ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

EVOLUTIONARY ENGINEERING OF POLYPHENOL RESISTANCE IN LACTIC ACID BACTERIA

Ph.D THESIS

Tarık ÖZTÜRK

Molecular Biology-Genetics and Biotechnology Department

Molecular Biology-Genetics and Biotechnology Graduate Program

NOVEMBER 2018

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To my spouse and children,

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ABBREVIATIONS

LAB	: Lactic Acid Bacteria
IS	: Insertion Sequence
EFSA	: European Food Safety Authority
QPS	: Qualitatively Presumed as Safe
EPS	: Exopolysaccharide
GI	: Gastrointestinal
MRS	: de Man, Rogosa and Sharpe
TNO	: Netherlands Organisation for Applied Scientific Research
DSMZ	: German Collection of Microorganisms and Cell Cultures
PBS	: Phosphate Buffer Saline
OD	: Optical Density
OE	: Olive Extract
CDW	: Cell Dry Weight
CFU	: Colony Forming Unit
EMS	: Ethyl Methane Sulfonate
MPN	: Most Probable Number
HPLC	: High Performance Liquid Chromatography
RI	: Refractive Index
TIM-1	: TNO *In vitro* Model One

SYMBOLS

°C : Degrees Celsius
g : Gram
mM : Millimolar
l : Liter
ml : Milliliter
rpm : Revolutions per minute
vol : Volume
w : Weight
µm : Micrometer
µl : Microliter
mg : Milligram
µ : Specific Growth Rate
kg : Kilogram
pH : Negative logarithm of hydrogen ion concentration

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EVOLUTIONARY ENGINEERING OF POLYPHENOL RESISTANCE IN LACTIC ACID BACTERIA

SUMMARY

The aim of this study was to obtain robust starter cultures for more efficient olive fermentations by developing stress-resistant lactic acid bacteria, more preferably *Lactobacillus plantarum* strains by using evolutionary engineering approach. Since resistance mechanisms in lactic acid bacteria involve numerous genes and many regulators, it would be difficult to obtain the desired resistant phenotypes by direct genetic engineering or over-expressing certain genes. Thus, evolutionary engineering could be a good alternative strategy to obtain the desired phenotypes. In this approach, the resulting strains are not considered as genetically modified organisms which makes them more acceptable for the food industry.

It was generally thought that lactic acid bacterial starter cultures for olive fermentations were not robust enough due to the presence of olive polyphenols and mainly oleuropein in fermentation brines. In the first part of this thesis, this hypothesis was tested by determining the resistance of various strains to stress factors that are commonly encountered during olive fermentations. The results revealed that NaCl, and not oleuropein, was mainly responsible for the decreased growth rates of lactic acid bacteria during olive fermentations.

For the evolutionary engineering studies, a *L. plantarum* strain (T129) isolated from table olive-producing companies in the Marmara Region was obtained from TUBITAK MAM Food Institute collection and a *L. plantarum* type strain DSMZ 10492 isolated from olive-processing brines in Portugal was obtained from DSMZ Culture Collection.

These strains were treated with EMS (ethyl methane sulfonate) to obtain two different, genetically diverse initial populations for selection. The NaCl resistance of these populations was increased by applying increasing levels of NaCl stress during successive batch cultures of selection. Mutant individuals were selected from the final mutant populations that resisted 10 % (w/v) NaCl. The survival rates of mutant individuals were compared with those of the wild-type at 10% (w/v) NaCl and 5 g/l oleuropein-containing 10% (w/v) NaCl. Most of the mutant individuals survived both NaCl and oleuropein stress, unlike their wild-type strains.

The cross-resistance of selected mutant individuals against commonly encountered stress factors during olive fermentations were determined to confirm the suitability of the mutants as robust olive starter cultures. One mutant strain derived from *L. plantarum* DSMZ 10492 (D2) and one mutant strain derived from the *L. plantarum* T129 (T8) were chosen for further studies. The NaCl resistance of the mutant strains during five successive cultivations in non-selective (stress-free) media were determined and the results confirmed that NaCl resistance was not an adaptation, but had a heritable genetic background. These genetically stable strains could therefore

be used as starter cultures for olive fermentations without losing their gained resistance.

Another aim of this thesis study was to obtain lactic acid bacteria that could be used as probiotic cultures for olive fermentation. Such a starter culture would not only ensure a standardized olive fermentation, but also provide added health benefits to its user. Thus, lactic acid bacteria need to be engineered to increase their survival during the passage through the upper digestive tract. It is difficult, however, to select a single stress condition to obtain efficient probiotic mutants.

Ingested microorganisms are faced with a series of stress factors during their passage through the gastrointestinal tract. Time-based decrease in pH and the presence of gastric enzymes are major stress factors in the stomach. While peristaltic movement of the small intestine prevents microbial colonization, weakened bacteria are faced with pancreatic enzymes, bile salts and nutrient deprivation in different sections of the small intestine. Thus making use of an *in vitro* model of the upper gastrointestinal tract was the novel evolutionary engineering strategy to select for more viable, robust and efficient probiotic strains.

It was hypothesised that mutant individuals with high survival rates would be enriched during the consequent passages through the *in vitro* model. By this strategy, it would be possible to isolate an individual with increased survival. The TNO *in vitro* model of the stomach and small intestine (TIM-1) is a computer-controlled dynamic model that accurately mimics the dynamic conditions in the upper parts of the human digestive system. TIM-1 consists of four compartments: the stomach, duodenum, jejunum and ileum. It mimics body temperature, peristalsis, intestinal passage, specific pH conditions, enzyme and electrolyte levels as close as possible to the physiological conditions. Therefore, the EMS-mutagenized rifampicin-resistant mutant population of T129 was introduced to this system for five successive times. At the end, an individual strain, J1, was selected with a survival rate close to 100%. The genetic stability of this strain was determined by reintroducing this strain to the TIM-1 system after stress-free cultivation. The survival in the upper gastrointestinal tract was preserved which showed that the mutant strain was genetically stable.

Finally the genetic background of the increased survival of the J1 strain was investigated by comparing the whole genome sequences of the wild type T129 and The mutant strain J1. It was confirmed that all mutations were either G to A or C to T, as expected typically for EMS mutagenesis. The whole genome was annotated and the genes that were affected by these mutations were determined as: phosphoribosylformylglycinamidine synthase subunit PurL, glutamine ABC transporter, permease protein, DNA repair protein RecN, ribosome small subunit-dependent GTPase A, molecular chaperone DnaK, DNA-directed DNA polymerase III, alpha chain PolC-type, phosphatidate cytidylyltransferase cell surface protein, Cardiolipin synthetase and ATP-dependent zinc metalloprotease FtsH. This revealed that changes in cellular metabolism, DNA repair, and cell wall composition might possibly have led to the increased survival of the mutant strain J1.

To conclude, novel selection strategies were successfully applied in this study to obtain stress-resistant and robust *L. plantarum* strains with improved probiotic properties for potential use in olive fermentations. Further transcriptomic and proteomic research would be necessary to understand the complex molecular basis of the desirable phenotypes.

LAKTİK ASİT BAKTERİLERİNİN POLİFENOL DİRENCİNİN EVRİMSEL MÜHENDİSLİK İLE GELİŞTİRİLMESİ

ÖZET

Bu çalışmanın amacı zeytin fermantasyonlarında starter kültür olarak kullanılabilecek, oleuropeine dirençli laktik asit bakterilerinin, tercihen *Lactobacillus plantarum* suşlarının evrimsel mühendislik yaklaşımı ile geliştirilmesidir. Laktik asit bakterilerinin direnç mekanizmaları pek çok gene ve düzenleyiciye bağlı karmaşık süreçler olduğundan, istenilen dirençli fenotiplerin klasik genetik mühendisliği teknikleri, ya da belirli genlerin aşırı ekspresyonu ile eldesi zordur. Bu nedenle evrimsel mühendislik, iyi bir alternatif strateji olabilir. Bu yaklaşımla elde edilen mikroorganizmalar genetik modifiye organizma olarak nitelendirilmediği için, gıda uygulamalarında daha çok kabul görmektedir.

