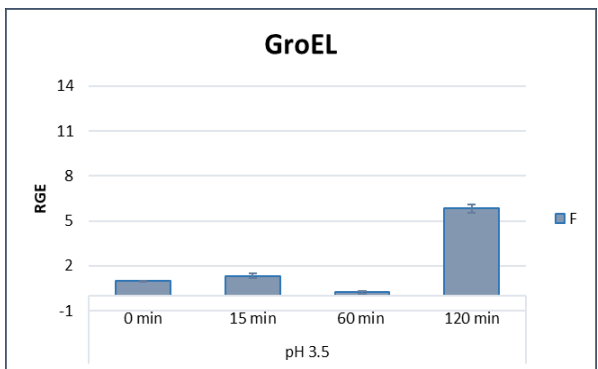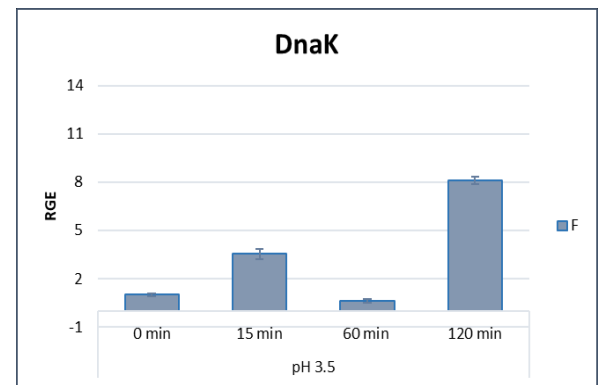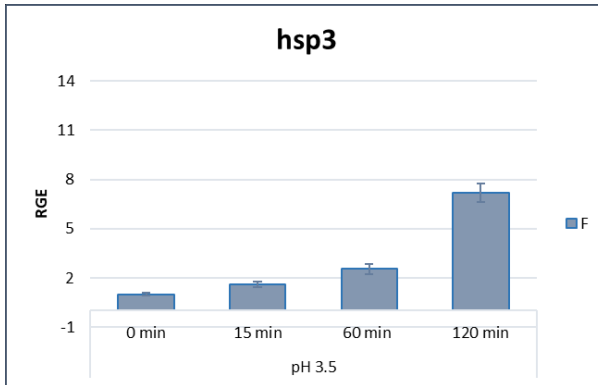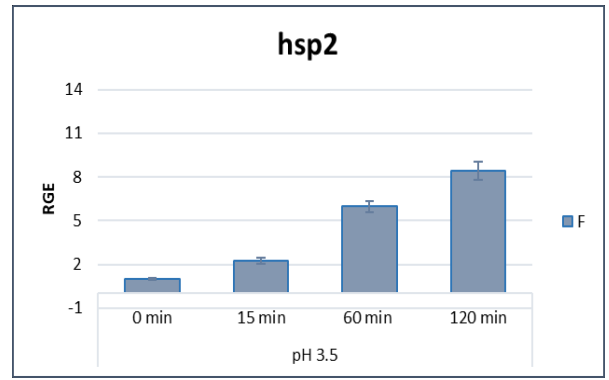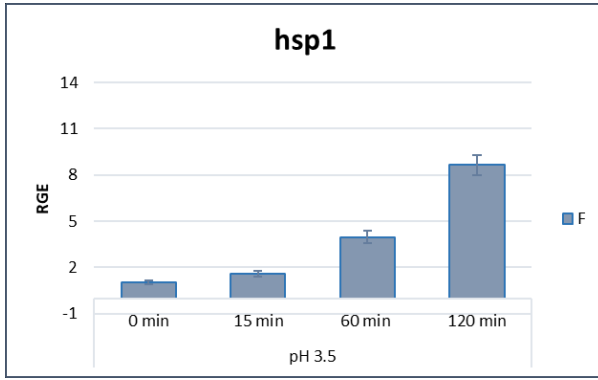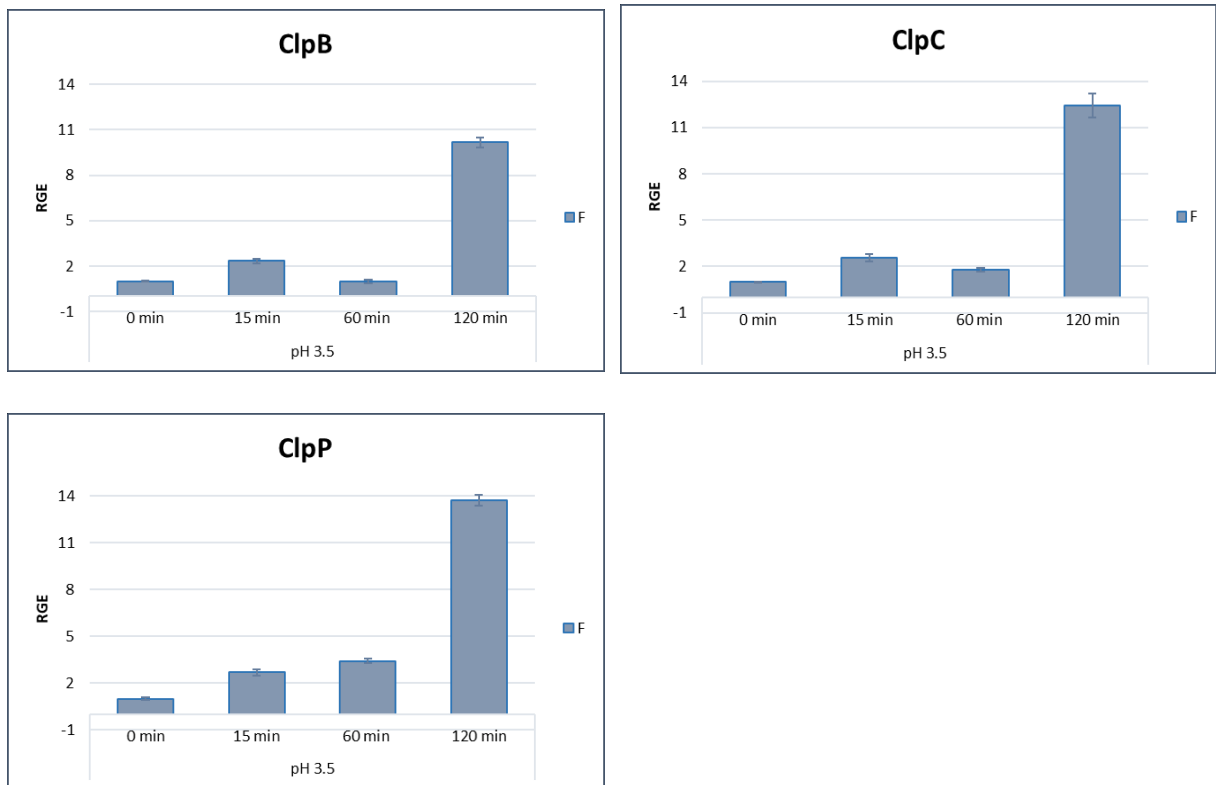
4.3.2 Oxidative stress

L. plantarum resistance was tested in presence of diamide and H_2O_2.

Oxidative stress conditions. The effect of oxidative stress due to diamide and H_2O_2 on *L. plantarum* strains analyzed was investigated at several time described in the materials and methods section, and the relative expression levels of 14 different stress response genes were monitored (Figures 2 and 3). Overall, the results reported suggest that:

• No significative differences in viability (measured as CFU/ml) between control and stress-induced strains were observed (data not shown).

• The expression of the stress responsive genes during oxidative stress and in all the strains analyzed, was lower compared to that previously observed during low pH stress.

• The expression of the stress genes analyzed during oxidative stress was, in general, linked to the strain analyzed and reported in **Fig.8** (diamide) and **Fig.9** (H_2O_2).

Fig 8. Example of relative gene expression (RGE) in a) *L. plantarum* A, b) *L. plantarum* B, c) *L. plantarum* C, and d) *L. plantarum* D, e) *L. plantarum* E and f) *L. plantarum* F under oxidative stress caused by diamide, of genes analyzed determined by quantitative real-time RT-PCR. The relative levels of expression were calculated normalizing the levels of *ldhD* gene, and the respective gene expression level under unstressed condition. The results are represented with their standard deviations indicated by vertical bars.

a)

b)

c)

GroEL

CtsR

hsp33

ClpE

ClpB

ClpC

ClpP

trxA2

d)

e)

ClpB

ClpC

ClpP

trxA2

trxB1

ftsH

f)

hsp1

hsp2

hsp3

DnaK

GroEL

CtsR

hsp33

ClpE

ClpB

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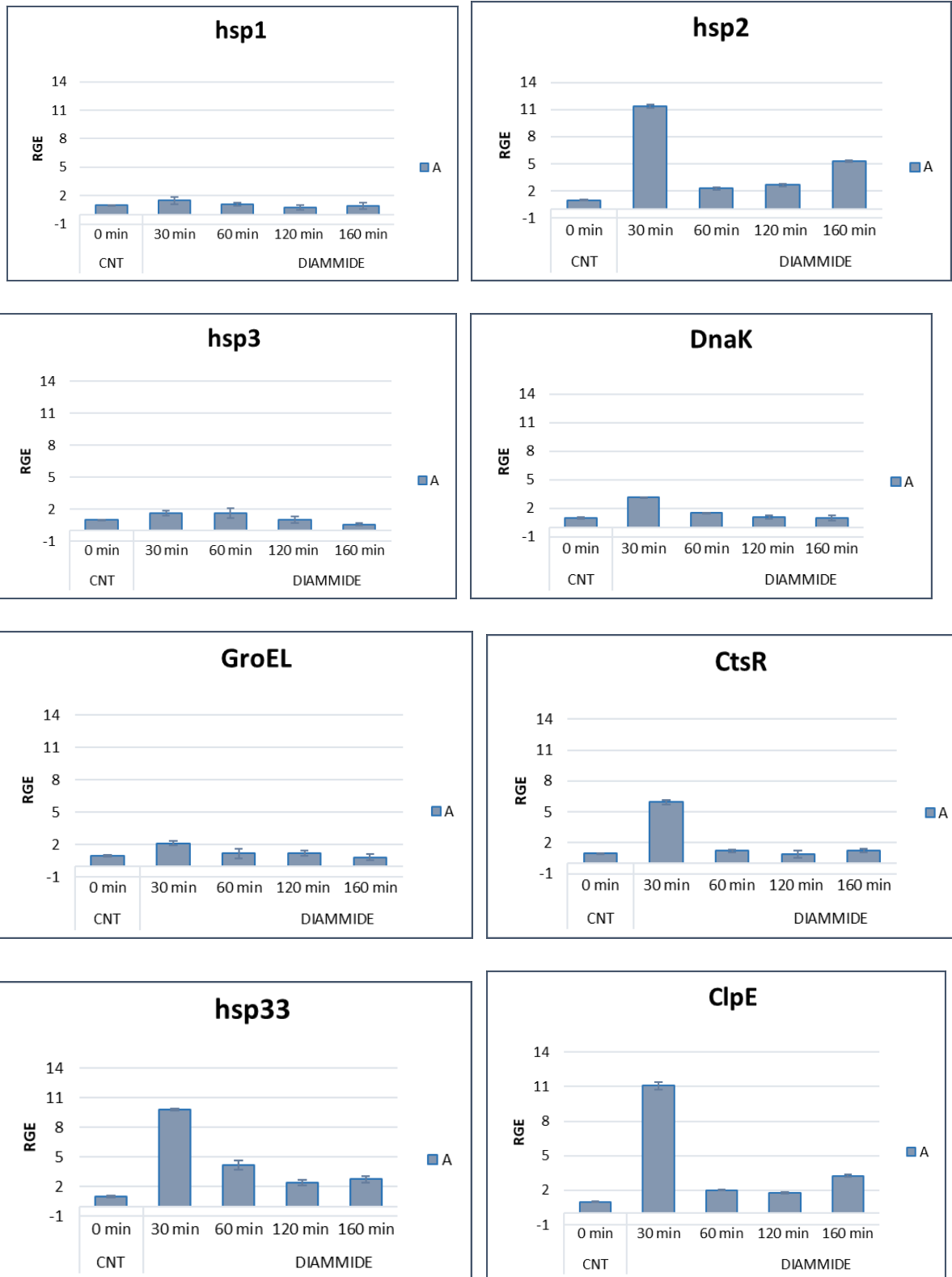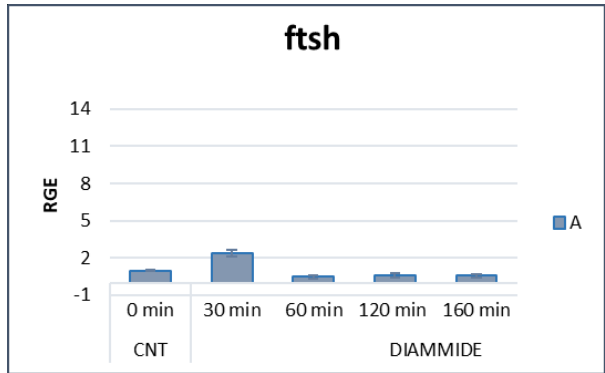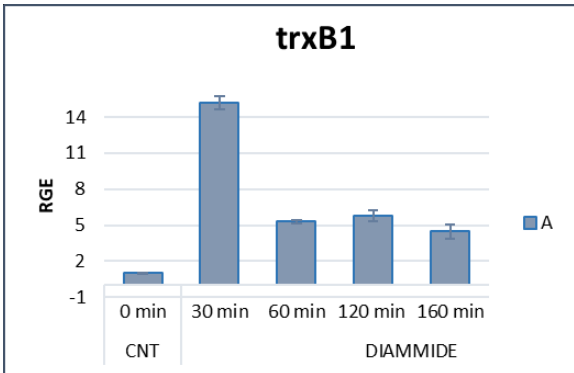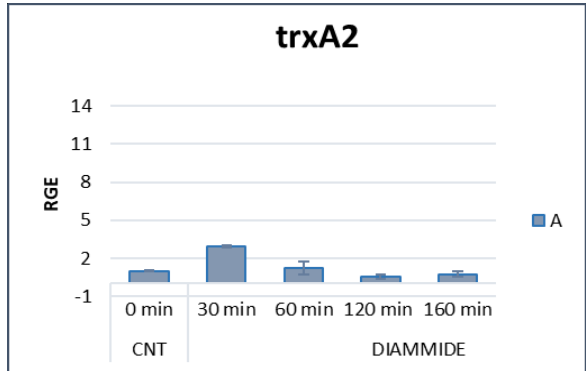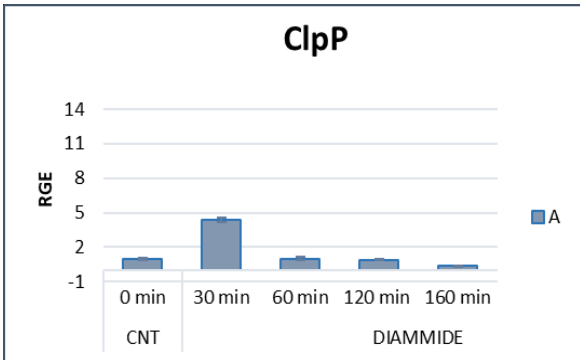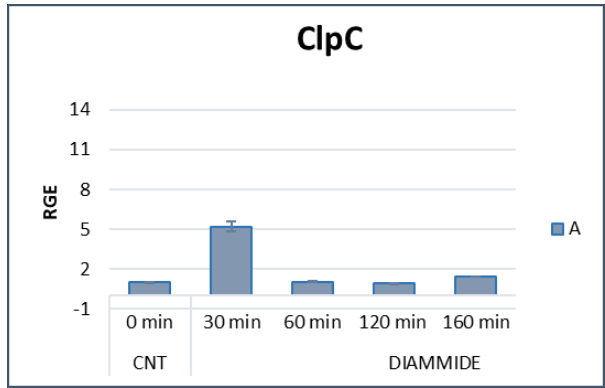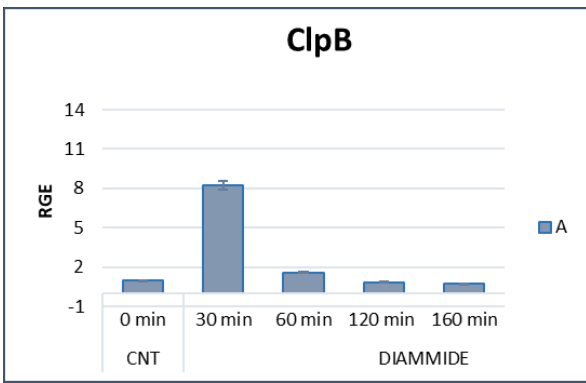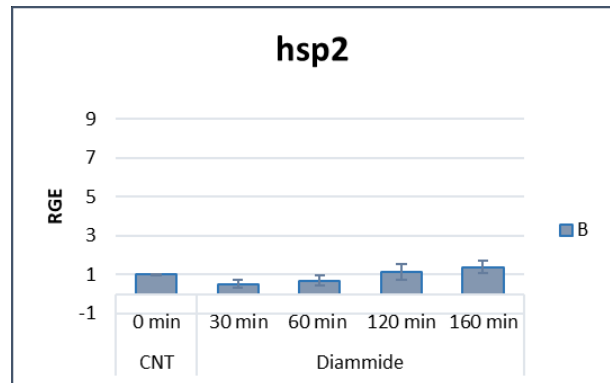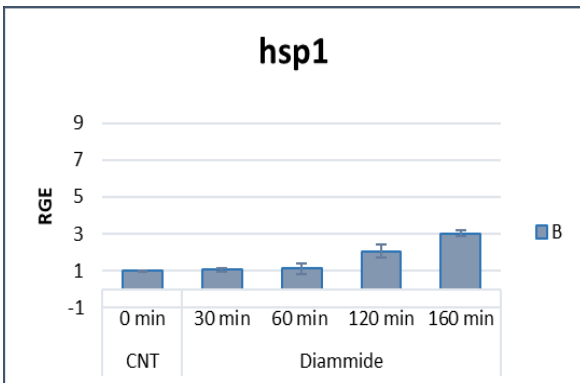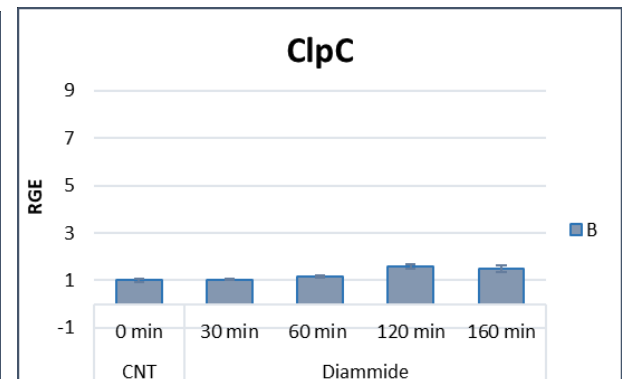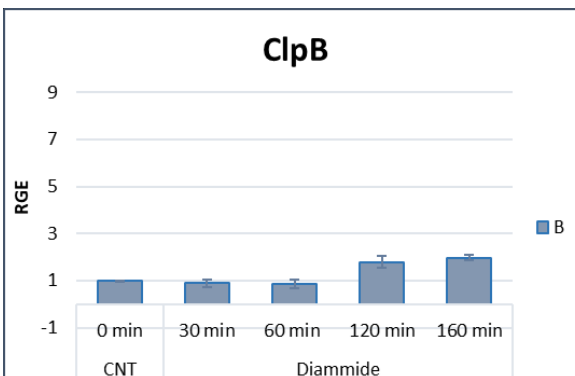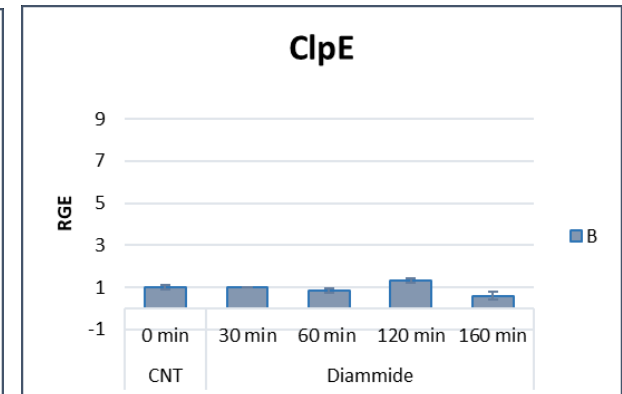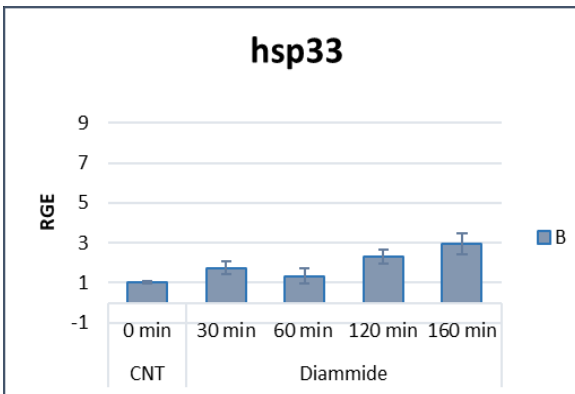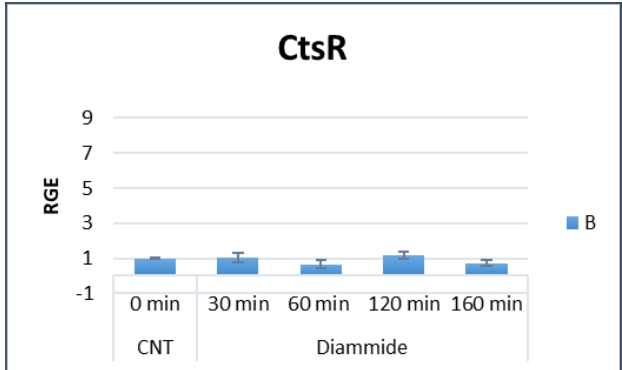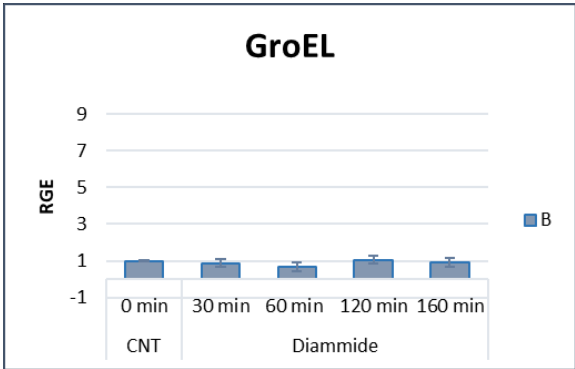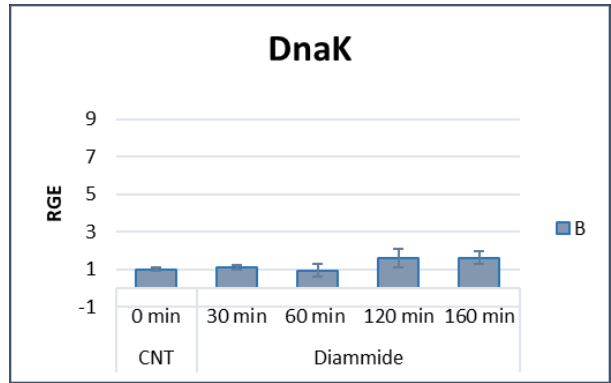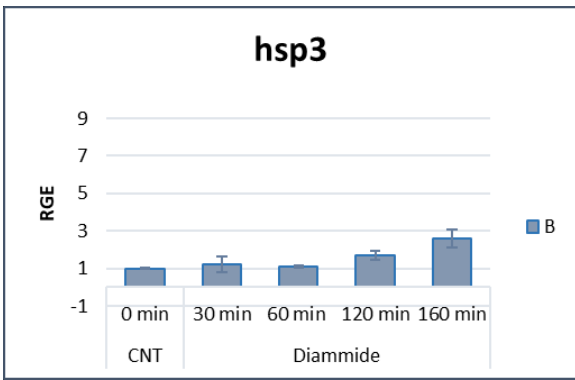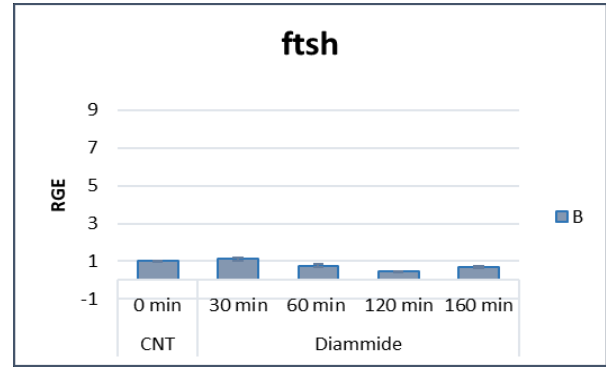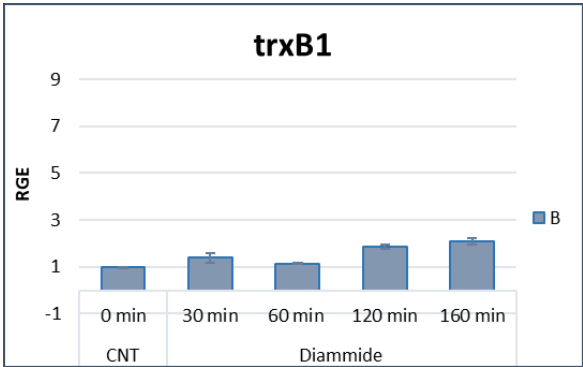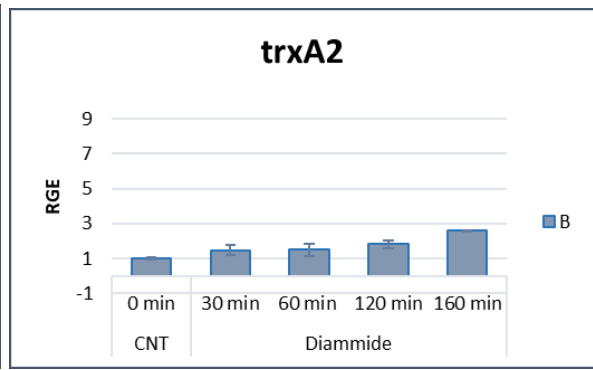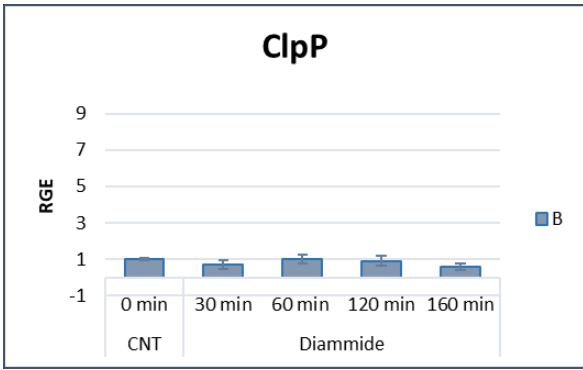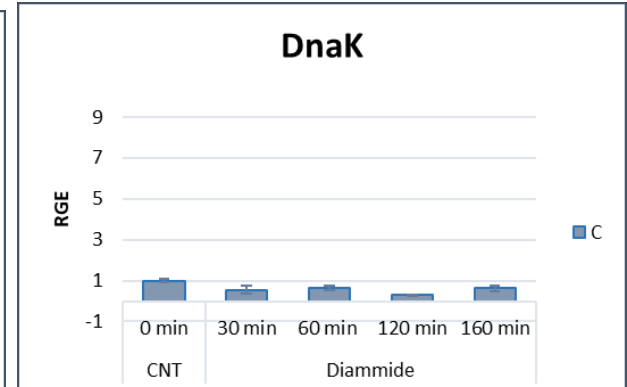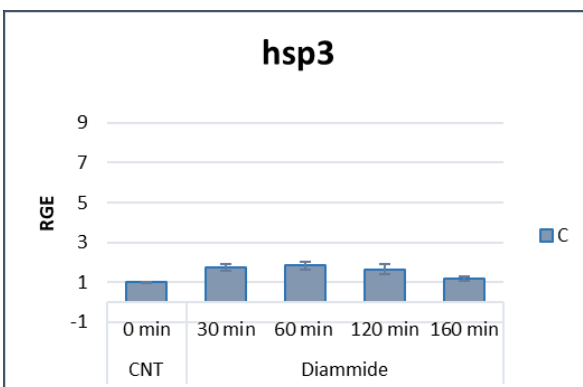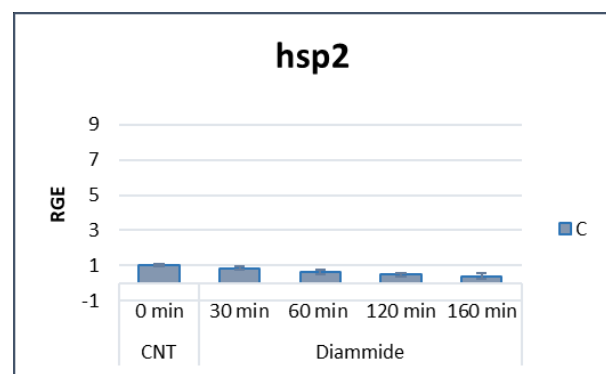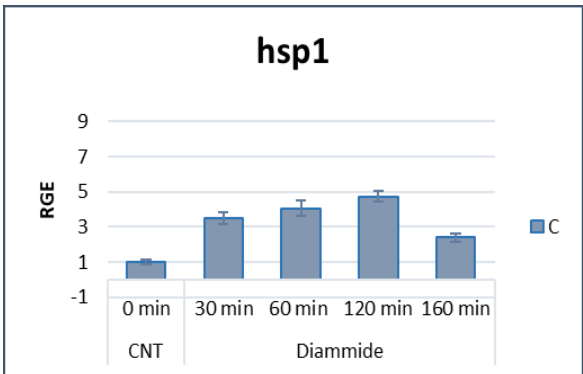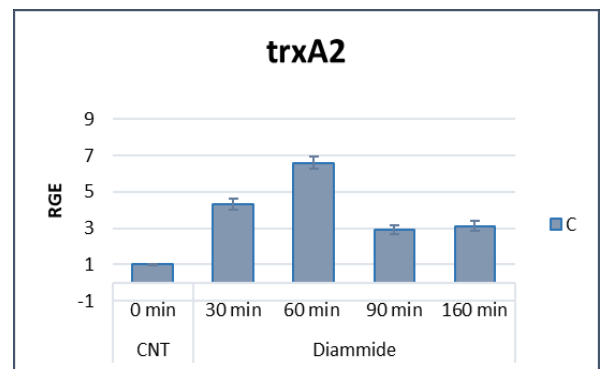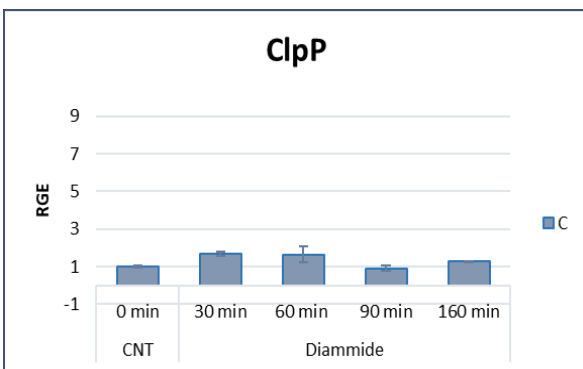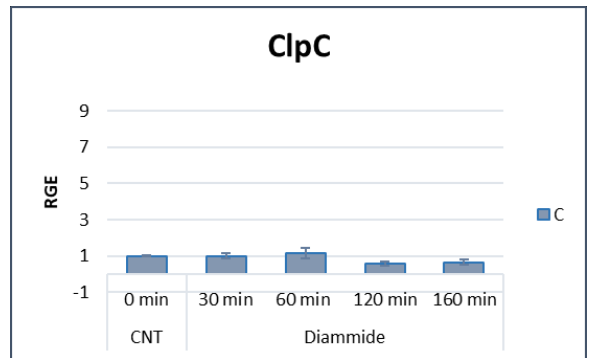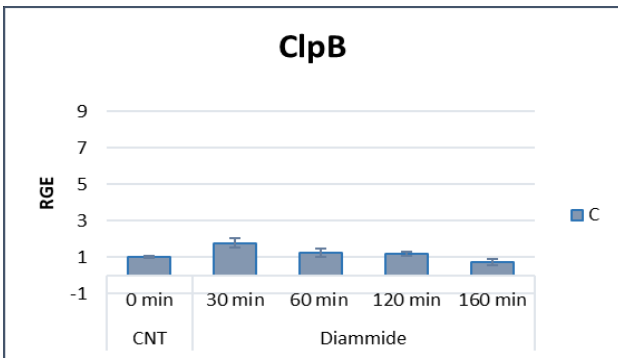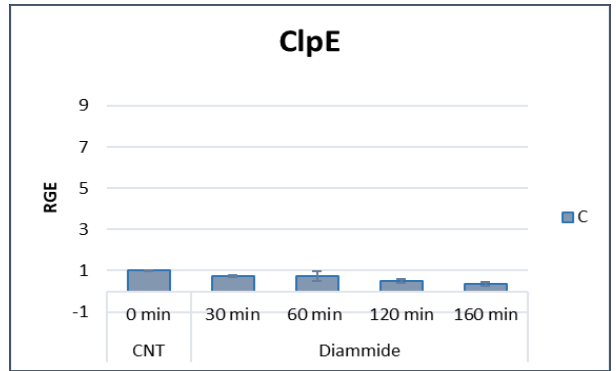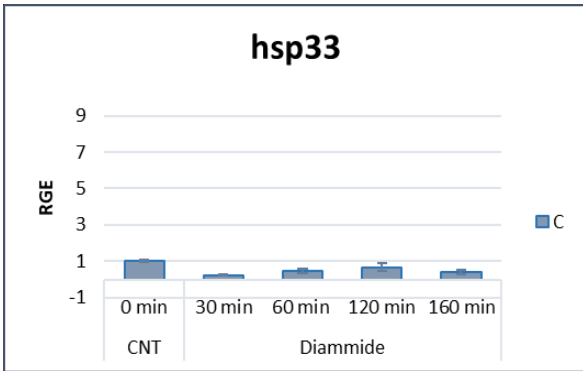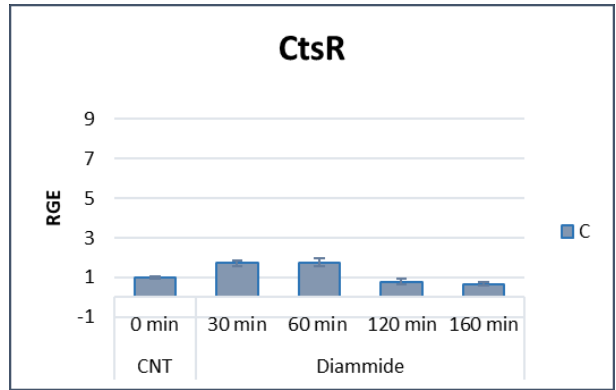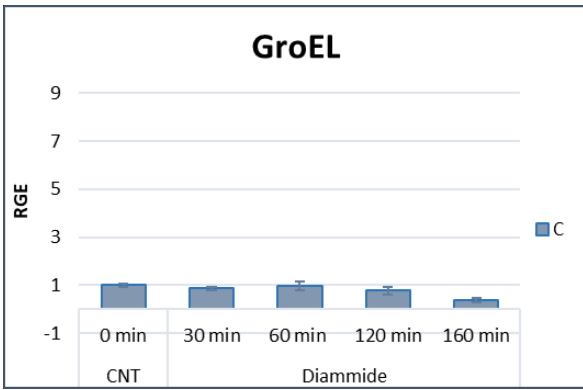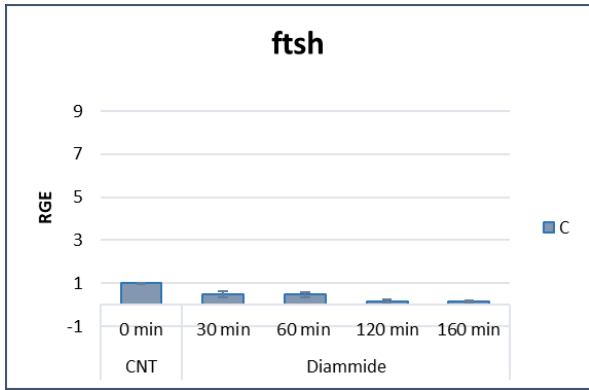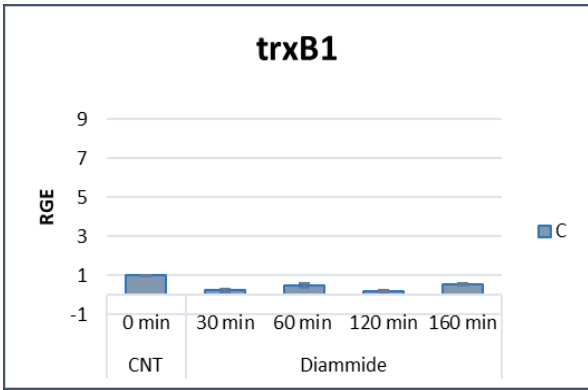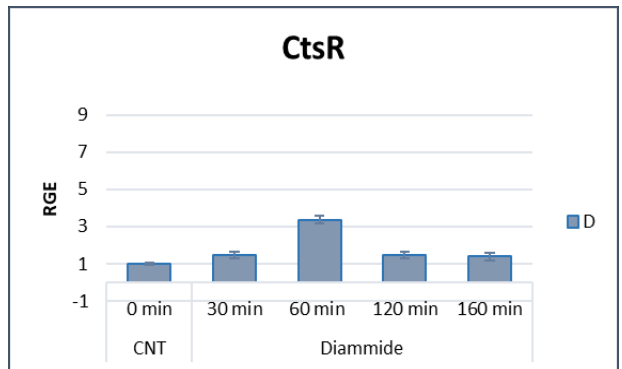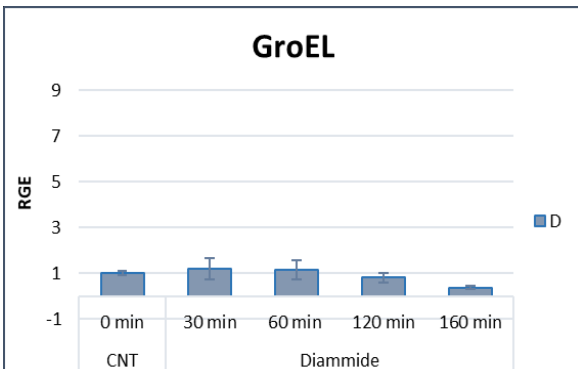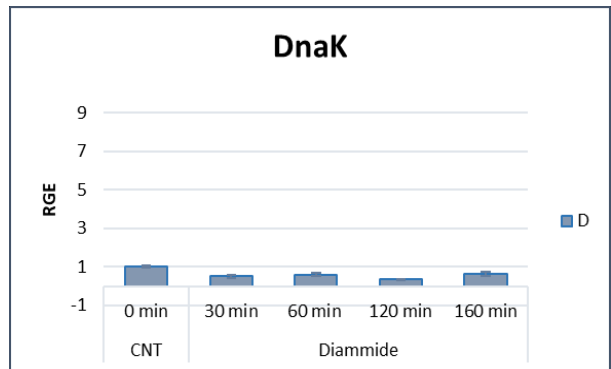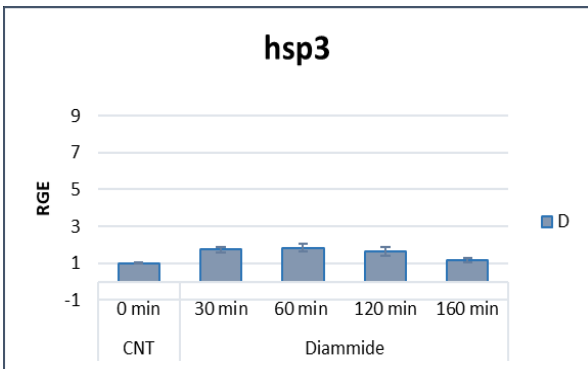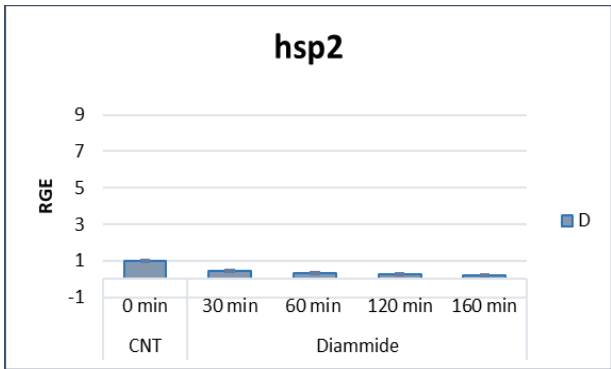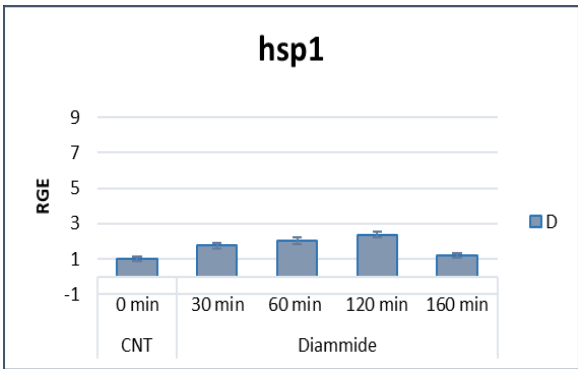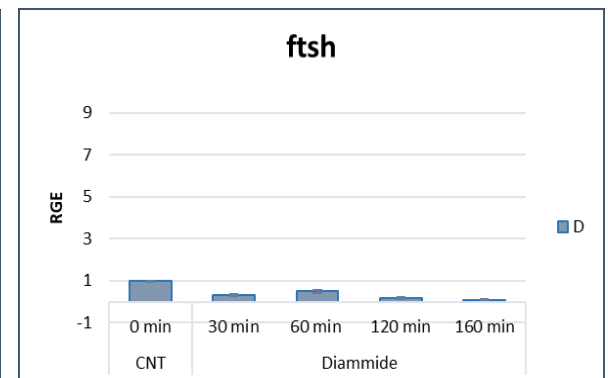