Çalışmanın ilk aşamasında; zeytin fermantasyonlarındaki ana stres unsurunun, zeytinde en çok bulunan polifenol olan oleuropein stresi olduğu fikrinden yola çıkıldı. Ancak gerek izole edilen kültürler ile yapılan denemelerde, gerekse de kültür koleksiyonlarından elde edilmiş tip kültür çalışmalarında, oleuropeinin zeytinde bulunabilecek konsantrasyonlarda tek başına bakteri gelişimini inhibe edici etki gösteremeyeceği, ancak tuz (NaCl) varlığında sinerjik bir antimikrobiyal etki gösterebileceği belirlenmiştir. Bu sonuçlara göre, zeytin fermantasyonlarındaki asıl stres kaynağının oleuropein değil tuz olduğu anlaşılmıştır. Yapılan çalışmalar, suşların canlılığının %0-3 NaCl varlığında değişmediğini ancak % 8 NaCl varlığında oldukça azaldığını göstermiştir.

Tuza ve zeytindeki oleuropeine dirençli suşların evrimsel mühendislik yaklaşımı ile geliştirilmesi için, T129 ve DSMZ 10492 adlı yabanıl tip *L. plantarum* seçilmiştir. Bu suşlardan T129 Marmara bölgesindeki zeytin işleyen bir işletmenin zeytin salamura suyundan daha önce izole edilmiş olup, TÜBİTAK MAM Gıda Enstitüsü Koleksiyonundan temin edilmiştir. DSMZ suşu ise, Portekiz'deki bir zeytin işleme tesisindeki salamura suyundan daha önce izole edilmiş ve DSMZ Kültür Koleksiyonundan temin edilmiştir.

Her iki suş da farklı konsantrasyonlarda etil metan sülfonata (EMS) maruz bırakılmış ve böylece genetik çeşitlilikleri arttırılmıştır. Sonrasında ise canlılıklarına bakılarak 0.37 M EMS ile 30 dakika muamele edilen mutant popülasyonlar en uygun bulunmuştur. Her iki mutant topluluğu 32 ardışık kesikli kültür işlemi boyunca kademeli olarak artan NaCl konsantrasyonlarına alıştırılmış ve sonunda %10 (w/v) NaCl' ye dirençli mutant popülasyonlar elde edilmiştir. % 10 tuz direnci zeytincilik uzmanları tarafından zeytin işlemek için yeterli görüldüğünden % 10 tuz varlığında direnç kazandırma çalışmaları tamamlanmıştır. Mutant topluluklarından onikişer mutant suş izole edilmiştir. İzole edilen mutantlar MRS, %10 (w/v) NaCl içeren MRS ve %10 (w/v) NaCl ve 4 g/l oleuropein içeren MRS'de üretilip, her 24 saatte bir katı besi yeri ortamına ekilerek sayılmıştır.

Yapılan sayımlar sonucu mutant suşların büyük bir bölümünün yaban tiplerine göre hem NaCl, hem de NaCl ve oleuropein dirençlerinin çok arttığı belirlenmiştir Gerek T129, gerekse DSMZ 10492 yaban tip suşlarından elde edilen mutant suşların, büyük çoğunluğunun her iki yaban tipin de üreyemediği %10 (w/v) NaCl ve %0,4 (w/v) oleuropein varlığında rahatlıkla üreyebildikleri ve bu koşullara kayda değer düzeyde direnç kazanmış oldukları dikkat çekmiştir.

Mutant popülasyonların her ikisinden de seçilen onikişer mutant suşun, zeytin üretiminde oleuropein dışında karşılaşılabilecek diğer stres koşullarına karşı çapraz direnci de çalışılmıştır. pH 9'da alkali stresi, NaOH ile acılığı giderilen zeytinlerde yetersiz yıkama nedeni ile sıklıkla NaOH kalıntıları kaldığından seçilmiş; mutantların canlılığı pH'sı 9'a ayarlanmış olan, %50 seyreltilmiş MRS besi yerinde incelenmiştir. Zeytinlerde istenmeyen mikroorganizmaların üremesini engellemek için zeytin işleme sularına laktik asit ve asetik asit ilave edilmektedir. Bu stres koşullarına direncin de araştırılması için, mutant kültürlerin pH değerleri asetik asit ve laktik asit ile 4'e ayarlanmış, iki farklı %50 seyreltilmiş MRS besiyeri ortamındaki canlılıkları incelenmiştir. Siyah zeytinlere renk vermek amacı ile, %0.1 (w/v)'lik demir glükanat uygulaması yapılabilmektedir. Bu nedenle mutantların %0.1(w/v)'lik demir glükanat varlığındaki gelişmeleri incelenmiştir. Son olarak ise, çoğu starter kültür liyofilize toz formda satışa sunulduğu için suşların liyofilizasyon ile kurutmaya karşı dirençleri incelenmiştir. Yapılan çalışmalar sonrası bütün suşlar %0.1 (w/v)'lik demir glükonat varlığında yüksek gelişme göstermişlerdir. T129 suşu ve mutantlarının neredeyse tamamı pH 9 daki NaOH'a dayanırken, bu direnç DSMZ 10492'nin bazı mutantlarında kaybolmuştur. pH 4'te asetik asit veya laktik asit stresi bütün suşları zorlamıştır. Liyofilizasyon stresine direnç ise bazı mutantlarda artmış, bazılarında ise azalmıştır. Sonuç olarak tuz direnci ile ciddi ölçüde birlikte gözlenen bir direnç belirlenememiştir.

Yapılan çapraz direnç çalışmaları sonrasında T129 suşunun T8 kodlu mutantı ile DSMZ 10492 suşunun D2 kodlu mutantı fizyolojik çalışmalar için seçilmiştir. Seçilen suşlar tuzsuz ve %6,5 (w/v) tuz içeren seyreltilmiş MRS besiyerinde geliştirilmiş ve gelişimleri, glukoz tüketimleri ve laktik asit üretimleri incelenmiştir. Yapılan fizyolojik çalışmalar her iki mutantın yüksek tuz konsantrasyonlarına adapte olmak için muhtemelen farklı moleküler mekanizmalardan yararlandıklarına işaret etmektedir. T8 suşunda yüksek tuz konsantrasyonuna direnç muhtemel olarak hücre duvarında meydana gelen değişiklikler kaynaklı olabilecekken, D2 suşunda ise tuz direnci, çözünen karşıt maddelerin hücrede depolanması ile gelişmiş olabilir.

Tez çalışmasının üçüncü aşamasında ise, zeytinde starter olarak kullanılabilecek probiyotik bir suşun geliştirilmesine çalışılmıştır. Böyle bir suş; hem zeytin fermantasyonunun kontrollü bir biçimde ilerlemesini, hem de zeytinlerin probiyotik kaynağı olarak tüketilebilmesini sağlayabilir. Bu amaçla zeytin işleme sularından izole edilmiş olan T129 suşundan, üst sindirim sisteminden yüksek canlılıkta geçen bir suş geliştirilmesine çalışılmıştır.

Ağız yolu ile alınan bir suş, sindirim sisteminden geçişi sırasında pek çok stres koşulu ile karşılaşır. Yutulan suş, mide ortamında giderek düşen pH ve mide enzimlerine maruz kalırken, mideden sonra kolonize olması da peristaltik hareket ile engellenir. Bir yandan da zayıflamış suş, ince bağırsağın farklı bölgelerinde pankreatik enzimler, safra tuzları ve besin eksikliğine maruz kalır. Bu nedenle, elde edilen EMS mutajenine maruz kalmış ve rifampisin dirençli mutant popülasyonu *in vitro* bir sindirim sistemine verilerek bu sistemden canlı kalanların tekrar sisteme