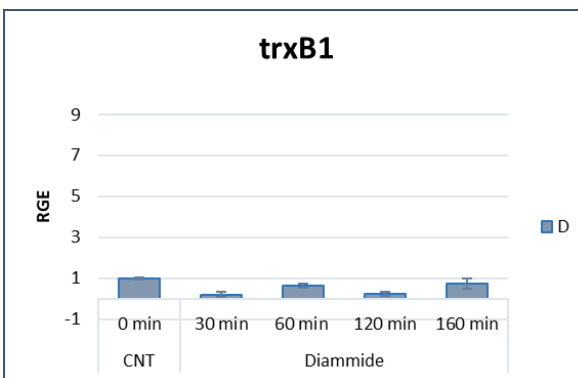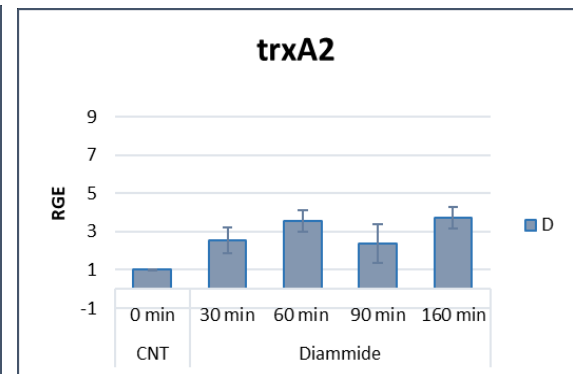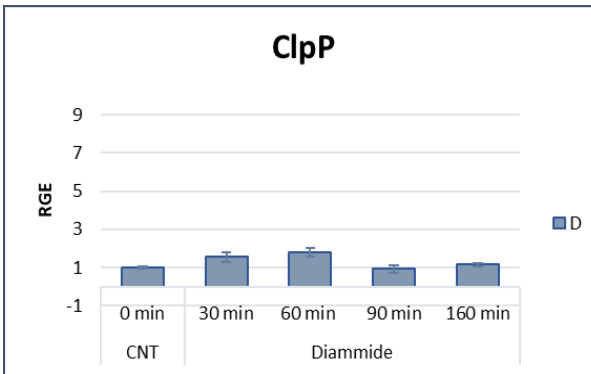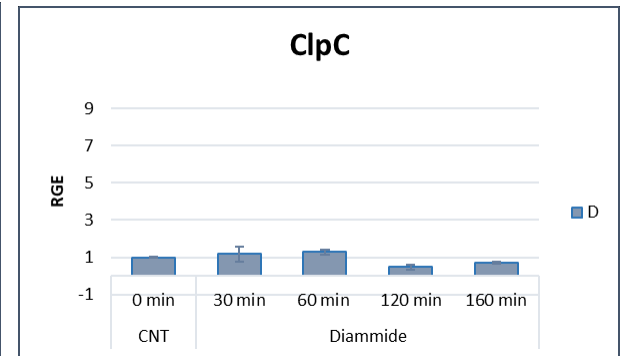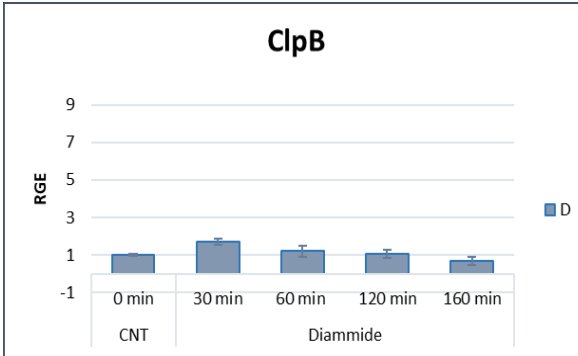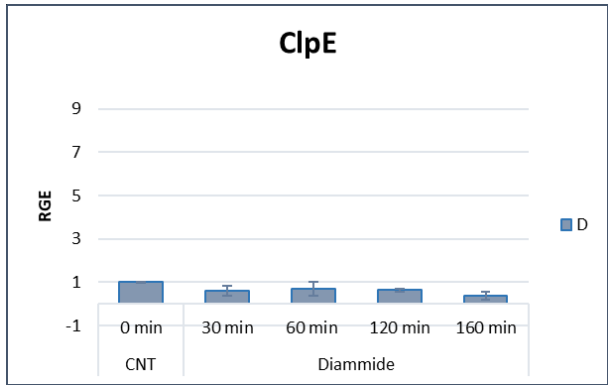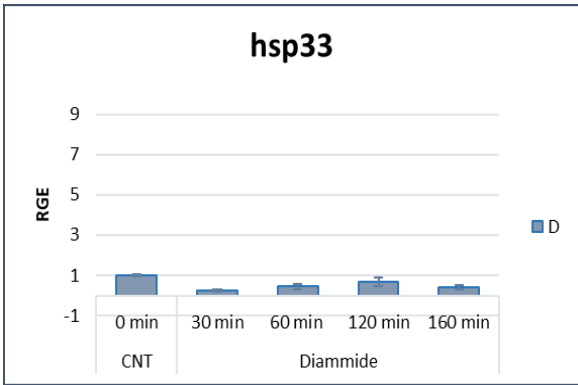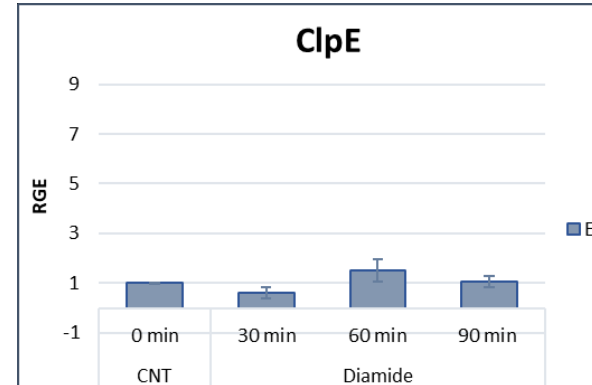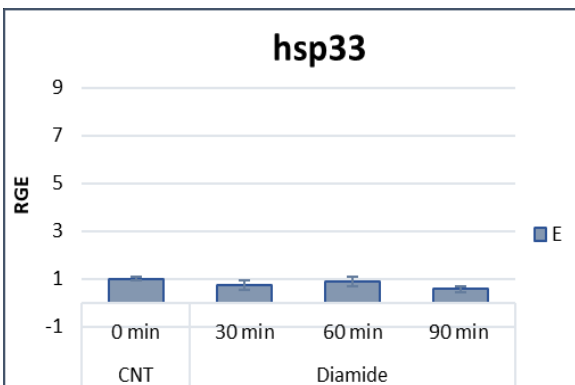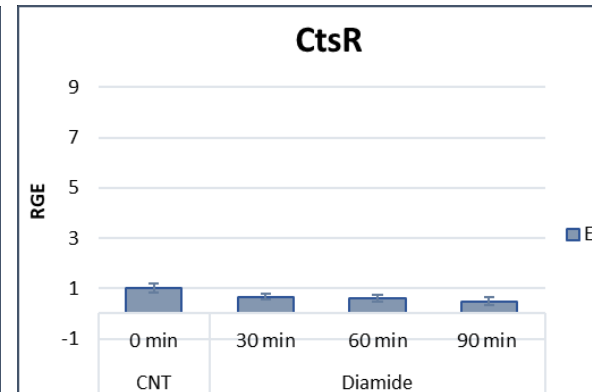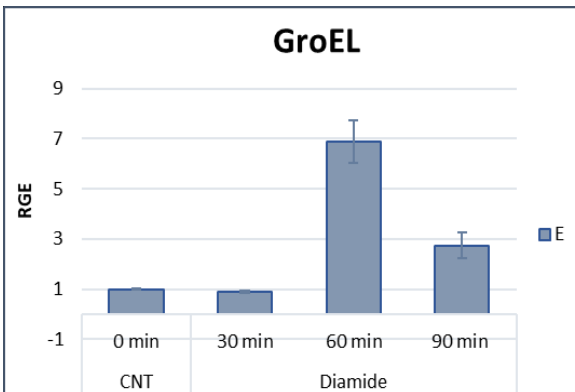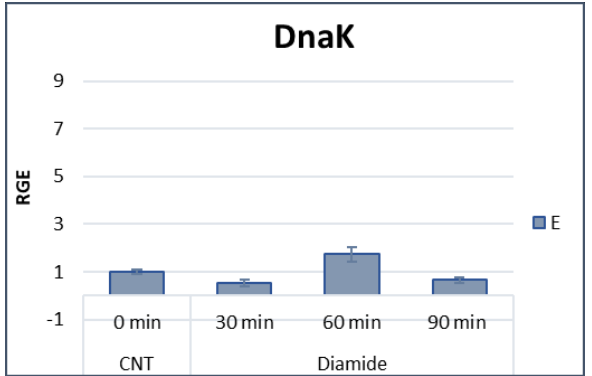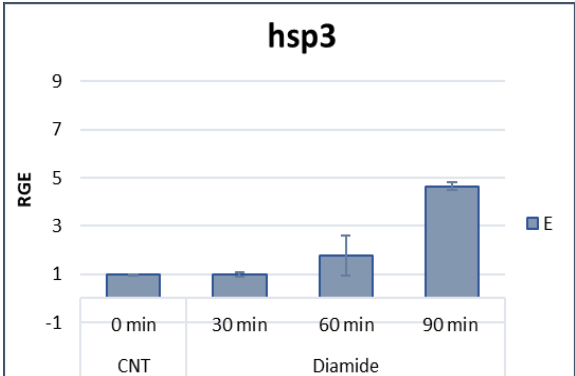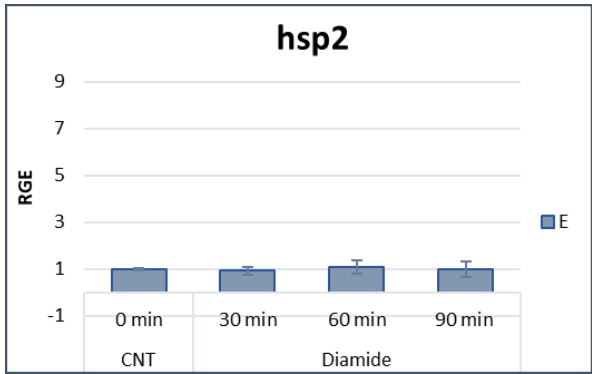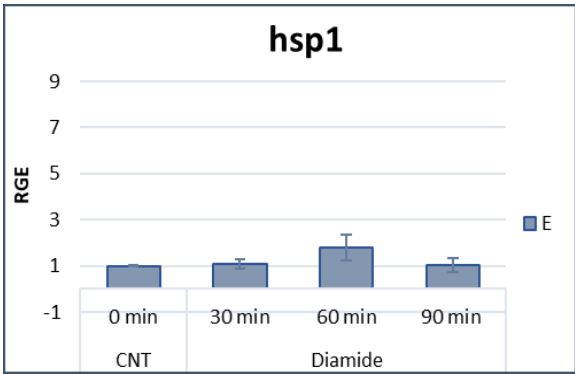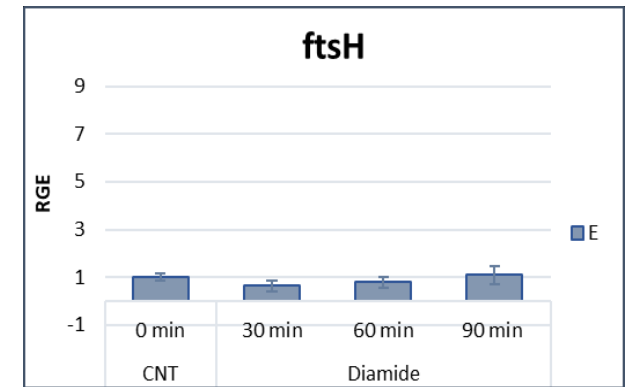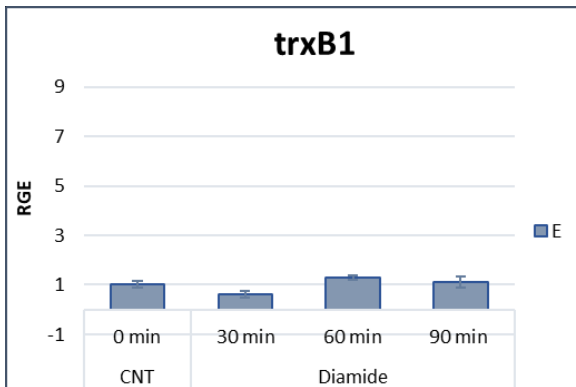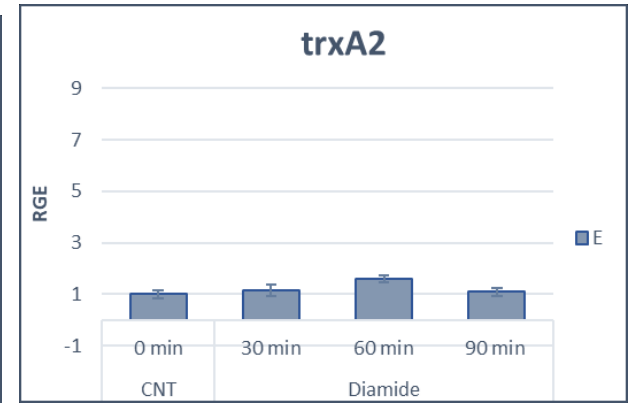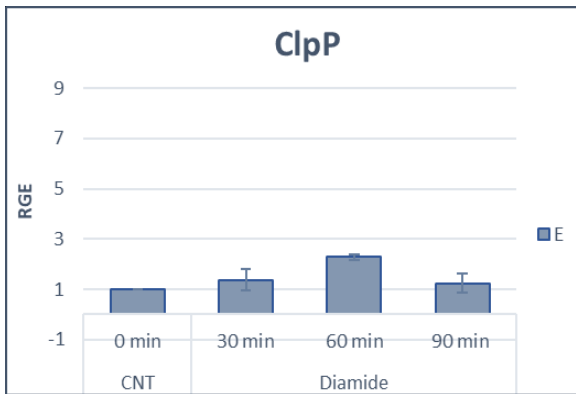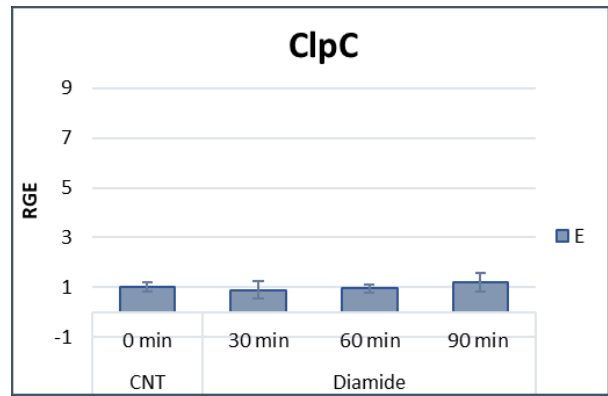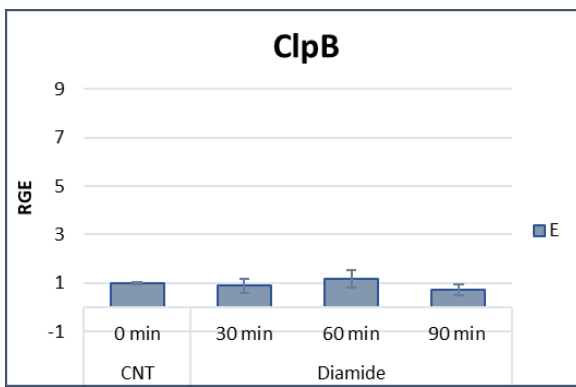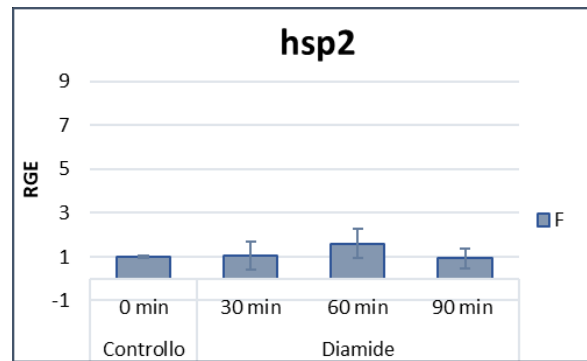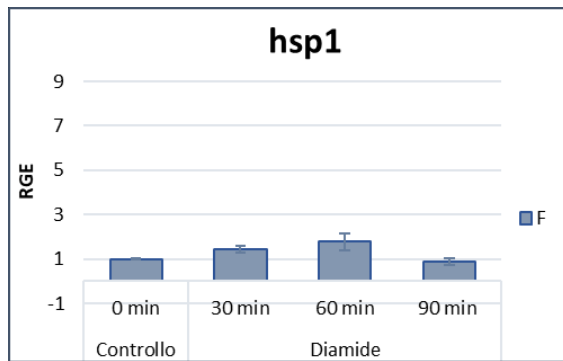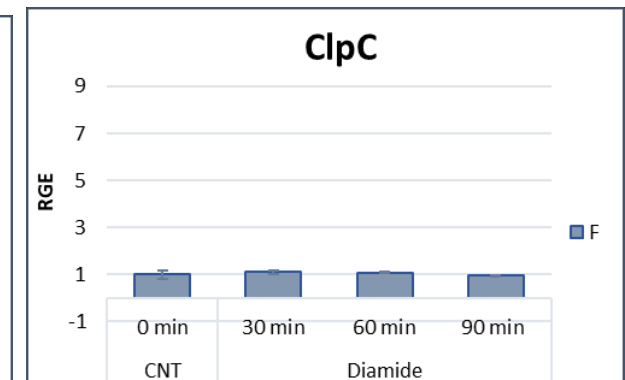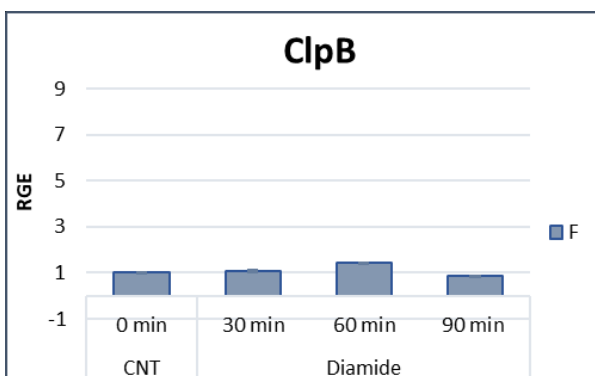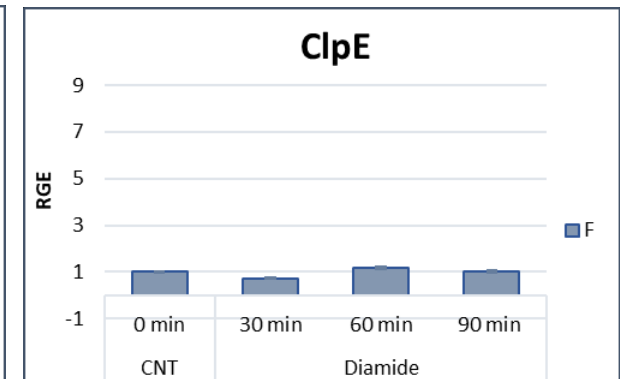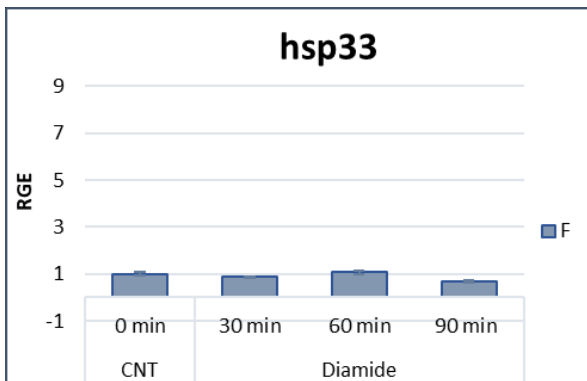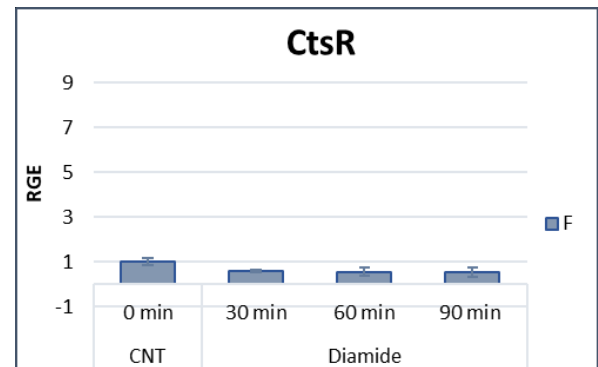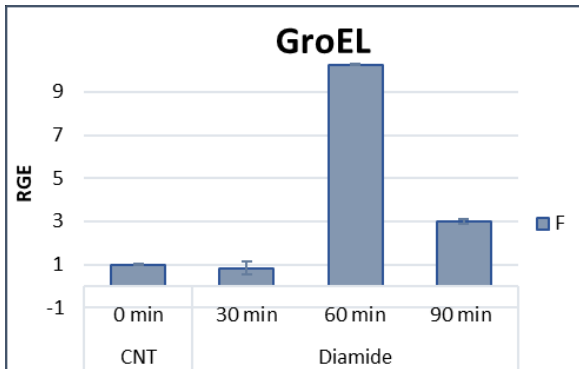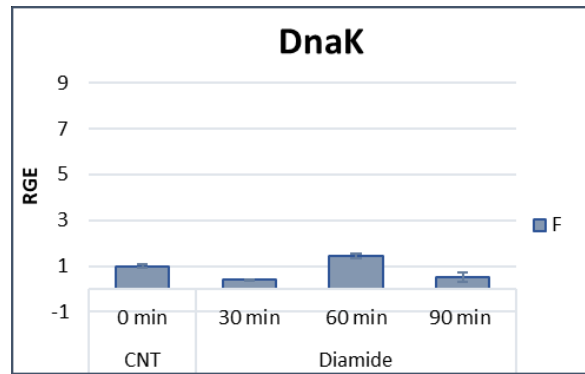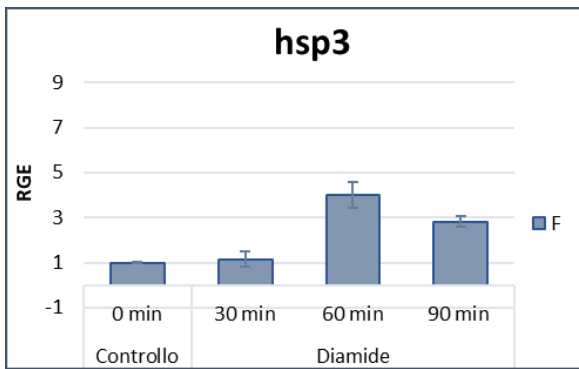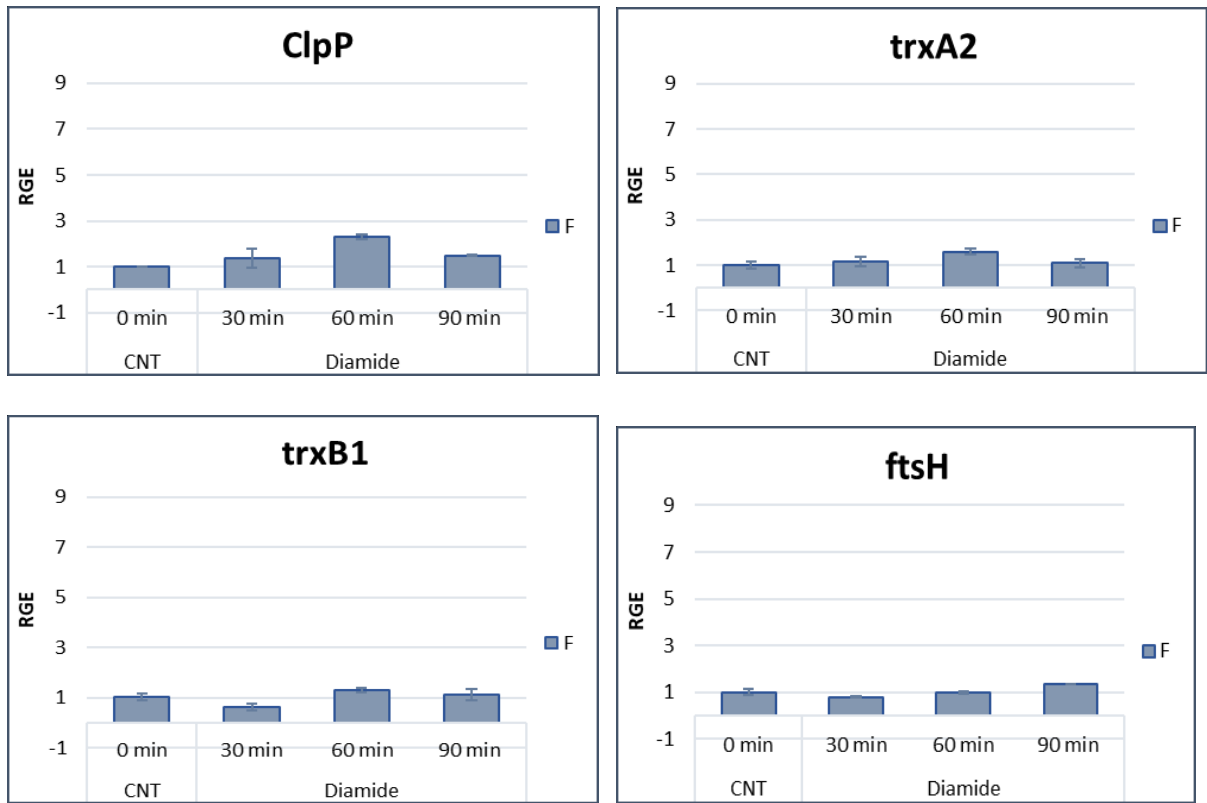
Fig 9. Example of relative gene expression (RGE) in a) *L. plantarum* A, b) *L. plantarum* B, c) *L. plantarum* C, d) *L. plantarum* D, e) *L. plantarum* E and f) *L. plantarum* F under oxidative stress by H2O2, of genes analyzed determined by quantitative real-time RT-PCR. The relative levels of expression were calculated normalizing the levels of *ldhD* gene, and the respective gene expression level under unstressed condition. The results are represented with their standard deviations indicated by vertical bars