verilmesi ile, sindirim sistemindeki bütün streslere dayanan bir mutant bulunabileceği hipotezi kurulmuştur. Bu amaçla, bilgisayar kontrolü ile insan üst sindirim sistemini dinamik bir biçimde doğru olarak taklit edebilen TIM-1 model sisteminin kullanılmasına karar verilmiştir. Sistemde bulunan dört bölme mide, onikiparmak bağırsağı, boş bağırsak ve kıvrım bağırsağı temsil etmektedir. Bu bölmelerde vücut sıcaklığı, peristaltik hareket, besin boşaltım hızı ve pH, enzim ve elektrolit seviyeleri gerçek fizyolojik koşullara mümkün olduğunca uygun olarak bölmeye özgü bir biçimde taklit edilmektedir. TIM-1 pek çok potansiyel probiyotiğin mide ve ince bağırsaktan geçişinde canlı kalma yüzdelerinin belirlenmesinde ve canlılığı sınırlayıcı faktörlerin ortaya konulmasında kullanılmış ve valide edilmiş bir sistemdir. Bu nedenle mutant T129 topluluğu sisteme beslenmiş ve sistemden çıkan popülasyonlar sisteme beş kez tekrar beslenerek sistemde yüksek oranda canlı kalan suşların seçimi yapılmıştır. Yapılan çalışmalar sonucu elde edilen mutant popülasyondan koloniler elde edilmiş ve tüp bazlı denemeler ile içlerinden J1 suşu seçilmiştir. J1 suşunun canlılığı TIM-1 sisteminde denenmiş ve suşun canlılığının yaban suşta %59,7 iken J1 mutantında %100'e yakın olduğu belirlenmiştir. J1 suşunun genetik kararlılığının belirlenmesi için J1 suşu seçici olmayan stressiz koşullarda art arda 3 kesikli kültürde toplam 88 saat büyütülmüş ve canlılığı TIM-1 sisteminde tekrar ölçülerek, suşun üst sindirim sisteminde canlı kalma yeteneğini koruduğu tespit edilmiştir. J1 suşunun üst sindirim sisteminde artan canlılığının genetik temellerinin araştırılabilmesi için J1 suşu ve T129 yabanıl tip suşunun genom dizileri yeni nesil dizileme yöntemi ile belirlenmiştir. Elde edilen dizilerin karşılaştırılmasının ardından iki genom arasında 32 nokta mutasyonu farkı olduğu görülmüştür. Mutasyonların hepsinin ya C'den T'ye ya da G'den A'ya nokta mutasyonları olduğu belirlenmiştir. Bu sonuç, oluşan mutasyonların, uygulanmış olan EMS mutajenez işlemi ile ilişkili olduğunu göstermektedir. Ayrıca; oluşan mutasyonlardan C'den T'ye 15, G'den A'ya ise 17 mutasyon saplanmış olması ve bakteri genomu ile kıyaslandığında oluşan mutasyon frekansı (10^{-5} olan literatür değeri ile uyumlu olduğu için), uygulanan mutasyon işleminin başarılı olduğunu göstermektedir. Nokta mutasyonların gen düzeyindeki etkilerinin belirlenebilmesi için genom anlamlandırması (annotation) yapılmıştır. Anlamlandırma çalışmaları sonrası nokta mutasyonların; genomda phosphoribosylformylglycinamidine synthase subunit PurL, glutamine ABC transporter, permease protein, DNA repair protein RecN, ribosome small subunit-dependent GTPase A, molecular chaperone DnaK, DNA-directed DNA polymerase III, alpha chain PolC-type, phosphatidate cytidylyltransferase, cell surface protein, cardiolipin synthetase ve ATP-dependent zinc metalloprotease FtsH genlerini etkilediği belirlenmiştir. Bu genlerde oluşan nokta mutasyonların protein düzeyindeki etkileri biyoinformatik incelemeler ile yorumlanmış ve J1 suşunun direnç kazanmak için muhtemelen hem hücre duvarı yapısını değiştirdiği, hem de DNA tamiri ve stres tepkisindeki önemli mutasyonların, hücrenin stres cevabını düzenleyerek üst sindirim sisteminde daha iyi adapte olacak şekilde geliştirdiği belirlenmiştir.

J1 suşunun bir diğer ilginç özelliği de hücre duvarı yapısında değişiklik olsa da hücre duvarındaki yapı değişikliğinin normal dışı hücre topaklanması gibi yapılara yol açmaması ve J1 suşunun stres cevabı ve DNA tamir sistemlerinde farklılıklar olsa da üreme hızının yabanıl tipten fark göstermemesidir.

Sonuç olarak, bu çalışmada; strese dirençli, genel anlamda dayanıklı ve probiyotik özellikleri de iyileştirilmiş *L. plantarum* suşlarının eldesi için, yeni seleksiyon stratejileri başarıyla uygulanmıştır. Elde edilen dayanıklı ve probiyotik özellikleri

iyileştirilmiş suşlar, zeytin fermentasyonlarında potansiyel olarak kullanılabilir. Stres direnci veya dayanıklılık gibi istenilen özelliklerin karmaşık moleküler mekanizmalarının anlaşılabilmesi için, transkriptomik ve proteomik düzeyde de kapsamlı çalışmaların yapılması gerekecektir.

1. INTRODUCTION

1.1 Lactic Acid Bacteria

Lactic acid bacteria (LAB) are a diverse group of bacteria. They can be characterized as bacteria that produce lactic acid as their main metabolic product, and they are gram-positive, non-spore forming and able to grow anaerobically. They are found in a wide range of environments ranging from animal and human body to plants. They play important roles in fermented foods made from many plant and animal products. Their ability to convert sugar to lactic acid has been exploited to conserve food by lactic fermentation since ancient times. Lactic acid bacteria are still used as starter culture in industrial or artisan food production. These cultures extend the shelf life and alter the flavor and texture of the product (Stiles 1996; De Vries et al., 2006; Khalid, 2011).

Lactic acid bacteria consist of several genera which are metabolically, physiologically, and phylogenetically relatively closely related. The main four genera among LAB are *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. Some other genera such as, *Aerococcus*, *Alloiococcus*, *Carnonbacterium*, *Dolosigranulum*, *Enterococcus, Globicatella, Lactococcus, Oenococcus, Tetragenococcus, Vagococcus* and *Weisella are* also part of Lactic acid bacteria group (Khalid, 2011).

Lactic acid bacteria are grouped in different genera by their morphology, mode of glucose utilization, growth at different temperatures, production of different lactic acid isomers, ability to grow at high salt concentrations and resistance to acid and alkali stress. Although phylogenetic analysis of lactic acid bacteria gives mostly consistent results, *Lactobacillus* and *Leuconostoc* genera can give inconsistent results due to high heterogeneity among these genera (Khalid, 2011).

Lactic acid bacteria optimally grow at slightly acidic pH, between 5.5-5.8. They depend on complex nutrients such as amino acids, peptides, nucleotides, vitamins, minerals, fatty acids and carbohydrates for growth (Khalid, 2011).

Lactic acid bacteria can convert sugar to lactic acid by two pathways. The first is glycolysis (Embden-Meyerhof) pathway which is frequently named as homolactic fermentation and the second is 6-phosphogluconate /phosphoketolase pathway, also called heterolactic fermentation. The main end product of homolactic fermentation is lactic acid. In contrast, the main end products of heterolactic fermentation are diverse as the name also dictates. Heterolactic fermentation results in production of ethanol, CO_2 and acetate nearby lactic acid. The fermentation characteristics of lactic acid bacteria are related to both species and environmental factors (Khalid, 2011).

Lactic acid bacteria do not only contribute to food fermentation but they also are present in the gut of animals. The number of lactic acid bacteria present in gut would be affected by animal species and age. *Lactobacillus crispatus, Lactobacillus gasseri,* and *Lactobacillus plantarum* are among few lactic acid bacteria species that are also present in human gut, in addition to being used in food fermentation (Çataloluk and Gogebakan, 2004).

1.1.1 Lactobacillus plantarum

L. plantarum can be found at many different sources. *L. plantarum* can contribute to positive or negative attributes depending on its environment. Positive attributes are its role in food fermentation and its probiotic effects, whereas negative attributes can be the spoilage of meat, orange juice or wine. A summary of products containing *L. plantarum* is given in **Table 1.1** (De Vries et al., 2006).

Table 1.1 : Products containing *L.plantarum.*

Source	Product	Reference
Plant Products	Olive	Quintana et al., 1999; Randazzo et al., 2004
	Cocoa beans	Ardhana and Fleet, 2003
	Cassava	Lei et al., 1999
	Sauerkraut	Stamer, 1971
	Togwa	Kingamkono et al., 1999
	Wine	Spano et al., 2004
Milk products	Stilton cheese	Ercolini et al., 2003
	Traditional feta cheese	Manolopoulou et al., 2003)
	Ricotta forte cheese	Baruzzi et al., 2000
Meat products	Fermented dry sausage	Cocolin et al., 2000; Enan et al., 1996, Gevers et al., 2003
	Fermented Italian sausage	Cocolin et al., 2000

The complete genome sequence analysis of *L. plantarum* WCFS1 has revealed that genes for uptake and utilization of various carbohydrates, peptide uptake, and synthesis of most amino acids were present. Kleerebezem et al. (2003) also predicted the presence of 217 surface anchored protein genes. Thus, it is predicted that this bacterium is able to adapt to many different circumstances (Kleerebezem et al., 2003)

A DNA-microarray based comparison study has revealed the difference among 20 *L. plantarum* strains. Differences were found to be at regions coding for plantaricin, non-ribosomal peptides, and exopolysaccharides beside prophages and IS-elements coding regions. The study found highest interspecies variability at a 200-kb region. This region was probably acquired by horizontal gene transfer and was coding for sugar metabolism. It was concluded that these differences in *L. plantarum* genomes could have served the strains to adapt to certain environmental niches (De Vries teal 2006).

1.1.2 Safety of Lactobacillus plantarum

L. plantarum has a long history of safe use in human consumption. It is utilized in large numbers at olive and sauerkraut fermentation. Although there are a high number of reports on safe consumption of *L.plantarum*, there are a limited number of cases where *L. plantarum* is accused to have caused infection. One *L. plantarum* strain that was able to *in vitro* coagulate human blood by aggregating platelets was isolated from infective endocarditis. The ability to *in vitro* coagulate human blood could be a pathogenic feature, but this is shared by many lactic acid bacteria and is not known to occur *in vivo* (Harty et al., 1994).

It has also been shown that no *L. plantarum* was present in blood and heart 96 hours after inter-venous admission of 10^8cfu of *L. plantarum* 299v strain to Sprague-Dawly rats. Thus, *L. plantarum* 299v strain did not possess a risk even if it could pass the intestinal barrier (Adawi et al., 2002).

L. plantarum NCIMB 8826 which was a human saliva isolate was administrated to mice and no abnormalities were observed in mice intestine. It was reported to neither cause macroscopic or histologic inflammation nor abnormal dislocations among mice intestine (Pavan, 2003).

Furthermore, European Food Safety Authority (EFSA) considers *L. plantarum* as Qualitatively Presumed as Safe (QPS). Thus, it is enough to show the absence of resistance to antibiotics of human or veterinary importance to safely use *L. plantarum* in food and feed applications (EFSA, 2013).

1.2 Starter Cultures

A starter culture can basically be defined as large numbers of at least one defined bacterium that is intentionally added to a product in order to alter the product properties in a specific manner. Lactic acid bacteria are widely used starter cultures. They are added to products mostly to enhance shelf life, increase microbial safety, improve texture and give the product a pleasant sensory profile.

1.2.1 Industrial importance of starter cultures

The starter culture market is in multibillion-dollar size. The dairy starter cultures have the largest share in this market. Yoghurt and cheese starter cultures are dairy starters with the highest economic value (Douillard and de Vos, 2014).

Backsloping which is defined as to use some successfully fermented end product to ferment the next batch, is the oldest example of the use of starter cultures. The fermentations were faster and stuck fermentations were less common by these technique, since this continuous re-cultivation of bacteria at similar conditions results in the dominance of best adapted strains. Backsloping is still employed at sauerkraut or sourdough fermentation. Backsloping is an important technique in the production of fermented products where the precise mechanism for successful fermentation is not well known. Backsloping is used in less developed countries as an alternative to commercial starter cultures. Developed countries make use of this technique to make small-scale traditional products with special gastronomic characteristics (Holzapfel, W. H. 2002).

Fermented foods are an important part of food industry. Large amount of food is fermented at industrial scale. Thus, direct addition of starter cultures is essential for processing a large amount of food under advanced control and obtain a standardized product with fixed sensorial and physical properties. Lactic acid bacteria, used in the fermentation of different products are shown in **Tables 1.2** (Leroy et al., 2004).

Table 1.2 : Lactic acid bacteria used in the fermentation of products.

Fermented Product	Lactic acid Bacteria
	Dairy products
Hard Cheeses without eyes	*Lactococcus lactis ssp. lactis, Lactococcus lactis ssp. cremoris*
Cheeses with small eyes	*Lactococcus lactis ssp. lactis, Lactococcus lactis ssp. lactis var. diacetylactis, Lactococcus lactis ssp. cremoris, Leuconostoc mesenteroides ssp. cremoris*
Swiss and Italian-type cheeses	*Lactobacillus delbrucki ssp. lactis, Lactobacillus helveticus, Lactobacillus casei, Lactobacillus delbrucki ssp bulgaricus, Streptococus thermophilus*
Butter and Butter milk	*Lactococcus lactis ssp. lactis, Lactococcus lactis ssp. lactis var. diacetylactis, Lactococcus lactis ssp. cremoris, Leuconostoc mesenteroides ssp. cremoris*
Yoghurt	*Lactobacillus delbrucki ssp bulgaricus, Streptococcus thermophilus*
Fermented probiotic milk	*Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus johnsonii, Bifidobacterium* lactis, Bifidobacterium* bifidum, Bifidobacterium* breve*
Kefir	*Lactobacillus kefir, Lactobacillus kefiranofeciens, Lactobacillus brevis*
	Fermented meats
Fermented sausage (Europe)	*Lactobacillus sakei, Lactobacillus curvatus*
Fermented sausage (USA)	*Pediococcus acidilactici, Pediococcus pentosaceus*
Fermented fish products	*Lactobacillus alimentarius, Carnobacterium piscicola*
	Fermented fruit and vegetable
Sauerkraut	*Leuconostoc mesenteroides, Lactobacillus plantarum, Pediococcus acidilactici*
Pickles	*Leuconostoc mesenteroides, Pediococcus cerevisae, Lactobacillus brevis, Lactobacillus plantarum*
Fermented olives	*Leuconostoc mesenteroides Lactobacillus pentosus, Lactobacillus plantarum*
Fermented vegetables	*Pediococcus acidilactici, Pediococcus pentosaceus, Lactobacillus plantarum, Lactobacillus fermentum*
Soy sauce	*Tetragenococcus halophilus*

Table 1.2 (continued) : Lactic acid bacteria used in the fermentation of products.

Fermented Product	Lactic acid Bacteria
	Fermented cereals
Sourdough	*Lactobacillus sanfransiscensis, Lactobacillus farciminis, Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus amylovorus, Lactobacillus reuteri, Lactobacillus pontis, Lactobacillus panis, Lactobacillus alimentarius, Weissella cibaria*
	Alcoholic beverages
Wine (malolactic fermentation)	*Oenococcus onei*
Rice wine	*Lactobacillus sakei*

1.2.2 Industrial development of starter cultures

Bacteria that are isolated from natural sources can have exceptional properties with industrial relevance. However, they are usually not fit for the market in their native state. Therefore, some of their properties have to be modified to make the most of their capacity. Alternatively, the exceptional properties of these native isolates could be useful to improve the performance of strains that are already on the market. The strategy for these improvements and modifications would be quite straightforward. The well-established techniques of recombinant DNA technology would enable such modification and transfer of the desired trait. However, recombinant DNA technology is not applicable to starter cultures intended for human or animal consumption, in view of the fact that recombinant DNA technology is not approved by most food authorities and consumer acceptance of genetic modified organisms is low. Hence, all strains that are intended to be used as food starter cultures should be improved by natural strategies such as random mutagenesis, directed evolution, dominant selection, or less common methods such as bacteriophage transduction, and natural competence (Derkx et al., 2014; Pedersen et al., 2005).

Random mutagenesis is a technique where random mutations are induced by diverse protocols to obtain a pool of random mutants. The mutant with desired property is later selected for use in desired application. This technique has widely been used in food industry for strain improvement. Although many successful results were

obtained, this technique lacks specificity and obtained strains can suffer from the negative effects induced by other simultaneous mutations (Derkx et al., 2014).

Directed evolution or adaptive evolution, is a technique where a strain is gradually forced to adapt to selective conditions. The individuals that can adapt survive this selection period whereas other individuals are eliminated during this process. Thus, the chance to isolate an individual with improved properties increases. However, it has a similar drawback as random mutagenesis that undesired mutations may also occur during this selection process (Derkx et al., 2014; Barrick and Lenski 2013).

Dominant selection technique depends on designing specific conditions that only enable the growth of the desired strain. This technique needs extensive knowledge on microbial physiology to be able to design such conditions. If the design is successful, it enables the selection of specific strains with a single mutation. The most important advantage of this technique is that the use of mutagenic agents can be omitted and accumulation of undesired mutations can be avoided (Derkx et al., 2014).

The only way of DNA transfer is not recombinant DNA technology. DNA is also transferred among bacteria by natural mechanisms such as bacteriophage transduction, natural competence and conjugation. Modification of the DNA by described natural mechanisms are not considered as genetic modification by the European Union (Derkx et al., 2014; Pedersen et al., 2005). An overview of strain improvement techniques are given in **Tables 1.3 and 1.4** (Derkx et al., 2014).