a)

b)

GroEL

CtsR

hsp33

ClpE

ClpB

ClpC

ClpP

trxA2

c)

hsp33

ClpE

ClpB

ClpC

ClpP

trxA2

trxB1

ftsH

d)

e)

f)

GroEL

CtsR

hsp33

ClpE

ClpB

ClpC

ClpP

trxA2
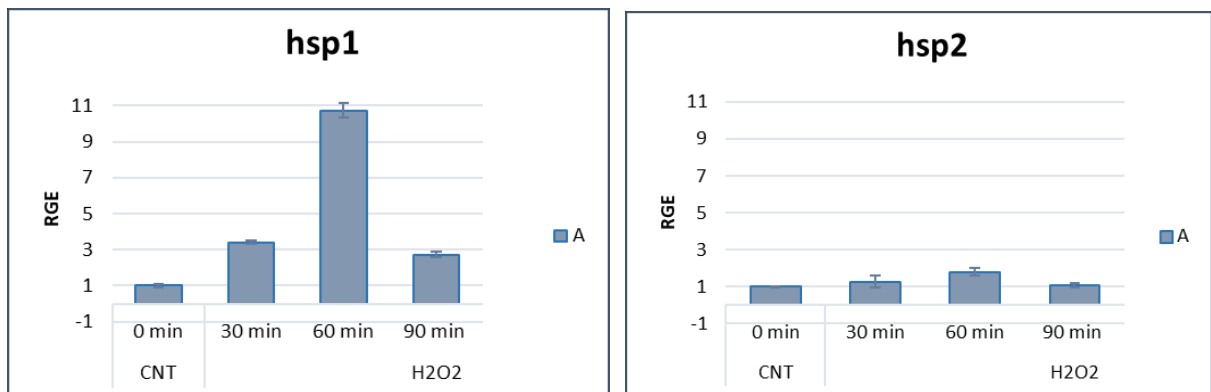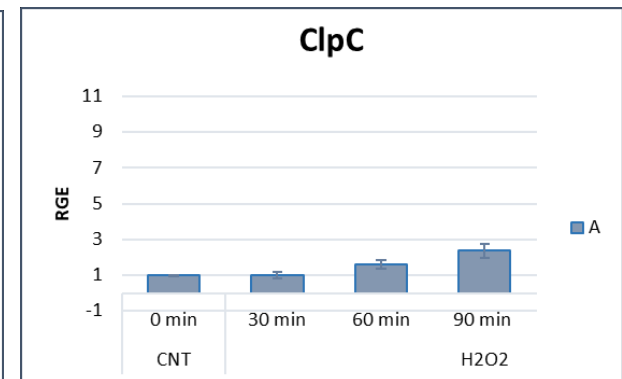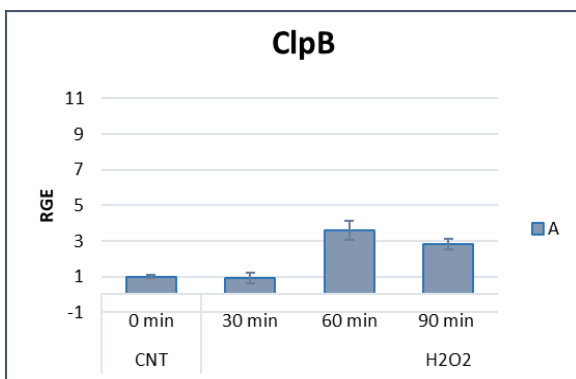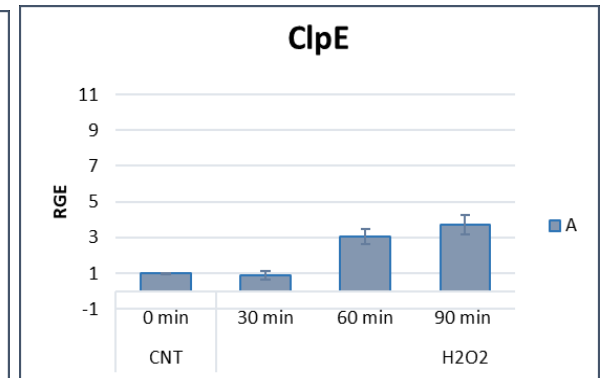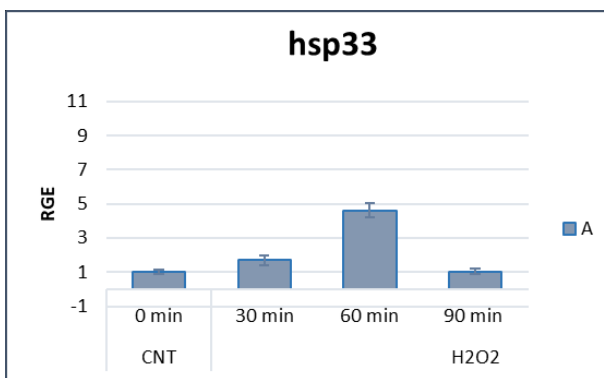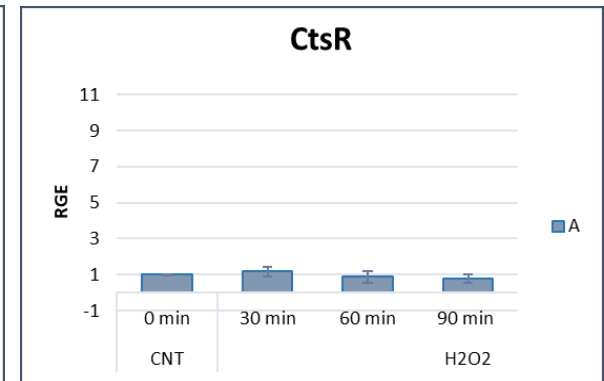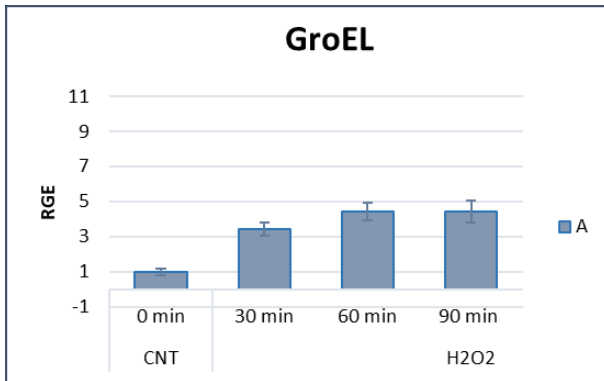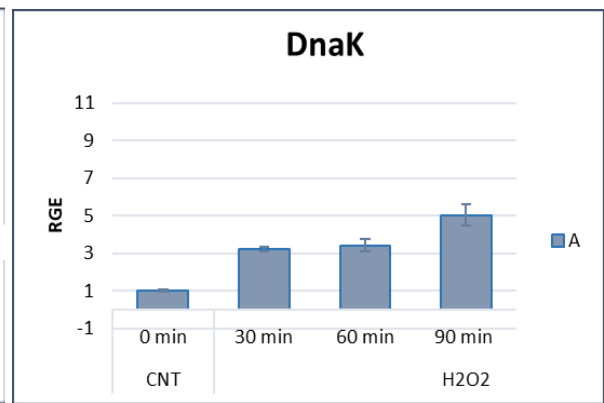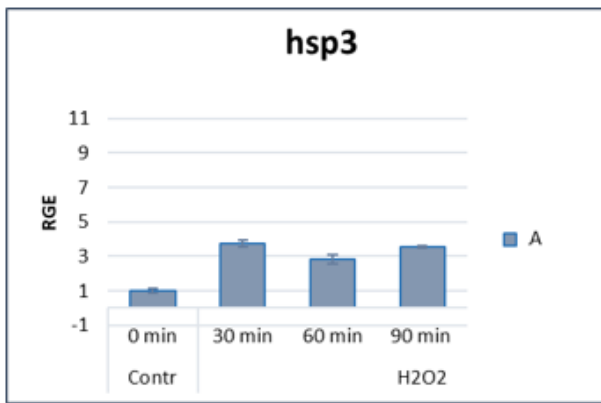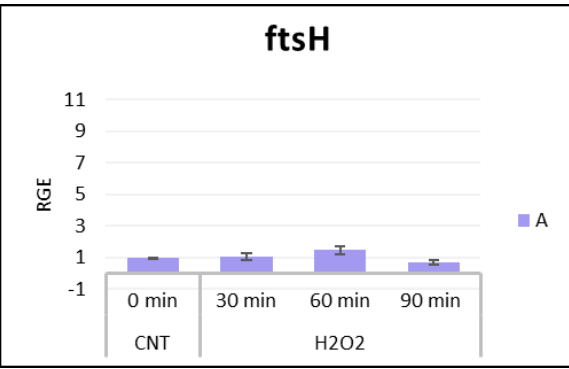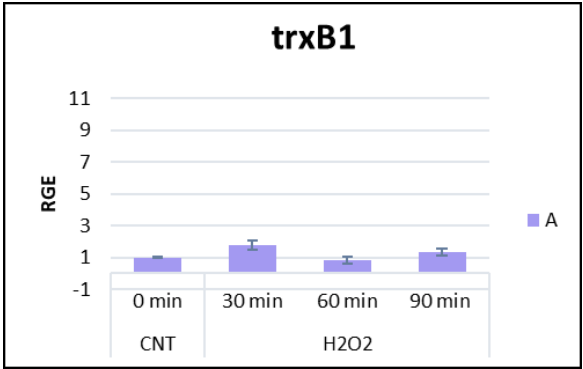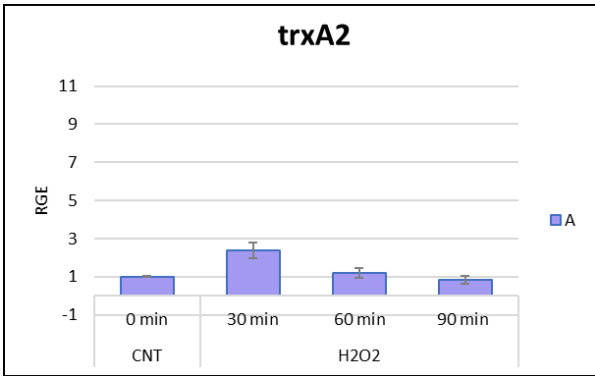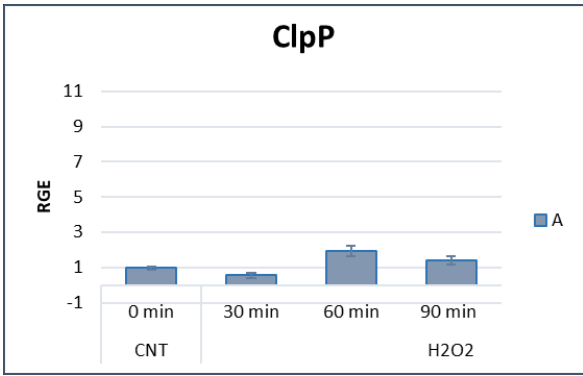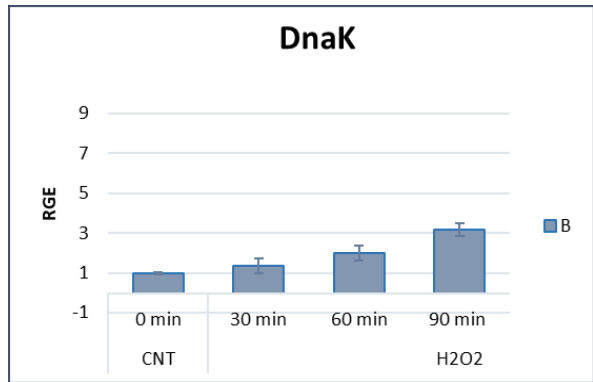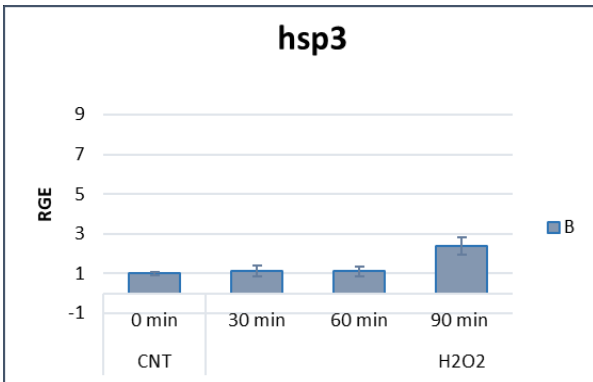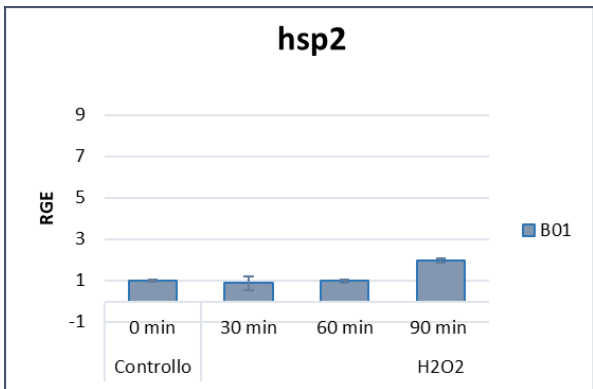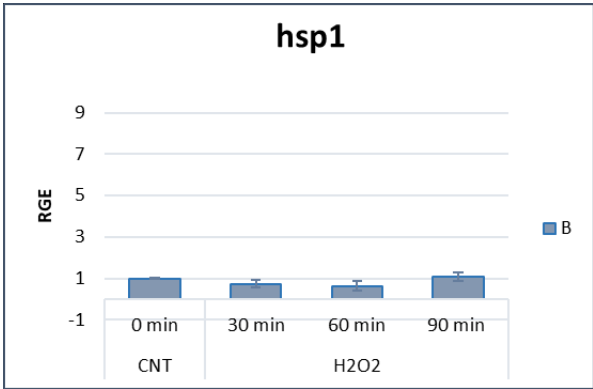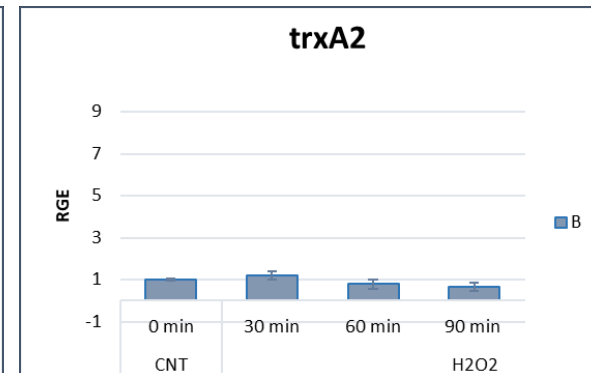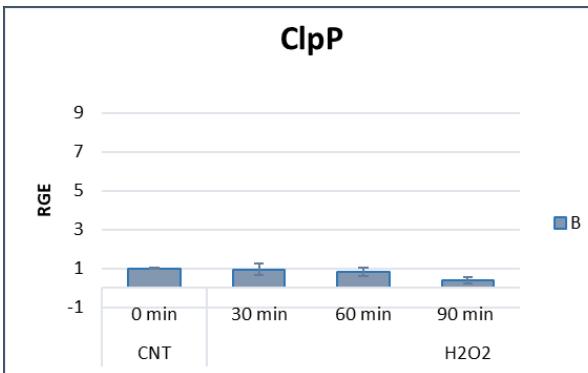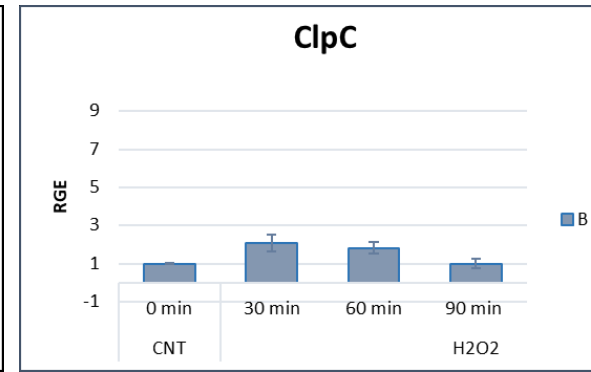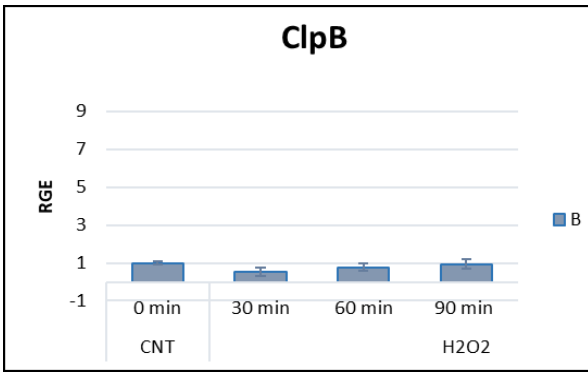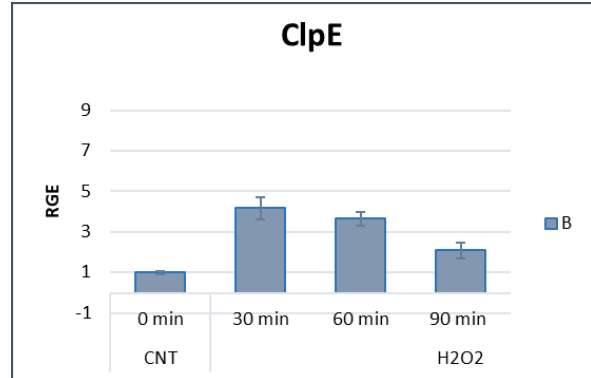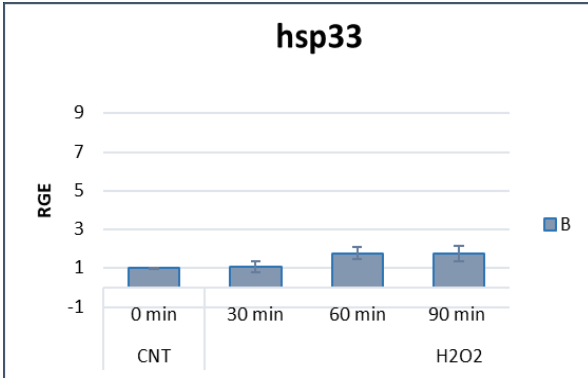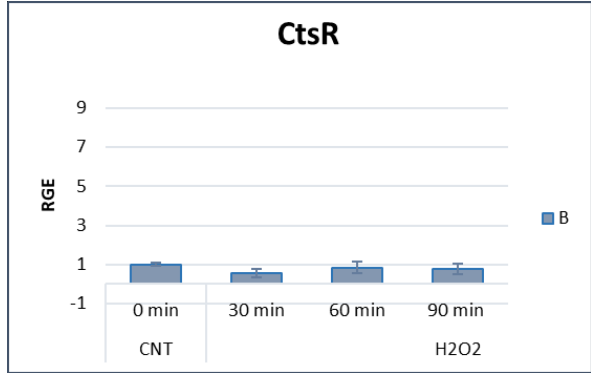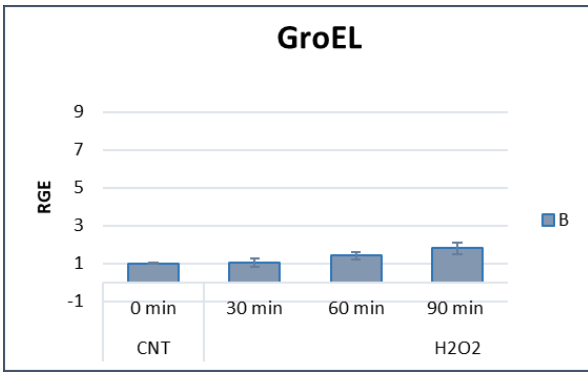