1.3 Olive Processing

Olives are the fruits of the *Olea europaea* tree. This fruits are inedible and bitter mainly due to the presence of the presence of an olive polyphenol, oleuropein. Thus, they have to be debittered. This is mainly done by removing the bitterness from the whole fruit to obtain table olives or the oil from this fruit is pressed to obtain an edible non-bitter oil since the main bitter compounds are left at the aqueous phase (Rivas et al., 2000; Papoff et al., 1996).

Table 1.3 : Industrially applied strain improvement techniques. I

Method used	Advantages	Disadvantages	Topic	Aim
Random Mutagenesis	Detailed knowledge on physiology is not a must. When direct selection is not achievable this technique is used to remove certain properties	Noxious agents are used to induce mutation. Concentration of mutations at certain parts in the genome Occurrence of undesired mutations along with desired ones. Need extensive screening.	Pyrimidine auxotrophy Elimination of antibiotic resistance Elimination of citrate metabolism Urease negative mutants of *S.thermophilus* Lactobacillus strains with low post acidification	Bacteriophage resistance Removal of certain properties Removal of certain properties Removal of certain properties Increase the stability of end product.
Directed evolution	Detailed knowledge on physiology is not a must. Possibility to select complex phenotypes. Use of mutagenic agents can mostly be omitted.	Undesired mutations along with desired ones can occur. Experimental design can be complex. Takes a lot of time. A population rather than a single strain is obtained. Individuals of this population should be screened	Rising the growth yield in fermentation	More efficient culture production

Table 1.4 : Industrially applied strain improvement techniques. II

Method used	Advantages	Disadvantages	Topic	Aim
Dominant Selection	Mutagens are not used Generally only one mutation occurs.	Detailed knowledge on physiology is a must. Noxious analogues could be used.	Bacteriophage receptors	Bacteriophage resistance
			Conjugation	Bacteriophage resistance
			Modifying bacterial cell surfaces	Improving texture
			Optimizing the metabolic pathway of EPS	Improving texture
			Bacteriophage resistant mutants	Improving texture
			Lactobacillus strains with improved ethanol or bile tolerance	Improving survival and efficacy
			Lb. helveticus producing succinate	Improving flavor
			Altering acidification properties by adapting the carbohydrate metabolism	Overcoming effect of a mutation

Table olives are processed by two main strategies called the Spanish style and the Californian style. In the Spanish style, the unripe, green olives are treated with lye and oleuropein is broken down. The lye is then rinsed and the debittered olives are fermented mainly by lactic acid bacteria. In the Californian style, the ripe olives are fermented in brine solution under aeration until the bitterness is removed and then fermented in an aerated or non-aerated milder brine solution by lactic acid bacteria. The application of debittering steps prior to olive fermentation is due to the toxicity of oleuropein against many lactic acid bacteria. As it is the case with tannins, yeasts are significantly more resistant against phenols including oleuropein and therefore rapidly grow in undebittered olives. This makes the lactic acid fermentation of bitter olive fruits a difficult task and the debittering of olives prior to fermentation an almost essential step of the process. However, this strategy has also its risks since the incomplete removal of olive phenols or the loss of essential sugar and soluble nutrients by overtreatment causes insufficient fermentation of the debittered olives. Furthermore, chemical treatments are undesired by the costumers. There is a trend for non-lye-treated olives. On the other hand, the alternative Californian style treatment of olives results in extreme long fermentation times and olives with elevated salt levels. Customers are also aware of the potential health risks of elevated salt intake and do not prefer olives with too high salt content. Another strategy is to use specific yeast starters that degrade oleuropein and fasten the olive debittering. In addition, some strains of *L. plantarum* are able to partially degrade oleuropein by their beta glucosidase activity. However, it is not possible to obtain satisfactory results using the present state of art (Rivas et al., 2000; Brenes et al., 1992; Ciafardini et al., 1994).

1.4 Probiotics

Probiotics are defined as live microorganisms, which confer a health benefit on the host when administered at adequate amounts (FAO/WHO 2002). Although no internationally accepted criteria for defining a strain as a probiotic exist, four general criteria have to be fulfilled by a potential strain.

i. The effects have to be defined specifically for genus and strain. Research on one specific probiotic strain does not apply on any other strain of the genus.
ii. The strain should be alive.

iii. Strain should be delivered in adequate dose until the end of its shelf life.

iv. The strain has to be shown to be effective in human studies.

Lactic acid bacteria are potential candidates for probiotic products, due to their natural presence in human colon and long and safe use in food fermentations (Lebeer et al., 2008).

Lactic acid bacteria cover a diverse group of bacteria and are defined as “non-pathogenic, non-toxigenic, Gram-positive, fermentative bacteria that are associated with the production of lactic acid from carbohydrates” by the World Gastroenterology Organisation. Examples to some commercial probiotic lactic acid bacteria strains that are used in probiotic products on the market are given in **Table 1.5** (Costa and Miglioranza, 2012).

Table 1.5 : Commercially available probiotic lactic acid bacteria strains.

Species	Strains
Lactobacillus acidophilus LA-1™	La-5™; NCFM; DDS-1; SBT-2062; La-14™
Lactobacillus casei Shirota™	LC™; DN1114001™; Immunitas™
Lactobacillus casei shirota	Yakult™
Lactobacillus casei ssp. defensis	Danone™
Lactococcus lactis	L1A
Lactobacillus fermentum	RC-14
Lactobacillus helveticus	B02
Lactobacillus johnsonii	La1™
Lactobacillus paracasei	CRL 431™
Lactobacillus plantarum 299 Probi™	LP115™; Lp01
Lactobacillus rhamnosus GG	GR-1; LB21; 271Probi™
Lactobacillus reuteri	SD2112
Lactobacillus salivarius	Ls-33

1.5 General Stress Response in Lactic Acid Bacteria

Bacteria encounter stress conditions not only during industrial processes, but also in their natural environment. Thus, the rapid response against stress is crucial for their survival. Bacteria harbour multifaceted mechanism to sense and respond against

environmental changes and resulting stress conditions. It is almost impossible to make them resistant to a variety of stress conditions by the regulation of a tailored gene. Thus, these sense and response mechanisms are a part of regulators involving several genes and even other regulators. Bacterial stress response is regulated by synchronized expression of genes being part of cell division, DNA metabolism, housekeeping, membrane composition, transport, and other cellular processes. (Zheng and Stortz, 2000, Van der Guchte, M. 2002)

The identification of stress response regulation in LAB is critical for its control, prediction and engineering. The comparison of these processes with well-known model organisms such as *Escherichia coli* and *Bacillus subtilis* could be highly informative to identify the similarities and differences of LAB-specific stress response. These can reveal the LAB-specific responses that make it the dominant organism at specific environments and processes such as milk fermentation. The well-studied stress types are acid, heat and cold stress. However, the comparison of LAB with these model organisms is limited, since most studies focused on specific protein families rather than the whole response. However, even these limited data already suggest significantly different regulation of these responses in LAB (Van der Guchte, M. 2002).

Many genes and regulators are involved in stress response and these differ greatly among species and subspecies. Even some functionally conserved stress proteins such as heat shock proteins, Csp and their regulators like HrcA, CtsR are not conserved among all LAB species. Hence, it is expected that stress response in LAB and model organisms differs considerably. Few of the stress-related genes are essential since lineage-specific gene loss is a vital force of evolution among prokaryotes. One example is the complete absence of σB orthologue in *Lactococcus lactis ssp. lactis*. The role of this regulation factor is not taken over by a distantly related or unrelated protein but it is just absent. As expected, the cell membrane plays a central role in stress response, but this stress response is rarely due to the change of the lipid structure of the membrane, it is more related to integral membrane proteins. These proteins are mostly undetected firstly due to the technical weakness of the main technique used to identify stress response proteins. Although proteomic techniques are numerous, 2-D Gel Electrophoresis is mostly applied in these studies and it cannot detect membrane proteins easily. Secondly, the change in

protein composition occurs by a long-term adaptation process. However, short-term responses are mainly due to alterations in existing proteins such as activation or stabilization (Van der Guchte, M. 2002).

Genomic studies have revealed that LAB got adapted to certain environmental niches by horizontal gene transfer. Studies done on *Lactococcus lactis* strains from different origins revealed that most of the bacterial genome were well preserved whereas a region of 21 genes responsible for cell wall teichoic acid production was obtained by horizontal gene transfer by a plant-based *L. lactis* strain. Similar adaptations were also observed at different LAB including *L. plantarum* and *Lactobacillus rhamnosus* strains (Douillard and de Vos, 2014).