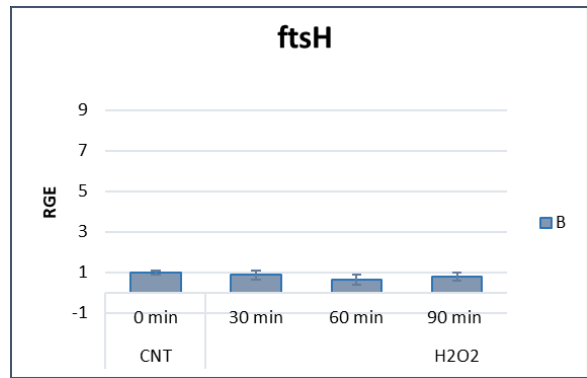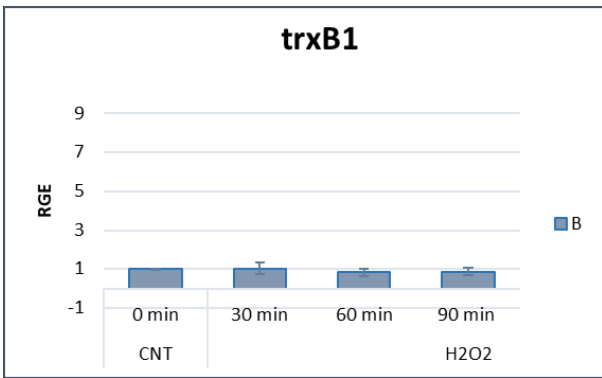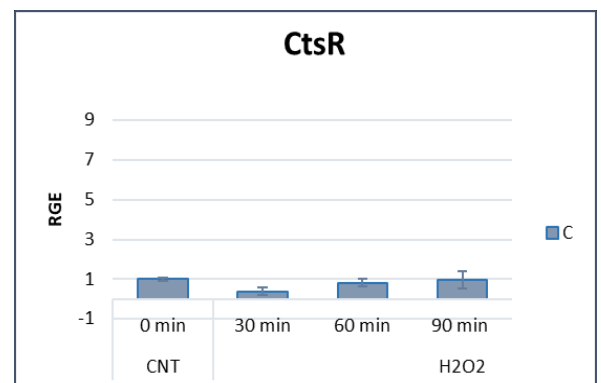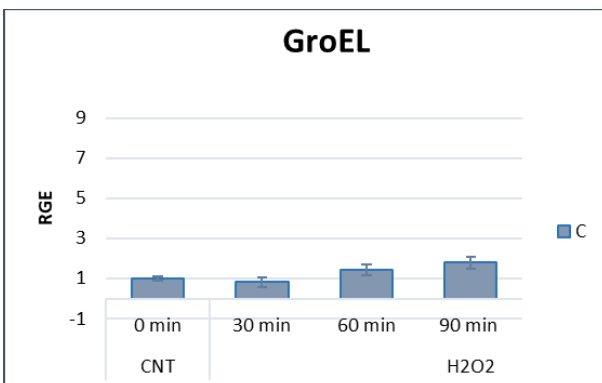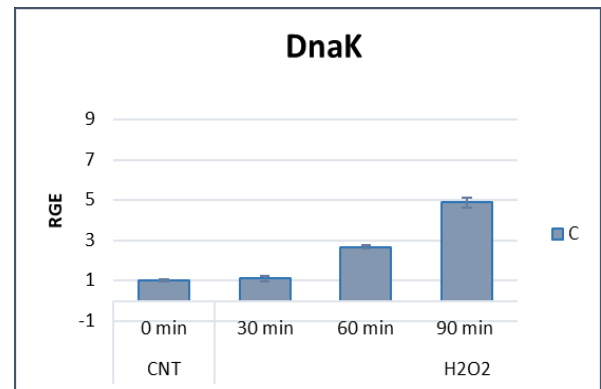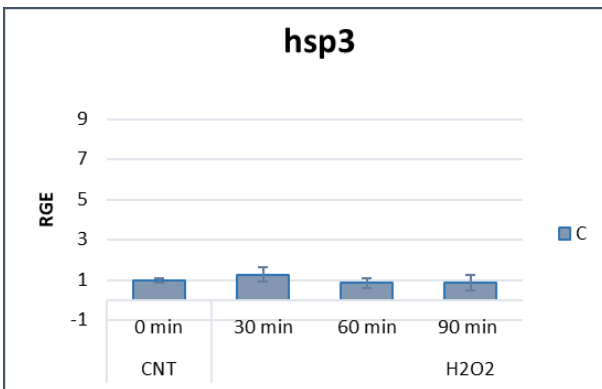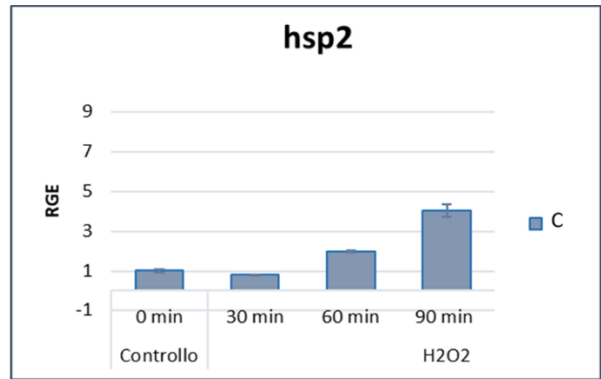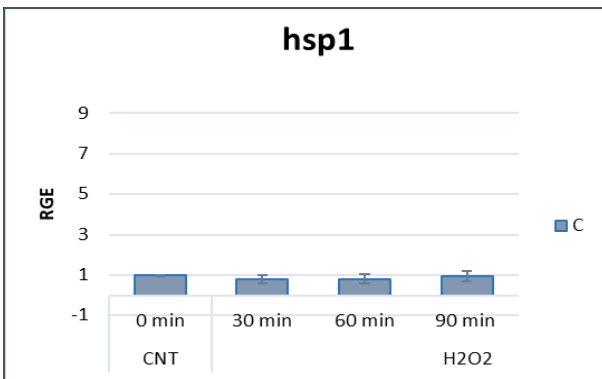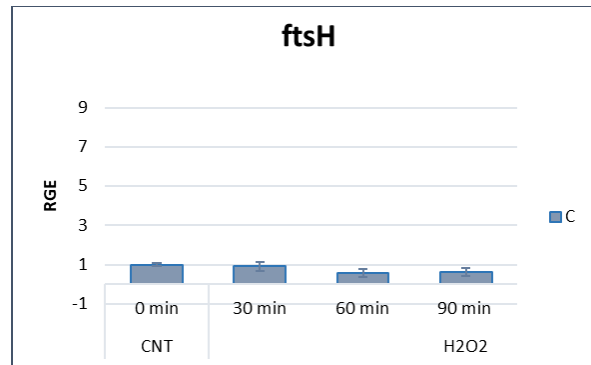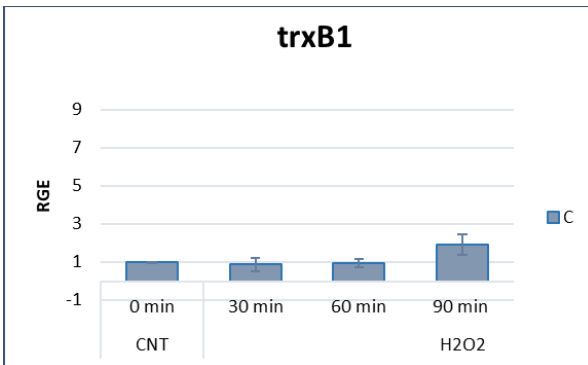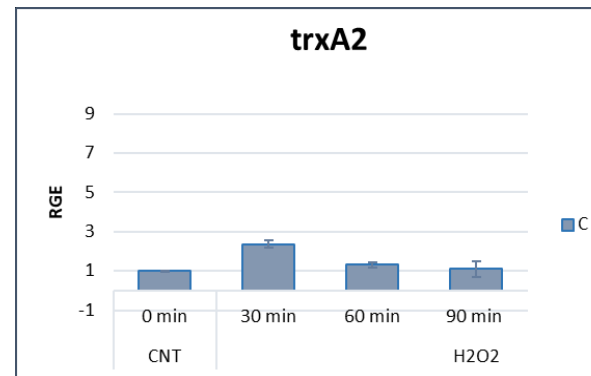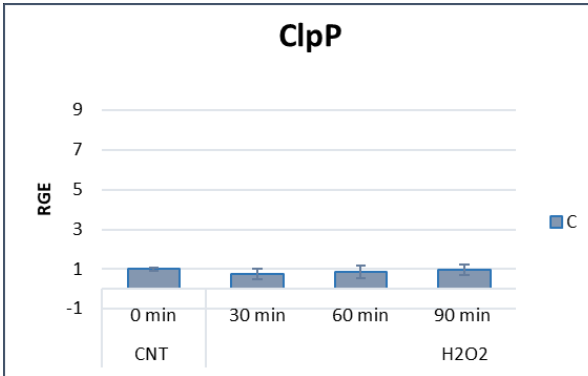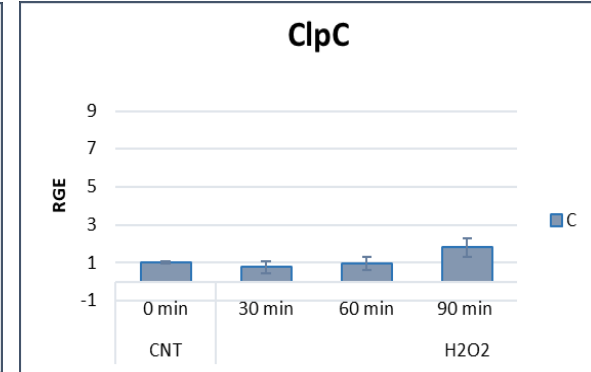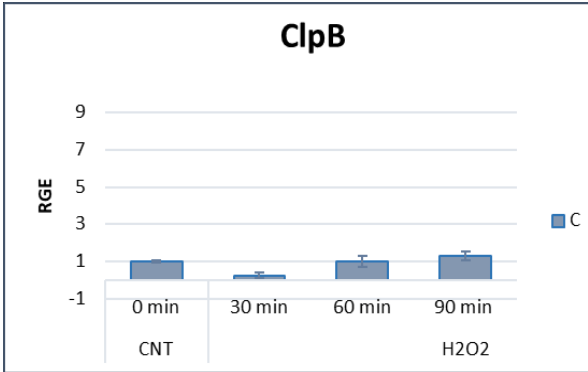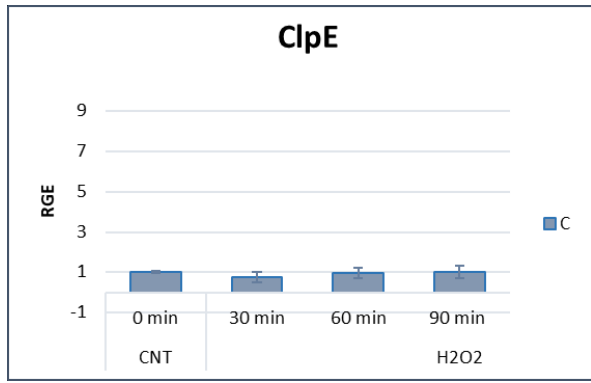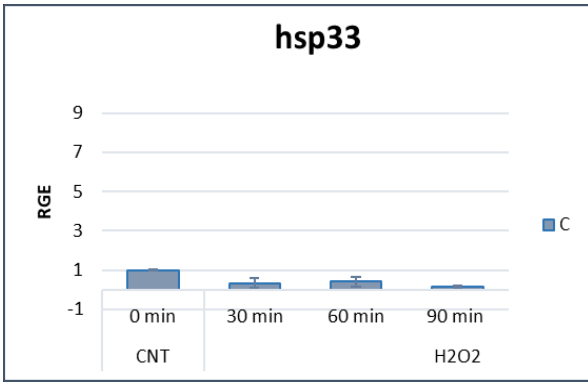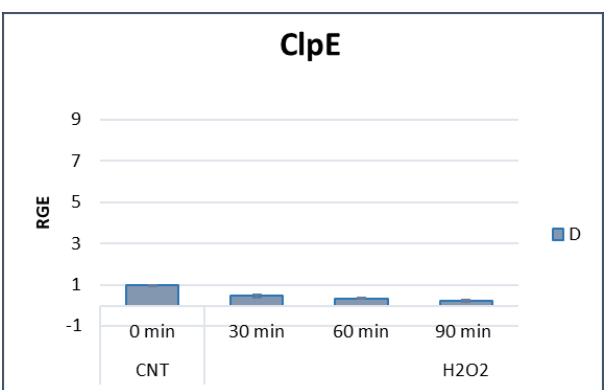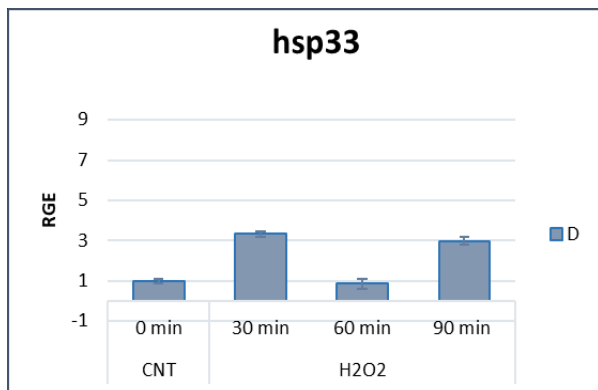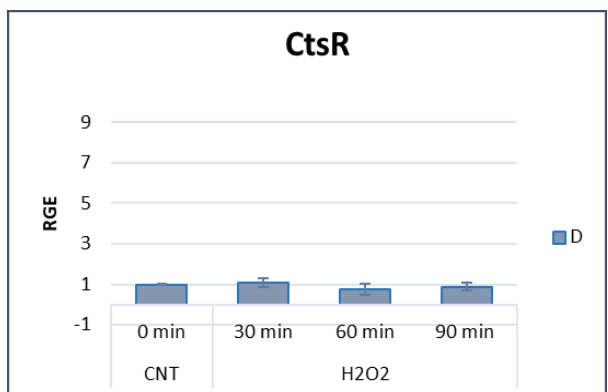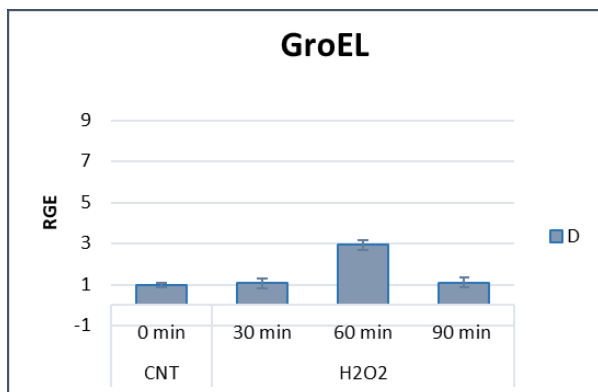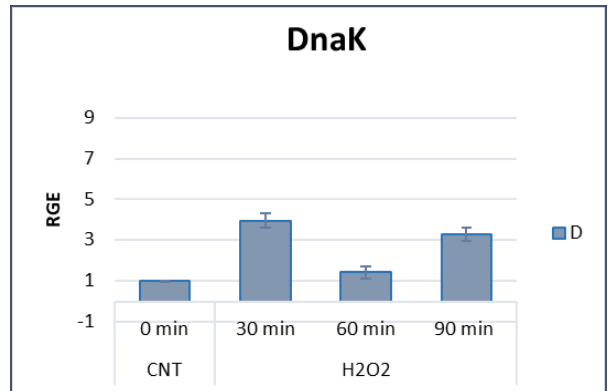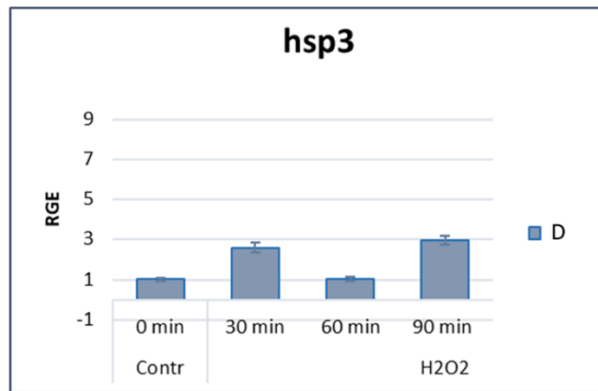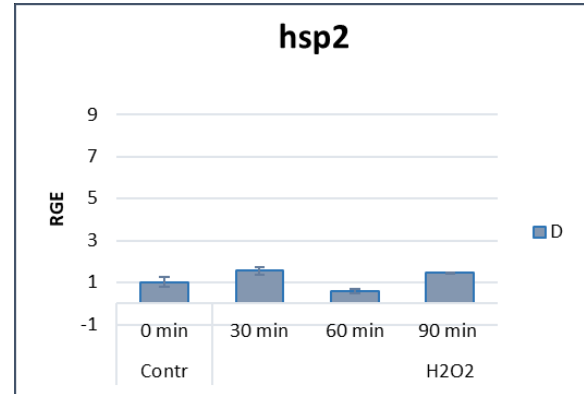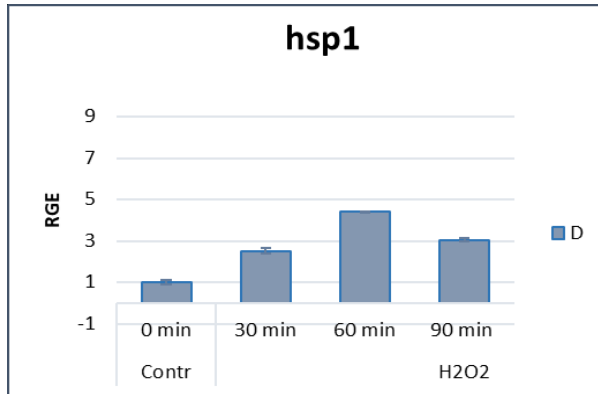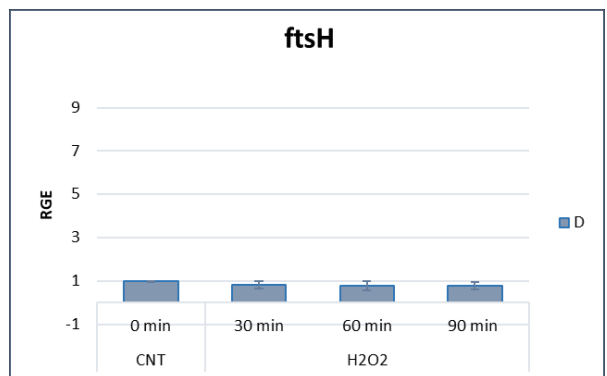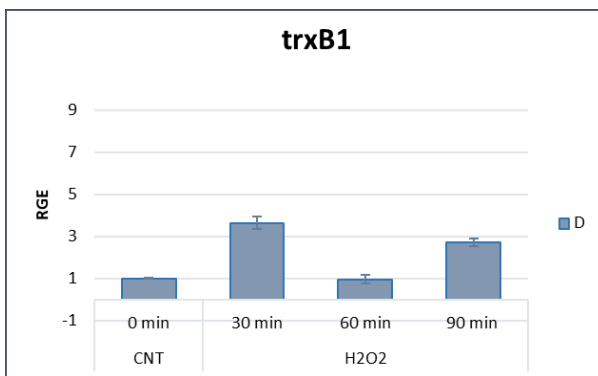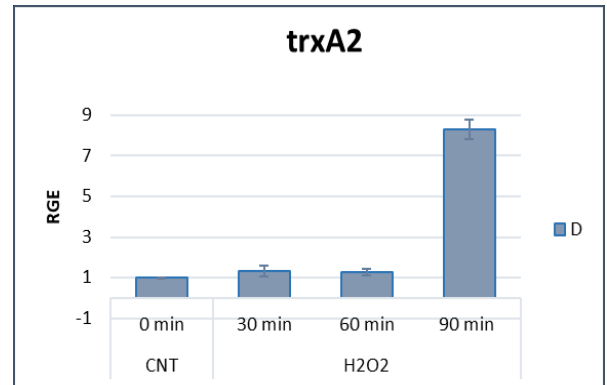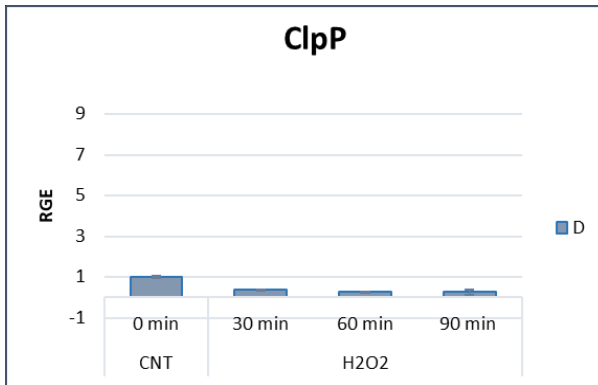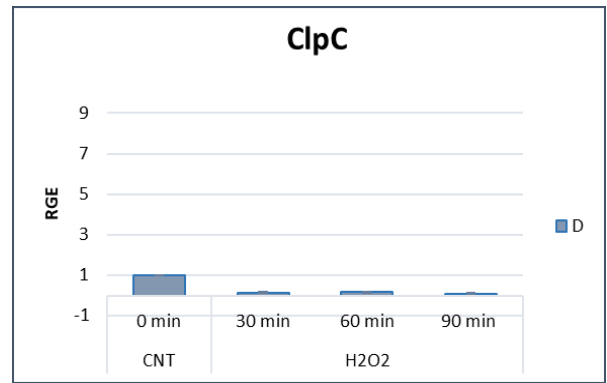