Regulation of metabolism is also a significant adaptation to stress. Genomic studies have revealed that CcpA (catabolite control protein) is present in all lactic acid bacteria and is the main regulator of lactic acid synthesis. The *ccpA* gene has 82 catabolite responsive element binding sites (*cre*). Thus, *ccpA* is thought to regulate the carbon utilization and metabolism under various environmental conditions. *glnR* is also important in controlling the metabolism of LAB. This gene is present in all lactic acid bacteria genomes and controls nitrogen metabolism. Beside general control of the carbon and nitrogen metabolism; a large number of membrane proteins, shock proteins and transporters are important in the stress response of LAB (Douillard and de Vos, 2014).

Complete genome analysis of *L. plantarum* WCFS-1 revealed more detailed insight into the stress response of this organism. Specific gene families for temperature stress, pH stress, osmotic stress and oxidative stress were suggested. Proteins that were not functional anymore were removed by three energy-dependent proteases: ClpP, HslV and Lon. In response to shifts in temperature, heat shock proteins were expressed from *groEl-groES* chaperonin, *hrcA-grpE- dnaK- dnaJ* operon, along with three small Hsp20 family proteins. Cold shock proteins coding sites were also present in the *L. plantarum* strain. These sides were coding for CspL, CspC and CspP proteins. Fof1-ATPase, and probably 10 sodium-proton antiporters mainly encounter acidic pH stress, while 3 paralogous alkaline shock proteins are encoded in response to high pH. Three osmoprotectants are known to be accumulated in *L. plantarum* in response to high osmotic stress.

The ABC transporters, opuABCD, and choSQ and other biosynthetic and transport genes are responsible for intracellular glycine-betaine, carnitine and choline accumulation in such a case (Douillard and de Vos, 2014).

Genes encoding catalase, thiol peroxidase, glutathione peroxidase, halo peroxidase, four thiopredoxins, four glutathione reductases, five NADH oxidases and two NADH peroxidases were present in *L. plantarum* WCFS-1 genome to cope with the oxidative stress. In addition to these genes, high Mn^{2+} levels (20-30mM) in the cytoplasm also have demonstrated antioxidant properties. Along with the 55 cation transporter genes, *L. plantarum* harbours one P-type manganese translocation ATPase and two natural resistance-associated macrophage proteins (NRAMP)-like transporters (Kleerebezem et al., 2003)

1.5.1 Olive-specific stress response

The main stress factors in olive fermentation are polyphenols and NaCl. Polyphenols are part of many plant tissues and play a central role in plant defence against microorganisms and higher fungi. Bacteria are inhibited at minimum inhibitory concentrations (MIC) of 0.012-1 g/L, whereas the MIC for fungi is usually higher than 0.5 g/L and also MIC values between 10-20 g/L are not uncommon. Yeasts are particularly more resistant and MIC between 25-125 g/L can be observed (Scalbert, 1991).

The antimicrobial activity of polyphenols is mainly attributed to the damage of the bacterial cell wall. Oleuropein was found to damage the peptidoglycan structure, cause leakage of inorganic phosphate, glutamic acid and potassium and caused deformation of *L. plantarum* strains (Juven et al., 1972, Ruiz-Barba et al., 1991). Although oleuropein has antimicrobial effect, studies have shown that nine phenolic compounds in olives were not antimicrobial to *L. plantarum* at concentrations found in olives (Landete et al., 2008).

Ruiz-Barba et al. (1991) considered the presence of both NaCl and oleuropein in olive-processing and found that 4%(w/v) NaCl and 0.4%(w/v) oleuropein showed antimicrobial activity against *L. plantarum*, whereas 0.4% oleuropein or 4% salt did not have antimicrobial activities when tested separately.

Further studies made with *L. plantarum* DSM 10492 strain that was resistant to high concentrations of oleuropein demonstrated that oleuropein was not the primary cause

of antimicrobial activity, but it decreased the resistance of the strain to NaCl. This sensitization was slightly reversed by glucose (Rozes and Peres 1996).

Transcriptomic analysis of *L. plantarum* gave a better insight into this phenomenon. *L. plantarum* WCFS-1 that was grown on 0.8M (approximately 4.7% w/v) NaCl showed an increased transcription of *ccpA* and a decreased transcription in *cre*-controlled cellular processes. This led to a total decrease in ATP but not to a change in the AMP/ATP ratio.

The osmotic shock protein-coding genes were not over-transcribed and even a decrease in transcription was observed. This phenomenon was also observed in other organisms and was related to the feedback inhibition of the previously over-transcribed proteins. The main response to high NaCl stress was a shift in cell wall composition from lipoteichoic acid to teichoic acid. This shift was supported by an increase in cellular processes responsible for DNA repair. DNA repair is an important process during NaCl stress, as NaCl causes double-strand breaks in DNA. Excinuclease ABC complex, recombination protein *recR*, exonuclease *sbcC* and *sbcD* and 2 exodeoxyribonucleases were over-transcribed in order to counteract NaCl by *L. plantarum* WCFS-1 (Pieterse, B. 2006).

Transcriptome analysis done on p-coumaric acid-exposed *L. plantarum* WCFS-1 strain gave a general insight of the response against plant polyphenols. The exposure resulted in a wide-ranged activation of cellular stress proteins that cover proteases, chaperones, heat shock proteins and alkaline shock proteins. This response was mainly regulated by Class I and III transcription activators, CtsR and HrcA. The cell entered growth arrest in response to p-coumaric acid exposure. As a result of this response, translation of purine, pyrimidine metabolism and cell wall and lipid metabolism-related genes decreased. The sugar transport and catabolism-related genes were also downregulated and products of pentose phosphate pathway were directed to glycolytic intermediates, while malate transport and metabolism genes were activated as an energy source. The main detoxification strategy was to decarboxylase coumaric acid by increased transcription of *pdc*, phenolic acid decarboxylase and multidrug transporter genes. The expression of cell wall-related genes was also affected by p-coumaric acid stress. An increased expression of peptidoglycan biosynthesis enzymes, Aad and HicD and various cell surface proteins was observed. These transcriptional changes were activated to counteract the cell

wall leakage. This phenomenon was also observed in response to other phenolic acids. Also a Na/H^+ antiporter was activated to overcome excess Na^+ from the cytoplasm (Reverón et al., 2012).

1.5.2 Stress response under gastrointestinal conditions

Ingested microorganisms face a series of stress factors during their passage through the GI tract. Time-based decrease in pH and the presence of gastric enzymes are major stress factors in the stomach. Peristaltic movement of the small intestine does not only prevent bacterial colonization, the weakened bacteria also face pancreatic enzymes, bile salts and nutrient deprivation at different sections of the small intestine (Marteau et al., 1997).

The viability of probiotics can widely vary on genus and strain basis. This variation has led to studies that investigated the basis of these differences in survival (Lebeer et al., 2008, Douillard 2013).

Bron et al. (2004) demonstrated that expression of certain genes of *L. plantarum* WCFS1 was changed during gastrointestinal passage in mice. Lebeer and colleagues (2008) compared the presence of some genes in the probiotic *L. rhamnosus* GG with non-probiotic *L. rhamnosus* strains. They found that luxS gene was important for survival in gastric passage. Although it was not possible to complement luxS gene by expression in a plasmid, an unknown mutation was able to restore the gastric survival on the expense of reduced adherence capacity (Lebeer et al., 2008).

Douillard and colleagues (2013) compared the whole genome of a probiotic *L. rhamnosus* strain with non-probiotic *L. rhamnosus* strains to determine the genetic basis of probiotıc activity. They suggested that *tauABC* locus is related with bile resistant *SpaCBA* pili gene cluster that is related to colonization in colon. They also found differences in carbohydrate metabolism and transport: most dairy based strains were able to utilize lactose whereas *L. rhamnosus* GG and related intestinal strains were not able to utilize lactose. On the contrary, they were able to utilize L-fucose which was not utilized by dairy strains (Douillard et al., 2013).

In vivo studies done to determine which genes were expressed by the probiotic *L. plantarum* 299v in human and WCFS-1 in germ-free mice have revealed that several genes were expressed in both mice and human by two different *L. plantarum* strains. This study confirmed previous findings of Lebeeret and Douillard. Sugar transport

and metabolism-related genes were expressed to optimally utilize the limited available nutrients, and cell survival was increased by the expression of an increased number of cell surface proteins and polysaccharides (Marco et al., 2010).

Bove et al. (2013), related certain stress response genes with gastric passage of *L. plantarum* WCFS-1. They reported that induction of genes was related to the conditions in their oro-gastrointestinal tract simulator. The induction of most stress-related genes was highest at the gastric compartment when the pH was 3. They reported high induction of stress-related genes, *dnaK, groEL, clpB, clp E, clpP, hsp1, hsp2, hsp3* and *ctsR.*

1.6 Metabolic Engineering

Metabolic engineering was first defined in conjugation with recombinant DNA technology by Bailey in 1991. He defined metabolic engineering as the rational alteration of cellular processes by means of recombinant DNA technology (Bailey, 1991). This engineering methodology gained importance since a large collection of knowledge on cellular processes became available by high throughput screening platforms and the emergence of next generation sequencing systems. However, cellular processes are of complex nature and mostly involve many different cellular processes. Generating a successful metabolic model of a cell requires knowledge on advanced mathematics, computation and biology. This is a very difficult combination of knowledge and has led to only a few advanced metabolic models of lactic acid bacteria. However, these metabolic models omitted many cellular processes and interactions, because of the high level of complexity. Even if it would be possible to specifically engineer strains by means of recombinant DNA technology, these strains could not be used as starter cultures due to the hurdles based on regulatory environment and consumer acceptance issues (Koffas and Cardayre 2005).

Thus, natural strategies were developed for strain improvement. Microorganisms can adapt to certain stress conditions by an evolutionary process based on random mutation and natural selection. This has also inspired researchers to use similar principles to develop mutant microorganisms with improved properties. Evolutionary engineering gives the opportunity to change the metabolism at diverse points and optimize the function of all changes by a nature-driven force. Optimization of changes by a rational design is a quite difficult task as it depends on the modelling of

all related metabolic pathways to be able to alter the genomic makeup of an organism. The only requirement in evolutionary engineering is, however, to make use of the right selection criterion that is easily screenable and gives rise to desired types of mutants (Koffas and Cardayre 2005).

1.7 Literature Review

1.7.1 Literature review on mutagenesis applied to probiotics

A literature review on mutagenesis studies made on probiotic strains is summarized in this section. Most studies employed gene deletion or insertion mutagenesis to determine responsible genes for a specified probiotic trait.

Rasinkangas et al. (2014) were the only group who had combined ethyl methane sulfonate (EMS) mutagenesis and whole genome sequencing to obtain pilus-deficient non-adherent strains of *L. rhamnosus* GG.

Živković et al. (2016) used insertion mutagenesis to investigate the activity of a strain-specific exopolysaccharide of *Lactobacillus paracasei subs paracasei* BGSJ2-8. They employed insertion mutagenesis to inactivate the expression of one subunit of produced exopolysaccharide to determine its effect on gut colonization and on communication with gut epithelium. They concluded that inhibition of glps-2198 gene by insertion mutagenesis made the mutant unable to produce subunit-2 of this exopolysaccharide and consequently decreased the ability to communicate with gut epithelium and reduce the proliferation lymphocytes in gastrointestinal associated lymphoid tissue.

Nzakizwanayo et al. (2015) used random transposon mutagenesis to understand the interaction of *E. coli* Nissle 1917 with intestinal epithelial cells. They identified that a mutation in kfiB gene, of the K5 capsule biosynthesis cluster increased the cellular attachment of the mutant to Caco-2 cells and had apoptotic and cytotoxic effects. By comparing their mutant with a mutant deficient of the gene KifC in K5 capsule biosynthesis cluster that also had no capsule, they determined that KifC deficient mutant did increase COX-2 expression in Caco-2 cells, but did not possess any cytotoxic or apoptotic effects. They concluded that genes in K-5 capsular biosynthesis cluster were important in the mode of interaction between *E.coli* Nissle 1917 with intestinal epithelian cells.

Hudson et al. (2014) used UV mutagenesis to develop uracil auxotrophic *Saccharomyces boulardii* mutants. They used these mutants to safely express therapeutically active proteins without the use of antibiotics for selection. They successfully introduced these mutant strains and expressed proteins of interest in gastrointestinal immune tissues of C57BL/6 mice.

Rasinkangas et al. (2014) employed EMS mutagenesis and immuno-dot blot analysis using antiserum against pilin proteins to obtain pilus-deficient *L. rhamnosus* GG strains. They isolated pilus-deficient individuals and investigated the underlying genetic mutations that led to the absence of pilus in these individuals. They classified the mutants based on the mutation that caused the absence of pilus. The first class had a mutation in pilus-specific sortase C, the second class had a deletion covering *spaCBA-srtCl* gene cluster and the third class had a mutation in the major pilus subunit SpaA. They concluded that pilus-deficient mutants were not able to adhere porcine intestinal mucus.

Douillard et al. (2014b) employed directed mutagenesis to confirm the significance of a triglycine structure in pilus proteins. They concluded that this triglycine motive could be active in the regulation of pilin-specific and housekeeping sortase activity in *L. rhamnosus* strain GG.

Jensen et al. (2014) employed ssDNA recombination to obtain defined nonsense mutants of *L. reuteri*. They determined that null mutants of srtA sortase had significantly lower adhesion ability to Caco-2 cells and mucus. They further identified that a null mutant of sortase-dependent protein hmpref 0536_10633 had also significantly decreased adhesion to Caco-2 cells and mucus. They concluded that this protein should be named as cell and mucus-binding protein A (CmbA), after the conclusion that cell adhesion could be restored and even increased by plasmid-encoded complementation of protein hmpref0536_10633 (cell-and mucus-binding protein A, CmbA).

Hemarajata et al. (2014) employed transposon mutagenesis to determine the genes active in histamine biosynthesis in *L. reuteri*. They identified that inactivation of a proton-chloride antiporter gene (eriC) resulted in suppressed histamine bioconversion. They concluded that this finding could be important since histamine is a biogenic amine with tumor necrosis factor repressing activity.

Ito et al. (2014) employed transposon mutagenesis to obtain *asnH, dnaJ, dnaK, dnaJ* and *dnaK* null mutants of *Lactobacillus casei* ATCC 27139. They investigated the ability of null mutants to enhance the host immunity against a *Listeria* infection and found that only the null mutant of *asnH* was deficient in enhancing mouse immunity during this infection. They concluded that *asnH* encoding asparagine synthetase was essential for peptidoglycan construction with immune-activating capacity.

Anwar et al. (2012) employed site-directed mutagenesis of inulosucrase residues of *L. reuteri* 121. They demonstrated that these mutations were able to alter the catalytic rate and specificity of the enzyme, without changing the beta(2-1) linkage in product.

Bron et al. (2012) employed double cross-over gene replacement to obtain *L. plantarum* WCFS1 mutants in genes active in cell wall biosynthesis. They constructed mutants with alternative wall and lipo-teichoic acids; such as mutants that lack wall teichoic acid (WTA), or have WTA with a ribiotol instead of glycerol backbone. They further concluded that *tarIJKL* gene cluster was essential for the production of alternative cell wall compositions and that the immune stimulation is affected by the cell wall structure.

Margolles and Sanchez (2012) employed random UV mutagenesis to obtain *Bifidobacterium animalis subsp lactis* strains with decreased acetic acid-producing activity. They identified a mutant with decreased acetic acid production and assessed by genome sequencing that this was due to a Phe-Ser substitution in the acetate kinase gene. They further found that this strain produced less ethanol and more yoghurt-related volatile compounds.

Thomas et al. (2012) employed targeted mutagenesis to determine genes active in histamine production in *L. reuteri* ATCC PTA 6475. They identified that histidine decarboxylase pyruvoyl type A (*hdcA*), histidine/histamine antiporter (*hdcP*), and *hdcB* were active in histamine production from histidine.

Alazzeh et al. (2011) employed EMS mutagenesis to obtain *L. reuteri* CF2-7F mutants with increased beta-galactosidase activity. They were able to isolate a higher beta galactosidase-producing strain by screening mutant populations on X-gal. They increased the beta galactosidase activity by 137.3% and concluded that EMS mutagenesis could be an effective method in enhancing enzymatic activity.

Ito et al. (2010) described a random mutagenesis system based on TN5 transposon complex and employed this to create a mutant library of probiotic *Lactobacillus casei* ATCC 27139. They concluded that this library was screenable for auxotrophs and the analysed mutations were random.