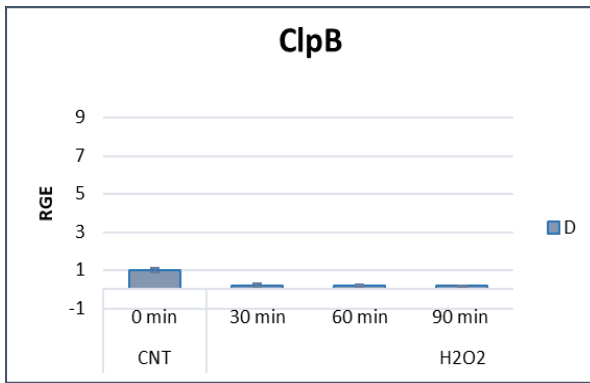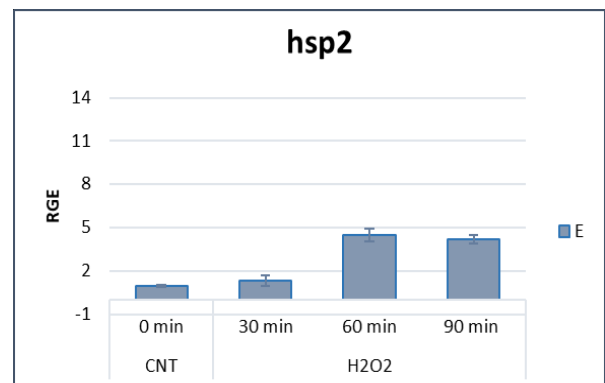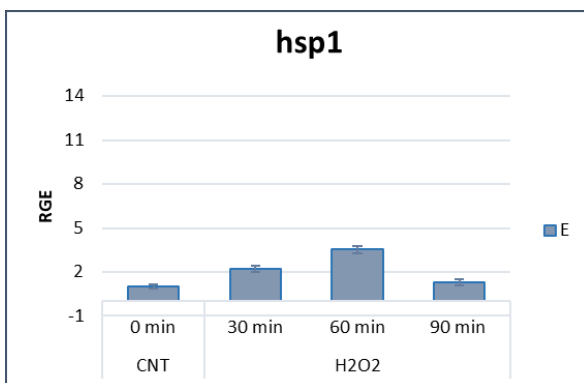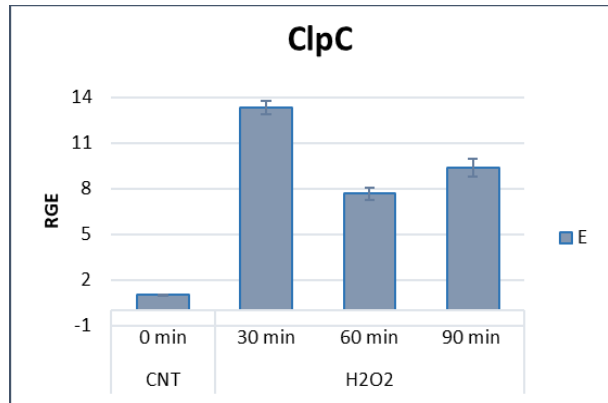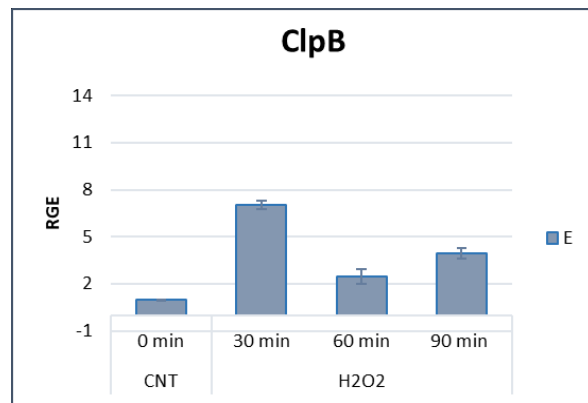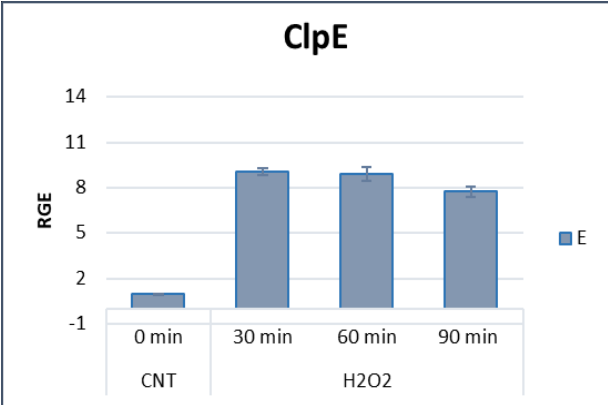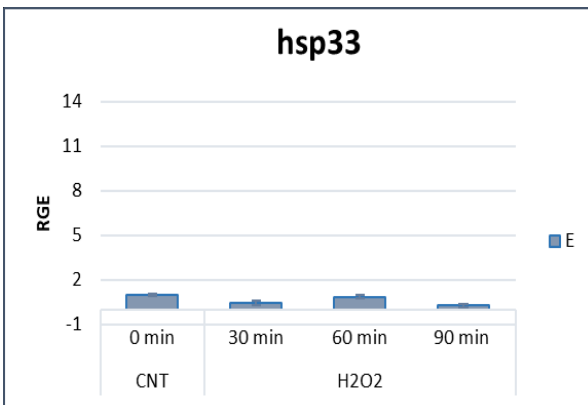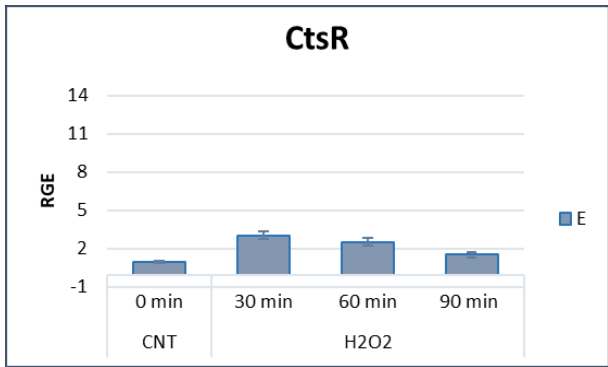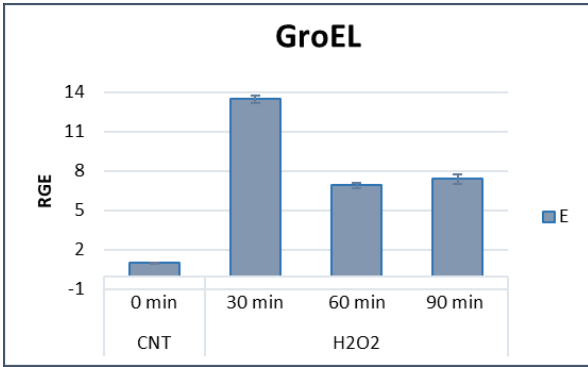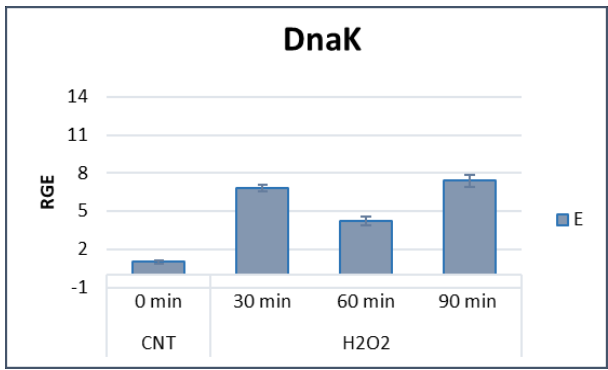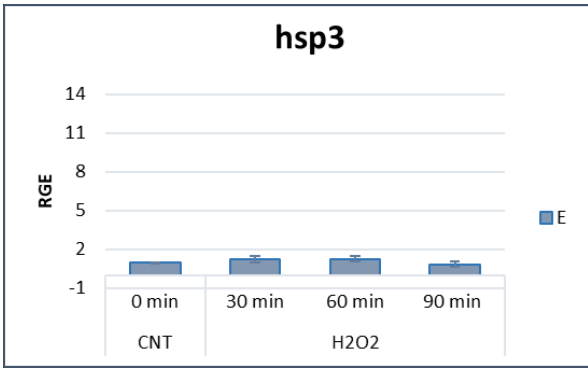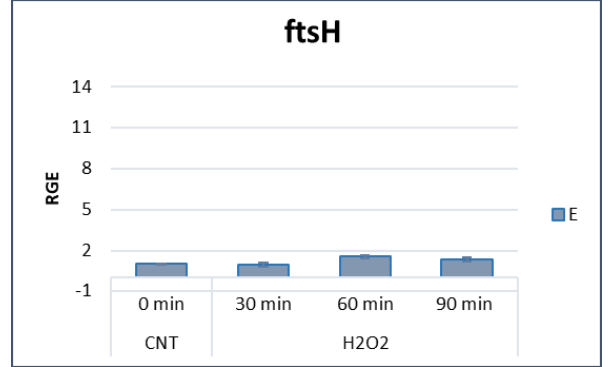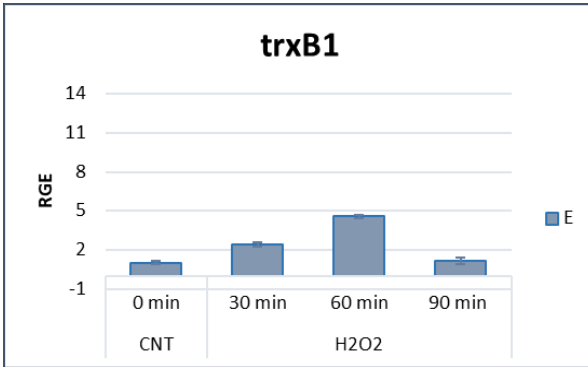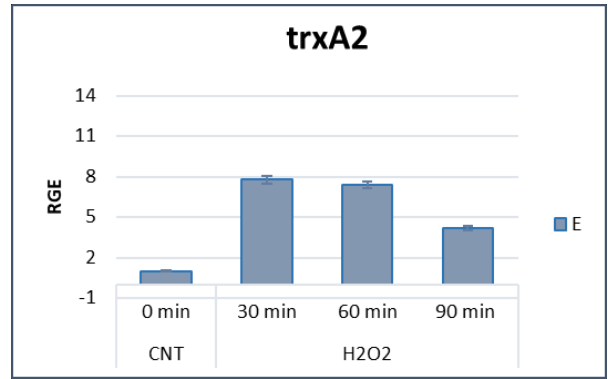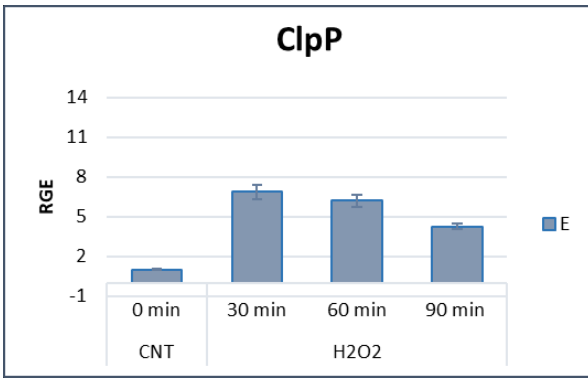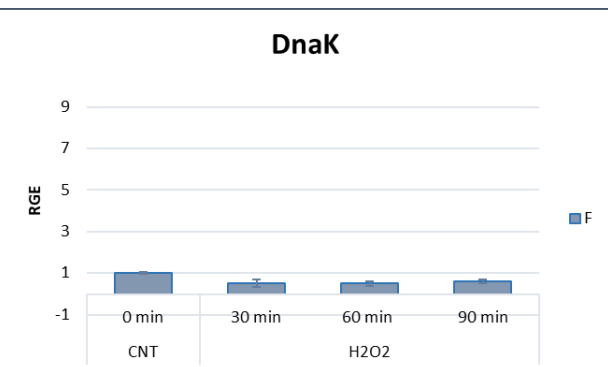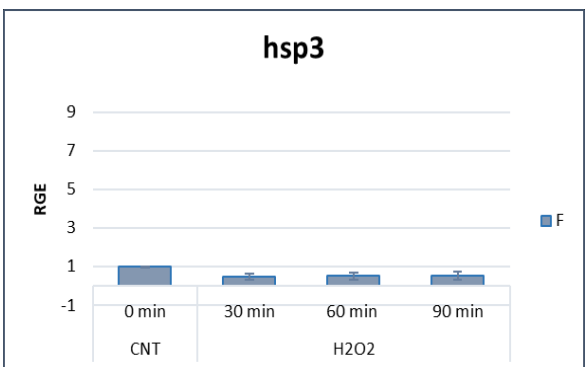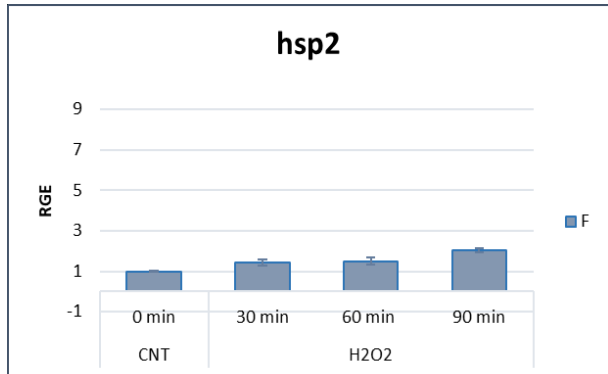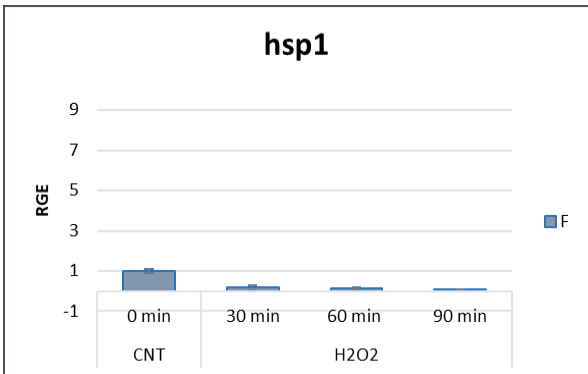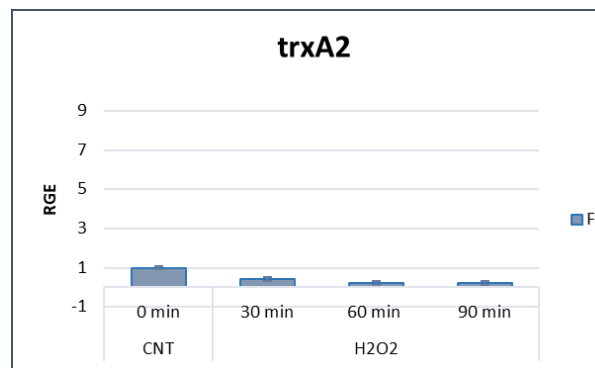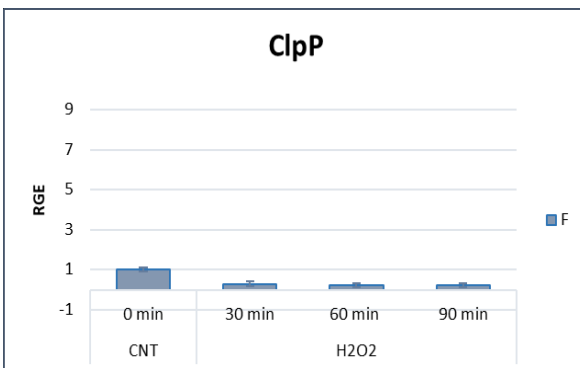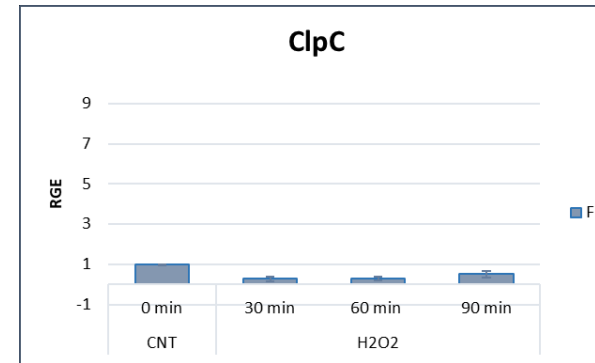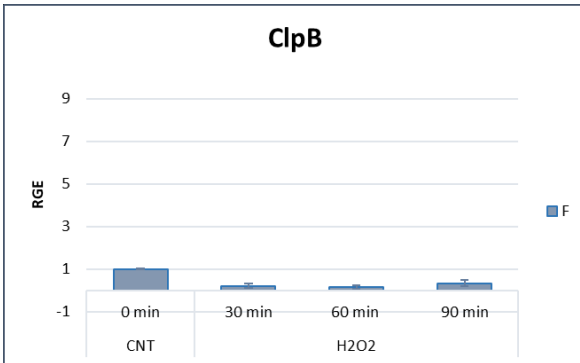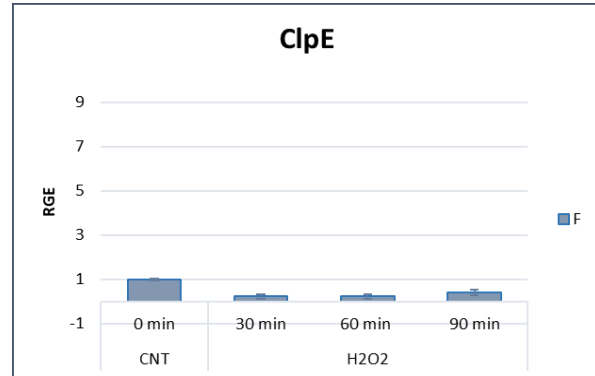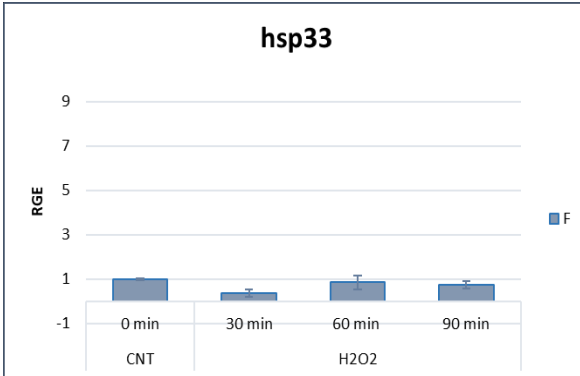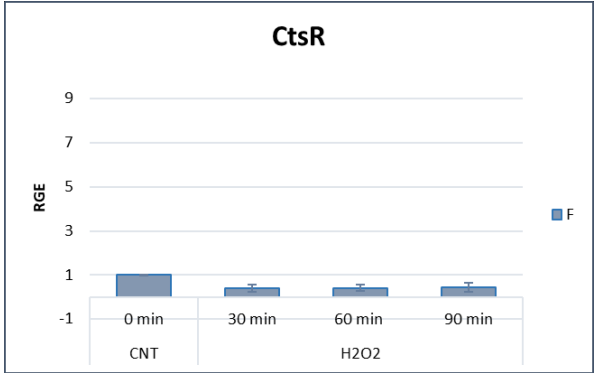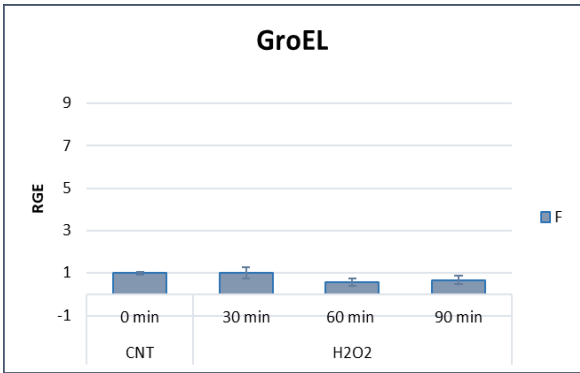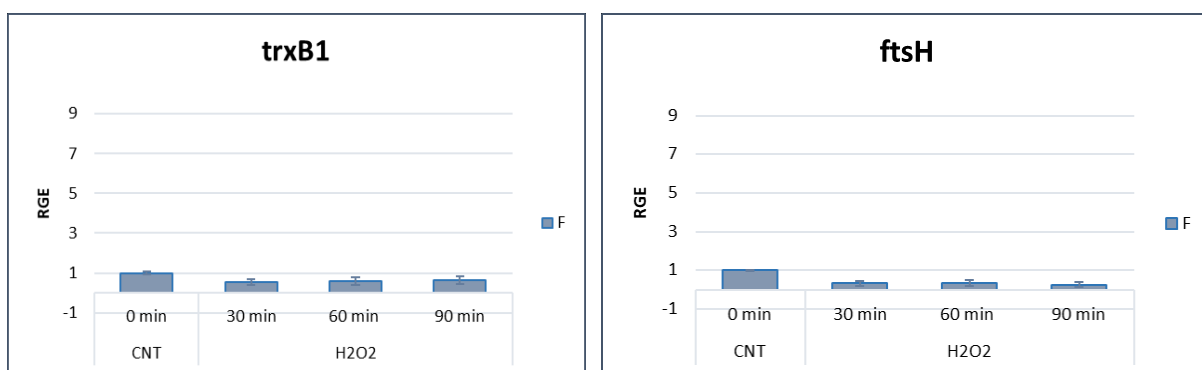
4.4 VOCs profiling by PTR-ToF-MS

4.4.1 Data processing and tentative molecule identification

The combination of the PTR-ToF-MS and the Autosampler gave the possibility to measure VOCs at a high-throughput level during fermentation processes. Six biological replicates for each bacterial strain were selected for evaluation of random biological variation of the bacteria volatilome. Furthermore, six technical replicates of bacteria substrate and six empty samples were evaluated for random noise associated with equipment.

Gaus et al. established the general detection efficiency, sensitivity, reproducibility of PTR-ToF-MS measurements [165]. The reproducibility of the used PTR-ToF-MS apparatus was controlled by periodic calibrations with a calibration gas standard (Ionicon Analytik GmbH, Innsbruck, Austria).

Wine is an interesting example of an alcoholic beverage in which the change in perceived aroma profile with time is well-documented. VOCs release occurs through mass transfer of the volatile molecules from the liquid to the gas phase. Real time monitoring of VOCs was performed to investigate the differences in volatilome of analyzed strains bacteria and to evaluate their effects on the sensorial quality of the wine. The composition of the wine in terms of molecules with olfactory significance appeared to be more complex. More than 400 peaks have been extracted from the average spectra associated to each time point, 50 have been tentatively identified (**Tab.4**). Univariate and multivariate analyses have been performed on the data matrix highlighting the volatilome evolution and strain specific features.

Three kinds of trends of VOC can be observe regardless of the strain and the moment of inoculation. The **Fig**.**10** shows examples of trends that grow, decrease or show small variations in the concentration of VOCs. Data show that PTR-MS represents a powerful bioprocess-monitoring tool (alcoholic and malo-lactic fermentation) in real time (**Fig.11**).

As is evident in the **Fig.12** the trend is similar for each category of compounds: ketones (m/z 59,0485, t. i. as Acetone), esters (m/z 115.075, t. i. as Caprolactone) and other fragrance agents (m/z 125,0976, t. i. as (Z)-1,5-octadien-3-one; while it is different for aldehydes, alcohols, acids and furans (data not shown).

Furthermore, it can be seen from the figures that the behavior of the M strain (isolated from saliva) (in yellow) shows the same trend as the sample containing only yeast (in brown) while is clearly different from the trends of the oenological strains*, which show a similar behavior to each other as demonstrated in numerous compounds.

The different trends of enological strains are not visible from the graphs, while they are evident from the ANOVA values referred to points 7 and 20 of the co-inoculum and point 20 of the sequential inoculum (**Tab.5**)

*Red = strain F; Green = strain G; Blue = strain H; Light blue = I; Violet = L; Yellow = strain M; Brown = must only with yeast; Black = only must.

a)
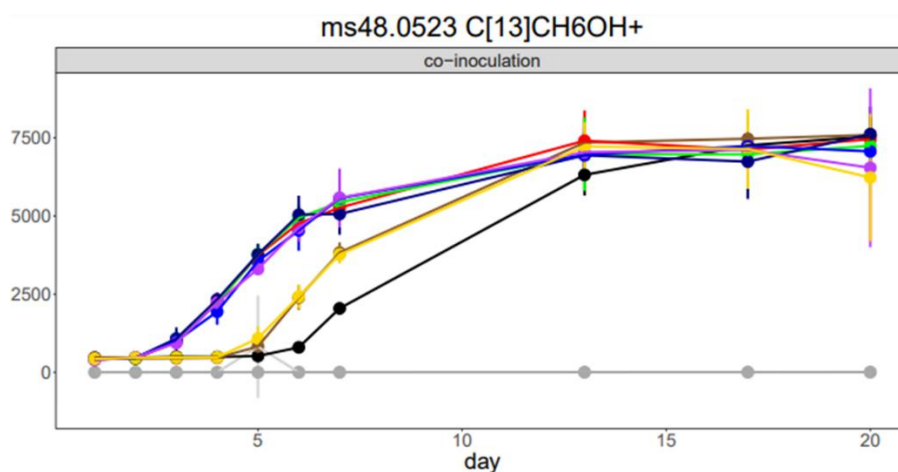
b)
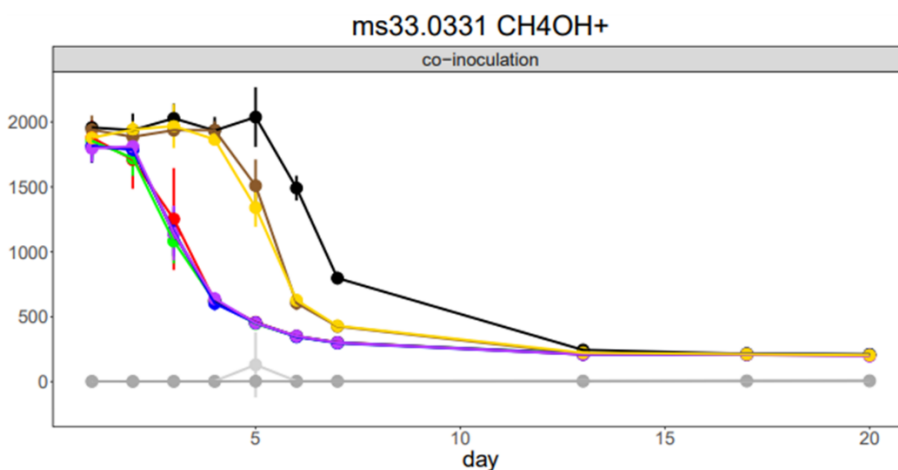
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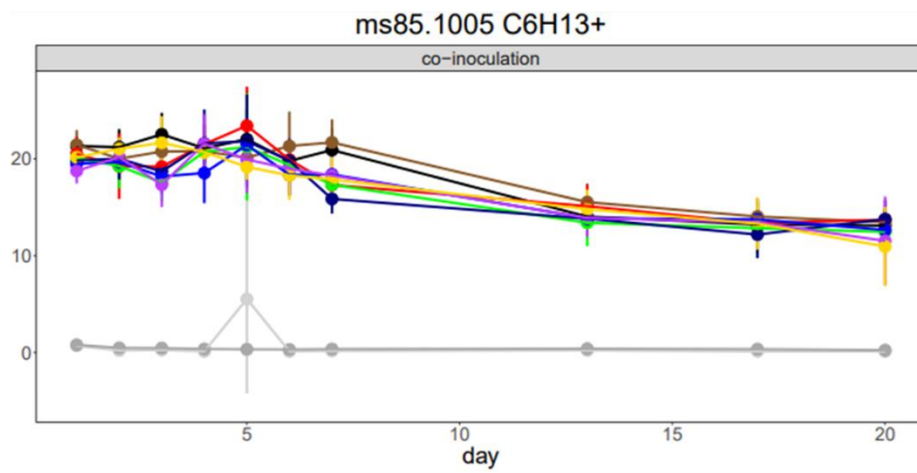
Fig.10 Different trends of volatile organic compounds: a) increasing, b) decreasing and c) almost unchanged
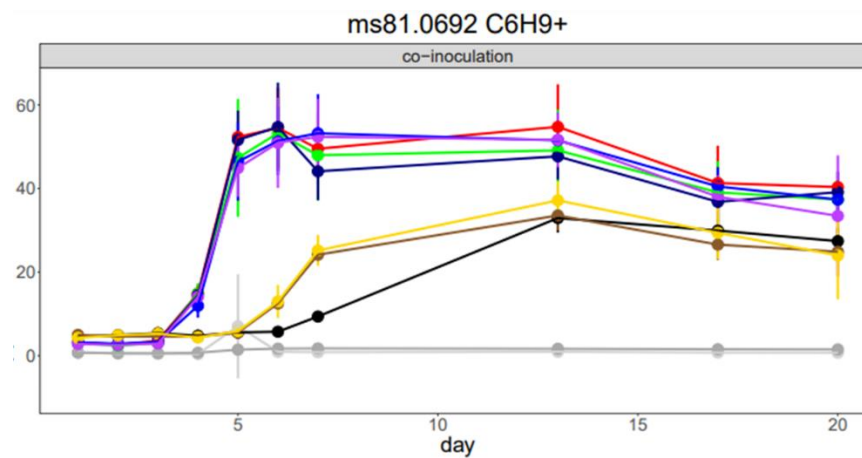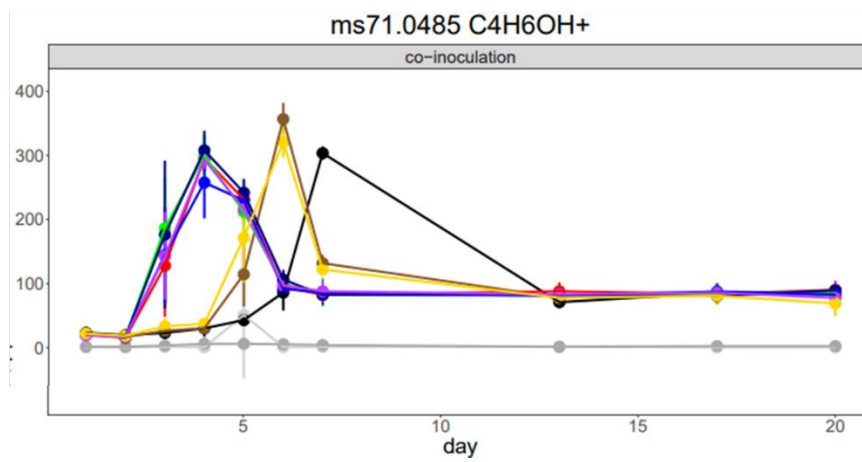
Fig. 11 PTR-MS as a powerful bioprocess-monitoring tool (alcoholic and malo-lactic fermentation) in real time

a)
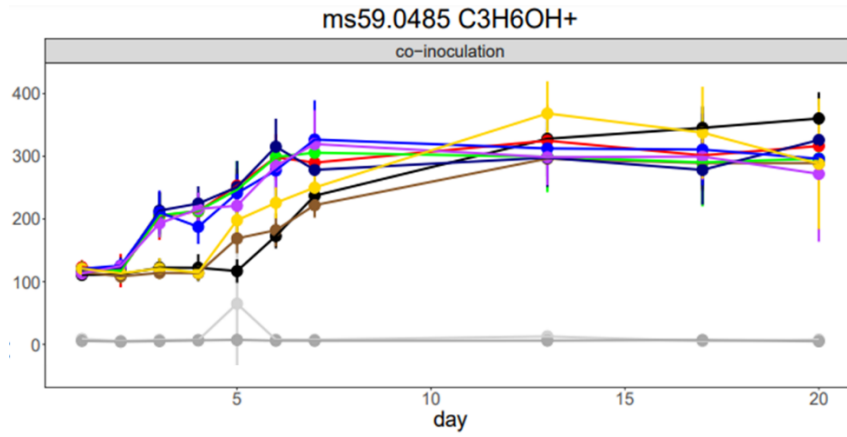
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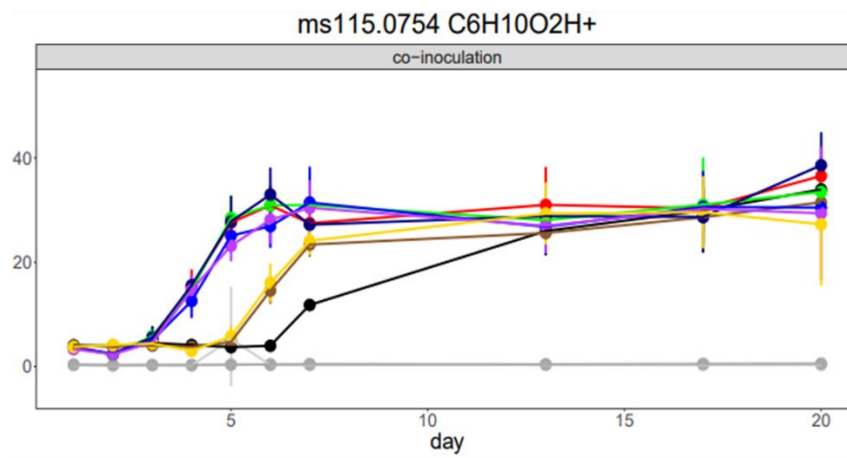
c)
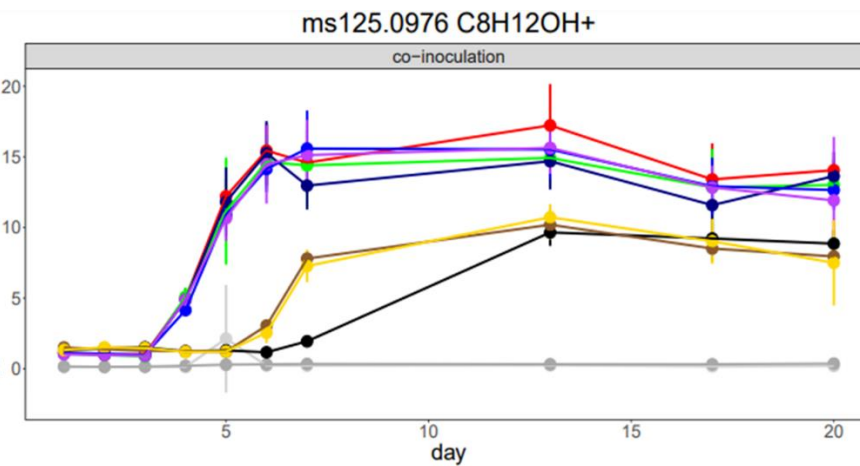
Fig.12 Similar trends category of: a) ketones (m/z 59,0485, t. i. as Acetone), b) esters (m/z 115.075, t. i. as Caprolactone) and c) other fragrance agents (m/z 125,098, t. i. as (Z)-1,5-octadien-3-one

Tab.5 For the bacterial strains the mean concentrations and standard deviations are reported. Statistically significant differences between experimental modes are established by means of one-way ANOVA ($p<0.05$): a) point 7 co-inoculum; b) point 20 co-inoculum; c) point 20 sequential inoculum.

a)

Meas. mass	Strain F	Strain G	Strain H	Strain I	Strain L	Strain L
44.057	19,91±12,8[b]	20,6±14,5[ab]	19,83±11,83[b]	20,6±16,83[ab]	21,3±23,16[ab]	23,26±31,83[a]
48.052	5384,5±19,5[ab]	5572±21,3[ab]	5162,2±16,3[ab]	5815±24,3[a]	5850±24,3[a]	3853,5±5,16[b]
55.017	290,5±20,3[ab]	230,2±11,1[b]	244,54±14,5[ab]	236±15,5[ab]	263,6±18,1[ab]	340,03±31,3[a]
62.031	178,17±21[ab]	178±23,16[ab]	165,3±13,3[ab]	181,2±22,6[ab]	185,7±25,3[a]	136,40±5,5[b]
67.054	171,0±19,5[ab]	178,4±21,5[ab]	161,9±14,83[ab]	189,9±24,5[ab]	189,1±24,6[a]	125,26±6[b]
69.032	10,05±18,5[ab]	10,4±21,16[ab]	10,06±16,16[ab]	11,42±24,3[a]	11,35±25,5[a]	8,27±5,3[b]
71.084	2994,3±19,1[ab]	3198±22,66[a]	2845,1±15,5[ab]	3382±24,5[a]	3331±25,1[a]	1962,81±4[b]
85.027	5,26±22,66[a]	5,22±22,66[a]	4,59±12,83[ab]	5,35±24,83[a]	5,28±24,5[a]	3,16±3,5[b]
90.062	800,98± 20[a]	882,6± 23,6[a]	719,22±14,8[ab]	874,6±24,1[a]	896,5±24,8[a]	459,63±3,5[b]
97.061	5,47±19,66[ab]	5,77±19,66[ab]	5,27±16,5[ab]	6,49±26,5[a]	6,20±23,6[a]	3,45±5[b]
99.078	42,4±21,6[a]	43,1±20,8[ab]	36,01±12[ab]	45,1±25,33[a]	46,05±26[a]	29,03±5,1[b]
101.05	24,3±18,1[ab]	26,01±22,7[a]	22,82±15,67[ab]	26,34±25[a]	26,5±25,17[a]	16,6±4,33[b]
103.07	87,1±19,3[ab]	96,9±23,67[a]	81,29±14,83[ab]	95,6±23,33[a]	97,90±24,5[a]	59,68±4,33[b]
109.06	3,3±20,17[ab]	3,7±23,17[ab]	2,92±13,3[ab]	3,49±23[ab]	3,66±25[a]	2,74±6,33[b]
111.06	2,90±20[ab]	3,07±21,33[ab]	2,68±15,33[ab]	3,31±24,17[a]	3,13±25[a]	1,75±5,17[b]
115.11	0,83±17[ab]	0,75±13,17[b]	0,71±11,83[b]	0,93±19,83[ab]	0,84±17[ab]	1,33±32,17[a]
117.09	346,12±20,5[a]	374±22,67[a]	307,1±14,17[ab]	369,4±24,8[a]	381,1±25,17[a]	210±3,67[b]
123.08	2,01±19,33[ab]	2,05±20[ab]	1,93±15,33[ab]	2,24±26,33[a]	2,20±24[ab]	1,63±6[b]
125.09	14,66±22[a]	15,4±21,83[a]	13,31±15[ab]	15,94±24,5[a]	15,28±24,17[a]	7,55±3,5[b]
127.11	64,55±21,67[a]	68,3±22,17[a]	59,67±15[ab]	69,5±24,3[a]	67,76±24,33[a]	34,38±3,5[b]
131.10	307,12±21[a]	330,2±22,8[a]	258,3±13,8[ab]	336,71±25[a]	337,4±24,83[a]	114,05±3,5[b]
136.13	25,90±17,83[ab]	29,2±22,17[ab]	25,86±16,67[ab]	31±24,17[a]	32,04±25[a]	17,42±5,17[b]
137.13	6,05±21,17[ab]	5,93±19[ab]	5,95±18[ab]	6,56±23[a]	6,94±25,67[a]	3,23±4,17[b]

141.08	0,63±19,33[ab]	0,67±22,67[ab]	0,58±14,5[ab]	0,67±25,33[a]	0,66±22,83[ab]	0,48±6,33[b]
143.10	8,82±21,33[ab]	9,03±21,17[ab]	7,97±14,17[ab]	9,73±25,5[a]	9,23±24,33[a]	5,95±4,5[b]
145.12	686,9±22,17[a]	701,6±21,6[a]	586,3±12,83[ab]	727,2±25,1[a]	737,62±25[a]	445,7±4,17[b]
147.12	5,43±19,5[ab]	5,62±21,33[ab]	4,68±14,33[ab]	5,77±23,67[a]	6,11±27,33[a]	3,74±4,83[b]
151.11	0,61±18,5[ab]	0,66±22,5[ab]	0,61±15,17[ab]	0,69±22[ab]	0,72±26,58[a]	0,49±6,25[b]
157.12	4,25±21,67[a]	4,32±20,83[ab]	3,83±15,5[ab]	4,71±25,83[a]	4,4±23,33[a]	2,72±3,83[b]
169.12	1,47±16,83[ab]	1,51±21,83[ab]	1,35±14,17[ab]	1,62±21,83[ab]	1,68±30[a]	1,01±6,33[b]
173.03	2,36±20,17[ab]	2,37±20,5[ab]	2,18±15,67[ab]	2,78±26,17[a]	2,58±23,83[a]	1,19±4,67[b]
177.11	40,94±25[a]	39,9±20,3[ab]	34,41±17,17[ab]	37,15±22,8[a]	36,92±21,5[ab]	15,96±4,17[b]
181.18	2,38±19,17[ab]	2,50±22,33[ab]	2,17±16,33[ab]	2,85±24,5[a]	2,87±24,17[a]	1,23±4,5[b]
187.17	5,20± 23,17[a]	5,14± 19,6[ab]	4,84± 16,00[ab]	5,40± 24,00[a]	5,29± 23,67[a]	3,17± 4,50[b]

b)

Meas. mass	Strain F	Strain G	Strain H	Strain I	Strain L	Strain L
41.038	455,8±19,1[ab]	434,82±6[b]	450,39±16,83[b]	452,7±16,33[b]	467,6±19,8[ab]	561,5±32,8[a]
44.057	16,58±17[ab]	16,08±8,5[b]	17,5±19,83[ab]	17±17,17[ab]	17,77±20[ab]	20,72±28,5[a]
62.031	170,8±28,1[a]	159,1±20,8[ab]	161±21,33[ab]	152,1±18,3[ab]	154±14,8[ab]	132,2±7,5[b]
81.069	40,27±26,3[a]	37±18,6[ab]	38,4±21,83[ab]	37,4±18,33[ab]	39,60±20[ab]	25,01±5,8[b]
99.078	34,29±27,1[a]	29,85±18,3[ab]	31,05±22[ab]	29,7±17,33[ab]	31,99±20[ab]	20,4±6,17[b]
107.039	3,53±22,5[a]	3,97±28[a]	2,91±18,83[ab]	2,77±14,5[ab]	4,18±23,67[a]	0,69±3,5[b]
109.047	1,22±28,17[a]	1,04±18,67[ab]	1,07±21,5[ab]	0,99±15,17[ab]	1,28±19,83[ab]	0,76±7,67[b]
125.097	14,16±26,6[a]	12,83±19,3[ab]	13,34±23[a]	12,55±17,3[ab]	13,81±20[ab]	8,02±4,67[b]
127.111	57±28,17[a]	49±19,17a[b]	51,75±23[a]	49,21±18,3[ab]	51,56±18[ab]	29,74±4,3[b]
143.107	8,27±27,17[a]	7,43±17,5[ab]	7,83±22,33[ab]	7,27±15,83[ab]	8,02±20,67[ab]	5,46±7,5[b]
145.123	544,92±28[a]	465,1±18,5[ab]	486,3±21,6[ab]	458±17,33[ab]	499,2±19,6[ab]	300,5±5,8[b]
147.128	4,60±27,83[a]	3,92±17,33[b]	4,02±21,17[ab]	3,97±17,5[ab]	4,21±19,17[ab]	2,83±8[b]
155.143	6,81±24,83[a]	6,02±19,5[ab]	6,47±20,83[ab]	6,19±23[a]	6,37±18,33[ab]	4,12±4,5[b]
171.139	16,19±28,1[a]	14,7±18,83[ab]	15,02±22,5[a]	14,40±17[ab]	15,30±19,67[ab]	9,21±4,83[b]

173.155	986,5±27,8[a]	870,4±19,6[ab]	903,2±23,33[a]	844,94±17[ab]	905,51±18,8[ab]	512,3±4,3[b]
177.110	68,02±26,6[a]	55,50±21[ab]	59,69±25[ab]	44,00±13[ab]	58,07±18,33[ab]	38,13±7[b]
191.161	24,16±27,8[a]	20,5±18,17[ab]	21,87±23[ab]	20,49±17[ab]	22,14±19,17[ab]	13,1±5,83[b]
201.187	109,7±25,6[a]	95,7±19,33[ab]	105,4±21,83[ab]	100,35±21[ab]	101,5±18,6[ab]	61,32±4,5[b]
219.192	39,47±26,5[a]	33,86±18,5[ab]	36,88±23,83[ab]	33,27±17[ab]	36±19,17[ab]	19,57±6[b]

c)

Mass	Strain F	Strain G	Strain H	Strain I	Strain L	Strain L
41.038	464,5±11,5[bc]	475±17,6a[bc]	502,70±26[a]	499±30,17[a]	482,81±20,83[ab]	448,25±4,83[c]
91.025	5,81±1,67[ab]	5,78±16[ab]	6,97±22,83[ab]	6,47±25,67[a]	6,69±25,33[a]	4,98±5,5[b]

Tentatively identification

Temporary identification of the isomeric ions produced (both molecules and fragments) was carried out by comparison with the reports of the previous PTR-MS literature.

The peak at m/z = 33.03 corresponds to [CH4O]H+ could be methanol, a secondary product of alcoholic fermentation, which can give problems if present in the final product. The peaks at m/z = 46.03, [C2H4O]H+, can be tentatively assigned to acetaldehyde, one of the main intermediates of alcoholic fermentation. The peak at m/z = 59.05, of formula [C3H6O]H+, might correspond to the isomer acetone which could have a negative impact on wine smell, giving solvent-like aromas. The peak at m/z = 73.06 of formula [C4H8O]H+ is probably 2-butanone which is important from the oenological point of view.

Hexanal, cis-3-Hexen-1-ol, (Z)-2-Hexen-1-ol (m/z = 83,081) and E)-2-Hexenal (m/z = 99,078) help to give a green, vegetable, grass and herbal aromas when present in wine.

The peak of m/z = 85,064 corresponds to [C5H8O]H+ could be (E)-2-Pentenal, which could give a pungent, green and fruity apple-like aromas. The peak at m/z = 90.06, C3[13]CH8O2H+, is tentatively identified as ethyl acetate, an ester produced during the fermentation of wine by the combination of acetic acid and ethyl alcohol and an important molecule involved in wine quality. The peak at m/z = 97.027 of formula [C5H4O2]H+, most probably corresponds to furfural, a molecule present in barrel-aged wines which can give almond-like aromas [146,162] Furthermore, furfural is an important intermediate of the Maillard reaction of pentose or ascorbic acid, relevant in bread aroma and color (Murata et al. 2007). The mass peak m/z = 101.09 corresponds to [C6H12O]H+ is probably 4-Methyl-2-pentanone. The peak at m/z = 115.07, of formula [C6H10O2]H+, can correspond to _-caprolactone, responsible for sweet and

coumarin-like smell in wine. The peak at m/z = 115.11, assigned to the formula [C7H14O]H+, might probably be 2-heptanone that could produce blue cheese. Numerous molecules could be responsible for the peak m/z = 127,112, of formula [C8H14O]H+: 1-octen-3-one, usually found in fruits, is the odorant that is responsible for the typical metallic smell ; 6-methyl-5-hepten-2-one and 2-Octenal (E) which has a green citrus peel smell. The peak m/z = 137.11, [C10H17]H+, is related to various monoterpenes, and might possibly be limonene or myrcene, both molecules can give pleasant smell to wine. There are several peaks associated to higher alcohols usually synthesized by yeast: m/z = 41.038, C3H5+, related to common fragment among them we tentatively identified as 3-methyl-1-butanol and 2-methyl-1-butanol, isobutyl alcohol and 1-butanol and others; m/z = 71.084, C5H11+, t.i. either 3-methyl-1-butanol or pentanol and m/z = 57.069, C4H9+, t.i. a dehydrated fragment of butanol isomers [139,146,162,166].

Tab.4 Volatile organic compound tentatively identified

Compounds identification	Ions	Meas. mass g/mol	Theor. mass g/mol	Ref
Aldehydes				
Acetaldehyde	C[13]CH4OH+	46,0368	45,061	[162,167–169]
Butenal	C4H6OH+	71,0485	71,099	[162]
Hexanal*,cis-3-Hexen-1-ol*, (Z)-2-Hexen-1-ol*	C6H11+	83,0817	83,154	[167,168, 170]
Furfural	C5H4O2H+	97,0276	97,093	[162,169, 171]
(E,E)-2,4-Hexadienal (1)*, (E,E)-2,4-Hexadienal (2)*	C6H8OH+	97,0619	97,137	
E)-2-Hexenal*	C6H10OH+	99,0788	99,153	[168]
Nonalactone	C9H16O2H+	157,123	157,233	
1-Nonanal*	C9H18OH+	143,145	143,25	[168]
Octanal*	C8H16OH+	129,127	129,223	[168,172]
Heptenal	C7H12OH+	113,099	113,18	[168]
3-Methylfurfural	C6H6O2H+	111,042	111,12	
Ketones				
Acetone*	C3H6OH+	59,049	59,088	[162,168]
2-Butanone*	C4H8OH+	73,064	73,115	[162,168]
Mercaptoacetone	C3H6OSH+	91,026	91,148	
4-Methyl-2-pentanone*	C6H12OH+	101,094	101,169	
2-Heptanone*	C7H14OH+	115,112	115,196	[168]
2-Methyl-3-Heptanone*,3-Octanone*, 2-octanone*	C8H16OH+	129,127	129,223	[168]
Alcohols				
Methanol	CH4OH+	33,033	33,05	[162,167–169]
Ethanol*	C[13]CH6OH+	48,052	47,077	[162,168]

Butanol*	C4H9+	57,069	57,116	[172]
3-methyl-1-butanol*, Pentanol*	C5H11+	71,084	71,143	[170,173]
Acids				
Acetic acid	C[13]CH4O2H+	62,032	61,06	[168–170]
Butanoic acid	C3[13]CH8O2H+	90,063	89,114	[168,170, 173]
Benzoic acid	C7H6O2H+	123,047	123,131	[168,170, 171]
Esters				
Ethyl Acetate	C3[13]CH8O2H+	90,0626	89,114	[171,173]
2-Butenoic Acid Methyl Ester*	C5H8O2H+	101,058	101,125	
Ethyl sorbate	C8H12O2H+	141,089	141,19	
Caprolactone	C6H10O2H+	115,075	115,152	[162]
Terpenes				
Various monoterpenes	C10H17+	137,113		[162]
Furans				
Furanone	C4H4O2H+	85,028	85,082	[168]
Methylfuran	C5H6OH+	83,047	83,11	[169]
Other compounds				
Maltol	C6H6O3H+	127,038	127,119	[169]
1-octen-3-one*,6-methyl-5-hepten-2-one*, 2-Octenal (E)*	C8H14OH+	127,112	127,207	[168]
(Z)-1,5-octadien-3-one	C8H12OH+	125,098	125,191	
(E)-2-Pentenal*	C5H8OH+	85,064	85,126	
Butyrolactone*	C4H6O2H+	87,043	87,098	
Isobutyl butyrate*, Butanoic Acid Butyl Ester*, Ethyl Hexanoate*, Hexyl Acetate*, Octanoic Acid*	C8H16O2H+	145,123	145,222	[174,175]

Isopropyl Butanoate*, Ethyl Isovalerate*, Isoamyl Acetate*, Amyl Acetate*, Hexanoic Acid Methyl Ester*	C7H14O2H+	131,107	131,195	[175]
Methyl Isovalerate*, Ethyl Butyrate*, Butyl Acetate*, 2,2-dimethyl-butanoic acid*, 4-Methyl Pentanoic Acid*, Hexanoic Acid*	C6H12O2H+	117,091	117,168	[169]
Methyl Butyrate*, Pentanoic Acid*, 2-Methyl Butanoic Acid*	C5H10O2H+	103,073	103,141	

Principal component analysis (PCA) was performed on the log transformed and mean centered data. Fig.12. The PCA analysis applied to the final data matrix showed 2 groupings for PC1 vs PC2.
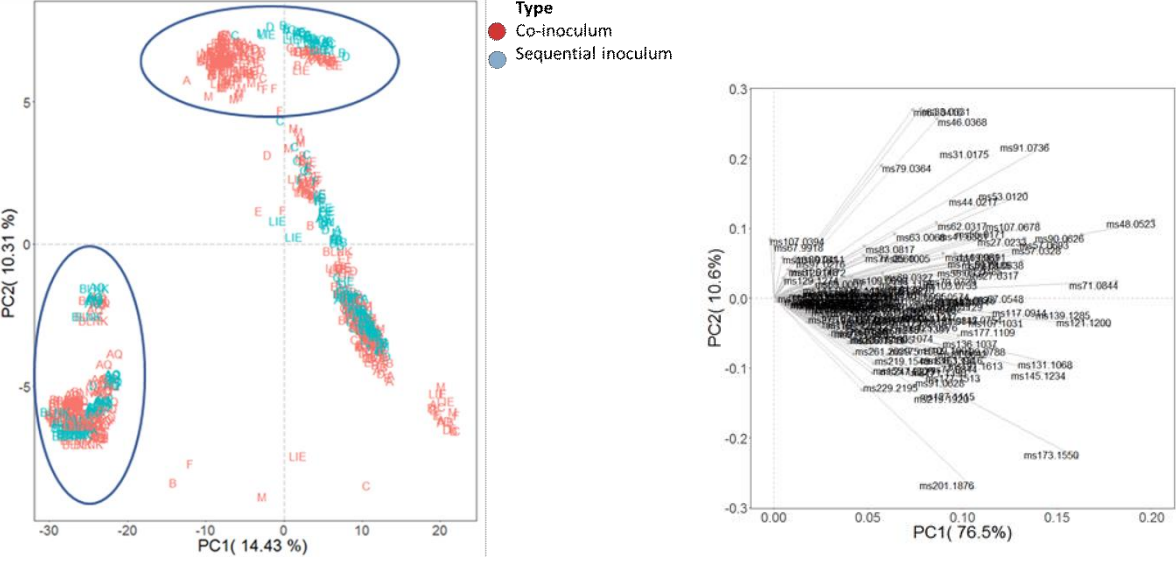
Fig.12 Principal component analysis (PCA)

5. CONCLUSIONS

The availability of complete genome sequences of industrial *L. plantarum* strains, may be useful to understand stress adaptation mechanisms at strain level and further identify cellular biomarkers.

The activities carried out, such as growth of *L. plantarum* strains, DNA extraction and related quantification, have allowed to obtain a DNA with a good quality and amount, ready to be used for whole genome sequence analysis. Then, the extracted DNA has been used for genome sequencing of the *L. plantarum* strains analyzed.

Acidic stress conditions. The observations reported led us to suggest that *hsp 2* gene may be a suitable candidate as stress marker to identify industrially important *L. plantarum* strains for fermented food on which low pH values are common (e.g. malolactic fermentation in wine).

Oxidative stress conditions. Apparently, the stress response of *Lactobacillus plantarum* strains A and C to oxidative stress was faster than strains D and B in terms of gene expression. In particular, the expression of *hsp 3* and *trxA2* genes was higher in *L. plantarum* strain A and C. *groEL* gene expression was also observed in strains A and C when low pH stress was applied. We can confirm that HSPs play a crucial role in a heat stress response, but they are also produced by different stress situations, e.g. acid or oxidative stress. In particular, the presence of H_2O_2 induces the expression of *hsp1, hsp2* and *groEL* genes in *L. plantarum* A, E and F respectively. The *groEL* expression was also observed in strains A and C when low pH stress was applied. The *trxA* gene, encoding a thioredoxin, was found to be strongly induced by the presence of H2O2 in strains D and F respectively after 90 and 30 min.

Apparently, the stress response of *L.plantarum* strains E and F to oxidative stress (H_2O_2) was faster than commercial strains in terms of gene expression. In particular, the expression of *groEL, clpB* and *trxA2* genes in strain F was observed after 30 min.

Preliminary observations suggest that, if we consider stress markers induction, within the commercial and autochtonoues strains analyzed, *L. plantarum* strains A and D seem to be the most robust among all commercial strains. Furthermore, a combination of *hsp2* and *hsp3* genes as stress responsive markers, may be suitable to identify the most resistant *L. plantarum* strains, to low pH value and oxidative stress.

Finally, *L. plantarum* volatilome changes was investigated and monitored in a rapid, real-time and nondestructive way during the whole fermentation process. PTR-ToF-MS coupled to a multipurpose GC autosampler guaranteed the high throughput, reproducibility and

automatization of the experiment and this approach may be useful to identify, even at VOCs level, markers suitable to improve robustness in *L. plantarum* strains, industrially important.

Appendix

List of scientific publications

M. P. Arena, V. Capozzi, **A. Longo**, P. Russo, S. Weidmann, A. Rieu, J. Guzzo, G. Spano, D. Fiocco. The phenotypic analysis of *Lactobacillus plantarum shsp* mutants reveals a role for hsp1 in cryotolerance. 2019. *Frontiers Microbiology*. (doi: 10.3389/fmicb.2019.00838). Impact Factor 4.259; H Index 88; Q1.

A. Longo and G. Spano. 2019. Stress responses of LAB. In: Food Molecular Microbiology. Chapter 9, 164-181. *CRC Food Biology Series press, Taylor & Francis Group.* Edited by Spiros Paramithiotis and Jayanta Kumar Patra (eds.) ISBN 978-1-138-08808-5.

D. Fiocco, **A. Longo**, M. P. Arena, P. Russo, G. Spano and V. Capozzi. 2019. How probiotics face food stress: they get by with a little help. *Critical Reviews in Food Science and Nutrition*. (https://doi.org/10.1080/10408398.2019.1580673). Impact Factor 6.704; H Index 135; Q1.

P. Russo, C. Fares, **A. Longo**, G. Spano and V. Capozzi. 2017. *Lactobacillus plantarum* with broad antifungal activity as a protective starter culture for bread production. *Foods*, 6, 110; (doi:10.3390/foods6120110). Impact Factor 3.011.

Participation to national and international congresses

M. P. Arena, P. Russo, **A. Longo**, G. Spano and V. Capozzi. 2018. Bread and 'microbiodiversity': safety, quality, and bioprotection. *XII° Convegno Nazionale sulla Biodiversità*, Giugno 2018, Teramo, Italy

A. Longo, M. P. Arena, D. Fiocco, C. Berbegal, V. Capozzi, G. Spano (2017). Oxidative and Acidic Stress response of exopolysaccharides-producing *Lactobacillus plantarum* Lp90. The *7th Congress of European Microbiologists, Valencia, Spain, from 9-13 July, 2017.*

A. Longo and G. Spano. Comparative genome analysis of *Lactobacillus plantarum* strains and identification of common and/or unique technological traits**.** *International Conference on Microbial Diversity (MD),* Ottobre 2017, Bari, Italy.

M. P. Arena, **A. Longo**, V. Capozzi, G. Spano and D. Fiocco. Small heat shock proteins characterization in a probiotic model. 2018. *XVIII Congresso Nazionale AIBG*, Ferrara, Italy

Participation to courses

Research activity and specific training on worker safety - average risk, in the Laboratories of the Research Unit (Sensory Quality) of the Food Quality and Nutrition Department of the Research and Innovation Center, Edmund Mach Foundation. San Michele all'Adige, Italy. 02/09 – 02/10/2018.

Exams: Applied biology (7 CFU, CdL medicine and surgery)

Marketing principles (8 CFU, Cdl Economy)

Training period on the use of PTR-ToF-MS. Laboratories of the Research Unit (Sensory Quality) of the Food Quality and Nutrition Department of the Research and Innovation Center, Edmund Mach Foundation. San Michele all'Adige, Italy. 16/04 - 24/04/2018.

Training course for the acquisition of 24 CFU in the anthropo-psycho-pedagogical disciplines and in teaching methods. December 2017 - May 2018.

Wine production and stabilization, Port wine sensory evaluation, 09/06/2017 at SAFE Department (Foggia University) with Fernanda Cosme (Assistant Professors in Food Science and Oenology della Trás-on-Montes and Alto Douro University (UTAD, Portugal)

Wine microbiology, 12/06/2017 at SAFE Department (Foggia University) with António Inês (Assistant Professors in Food Science and Oenology della Trás-on-Montes and Alto Douro University (UTAD, Portugal)

20-hour course on PROJECT MANAGEMENT delivered at the University of Foggia from 23rd March-9th May 2017 with Professor Aziz Houadria

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