Lee et al. (2010) employed random insertion mutagenesis and constructed a mutant library of *Lactobacillus acidophilus* A4 to determine genomic and proteomic factors that cause cholesterol reduction. They identified a mutant with deletion in the catabolite control protein A (*ccpA*) gene with reduced cholesterol uptake from media. They determined that the absence of this gene decreased the serum cholesterol-lowering activity of this bacterium *in vivo*.

İbrahim and O'Sullivan (2000) employed EMS mutagenesis and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) mutagenesis to obtain *Bifidobacterium breve*, *Bifidobacterium longum, Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus thermophilus* mutants with increased beta-galactosidase activity. They were able to isolate a higher beta galactosidase-producing strain by screening mutant populations. They determined that the beta galactosidase activity of mutant individuals isolated from mutated *L. delbrueckii ssp. bulgaricus; S. thermophilus; B. breve; B. longum* species were 137%, 104%, 70% and 222% of the wild type strains, respectively.

1.7.2 Literature review on mutagenesis applied to olive starter cultures

A literature review on mutagenesis studies made with lactic acid bacteria to improve their resistance to stress conditions related to the olive fermentation process is summarized in this section. However, only two studies were found where mutagenesis was applied to lactic acid bacteria which were intended to be used as olive starter cultures. These studies were not focusing on improving the stress resistance, but they were focusing on the determination of the genetic background of survival at brine conditions.

Perpetuini et al. (2016) employed transposon mutagenesis to *Lactobacillus pentosus* C11 in order to determine genes active in biofilm formation on olive skin during olive fermentation. They concluded that *enoA1*, *gpi* and *oba* genes were required for an ordered biofilm establishment on olive skin.

In a previous study, Perpetuini et al. (2013) employed transposon mutagenesis to *L. pentosus* C11 and constructed a mutant library in order to determine the genes that are essential for growth in olive brine medium. They identified that no growth was observed in olive brine in the absence of *enoA1*, *gpi*, and *obaC* genes.

1.8 Aim of the Study

The first aim of this study was to obtain oleuropein-resistant *L. plantarum* strains for more efficient olive fermentations, by using evolutionary engineering approach. Since stress resistance mechanisms in lactic acid bacteria generally involve numerous genes and many regulators, it would be difficult to obtain the desired resistant phenotypes by direct genetic engineering or over-expressing certain genes. Thus, evolutionary engineering could be an efficient alternative strategy to obtain desired phenotypes. The strains to be obtained might be potentially used as starter cultures in industrial olive fermentation, bioremediation of olive mill waste, and valorisation of waste products.

The second aim of this study was to obtain a probiotic culture that can be used in the fermentation of olives. It was started with a strain which already had NaCl resistance. To this strain, a novel evolutionary engineering strategy was applied to obtain a potential probiotic lactic acid bacterium that has an increased survival rate during the passage through the upper gastrointestinal tract, using a model system that mimics the upper gastrointestinal tract (TIM-1)

1.9 Hypotheses

The first hypothesis of this study was that lactic acid bacterial starter cultures for olive fermentation were not robust enough due to the presence of olive polyphenols, mainly oleuropein, in fermentation brines. It was thought that evolutionary engineering would be a suitable way to obtain lactic acid bacteria strains with improved polyphenol resistance.

The second hypothesis was that the major stress factor in olive fermentations was the high NaCl concentrations. Therefore, a strain that resists high NaCl concentrations would also resist the combined stress of NaCl and olive polyphenols. It was thought

that evolutionary engineering would be a suitable way to obtain lactic acid bacteria strains with improved NaCl and polyphenol resistance.

The third hypothesis was that a lactic acid bacterium isolated from olive fermentation brines could be improved by evolutionary engineering to have increased survival rate during passage through the upper gastrointestinal tract, and it could still preserve its initial NaCl resistance that would enable its use in olive fermentation processes as a potential probiotic culture.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains

Lactobacillus plantarum cultures were previously isolated from olives and brines from commercial table olive-producing companies in the Marmara Region, Turkey. They were previously investigated for their ability to produce dextran from glucose, to produce diacetyl, to grow at 5°C, 10°C, 15°C and 45°C and resistance against bile salts and acidity (pH 2.5). Properties of the selected *L. plantarum* strains are shown in **Table 2.1.** All cultures were stored in lyophilized state in the culture collection of TÜBİTAK Marmara Research Center, Food Institute, Kocaeli, Turkey.

L. plantarum DSM 10494 strain was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). This strain was isolated from an olive brine in Portugal. DSMZ 10492 strain has known resistance to 0.4% (w/v) oleuropein and up to 6% (w/v) NaCl (Rozes and Peres, 1996).

The *Enterococcus* strains used as indicators for bacteriocin production tests of the *L. plantarum* T129 strain were obtained from the Netherlands Organisation for Applied Scientific Research (TNO) culture collection. The bacteriocin-producing *Lactococcus lactis* TTC 03.0262 was also obtained from the TNO culture condition. The list of the *Enterococcus* strains used is given in **Table 2.2**.

2.1.2 Composition of culture media

de Man, Rogosa and Sharpe (MRS) Medium

Proteose Peptone No3	10 g
Beef extract	10 g
Yeast extract	5 g
Dextrose	20 g
Polysorbate 80	1 g
Ammonium citrate	2 g

Sodium acetate	5 g
Magnesium sulfate	0.1 g
Manganese sulfate	0.05 g
Dipotassium phosphate	2 g
Agar (for solid media)	15 g

Per 1 liter of distilled water

The medium is used after sterilization for 15 minutes at 121ºC.

Rogosa Agar Medium

Ammonium citrate	2 g
Caseine peptone	10 g
Ferrous sulfate	0.034 g
Dextrose	20 g
Magnesium sulfate	0.575 g
Manganous sulphate	0.12 g
Potassium dihydrogen phosphate	6 g
Sodium acetate	15 g
Yeast extract	5 g
Agar (for solid media)	15 g

Per 1 liter of distilled water

The medium is boiled and sterile 10 ml 10% (w/v) polysorbate 80 and 1.32 ml glacial acetic acid are added to fresh medium.

2.1.3 Composition of used solutions

Phosphate buffer saline (PBS)

Composition of phosphate buffer saline (PBS) 50 mM, pH 7.4

Sodium chloride	8 g
Potassium chloride	2 g
Disodium hydrogen phosphate	1.44 g
Potassium dihydrogen phosphate	0.24 g

per 1 liter of distilled water.

Table 2.1 : Properties of the isolated *L.plantarum* strains found in TUBİTAK culture collection

Strain number	Production of diacetyl	Production of dextran	Growth at				Resistance to	
			5°C	10°C	15°C	45°C	bile salt	pH 2.5
T813	+	-	-	+	+	-	-	-
T822	+	-	-	-	+	-	-	-
T831	+	-	-	+	+	±	-	-
T874	+	-	-	+	+	-	+	-
T880	+	-	-	+	+	-	±	-
T896	+	-	-	+	±	-	+	-
T957	+	-	-	+	+	-	-	-
T976	+	-	-	+	+	-	+	-
T026	+	-	-	-	+	-	+	-
T033	+	-	-	+	+	-	-	-
T068	+	-	-	-	+	-	+	-
T102	+	-	-	+	+	+	+	-
T109	+	-	-	+	+	+	+	+
T119	+	-	-	+	+	+	-	-
T123	+	-	-	+	+	+	+	-
T127	+	-	-	+	+	+	+	+
T129	+	-	-	+	+	+	+	+
T147	+	-	-	-	-	+	-	-
T150	+	-	-	+	-	+	-	-

± : the culture grew barely
+ : significant growth
- : no growth

Table 2.2 : *Enterococcus* strains obtained from TNO culture collection.

Strain	Species
TTC 98.0259	*Enterococcus faecalis*
TTC 99.0138	*Enterococcus hirae*
TTC 00.0173	*Enterococcus faecalis*
TTC 00.0251	*Enterococcus faecium*
TTC 00.0395	*Enterococcus faecalis*
TTC 00.0551	*Enterococcus faecium*
TTC 00.0632	*Enterococcus hirae*
2008.282	*Enterococcus faecalis*
2010.83	*Enterococcus pseudoavium*
2013.074	*Enterococcus faecium*

Peptone water

Universal peptone M66 1 g

per 1 liter of distilled water.

Physiological saline solution

Sodium chloride 8 g

per 1 liter of distilled water

2.1.4 Chemicals

Semi skim UHT milk (containing 1.5% fat) used in this study was the private label milk of Jumbo supermarket (the Netherlands). The list of the chemicals used is given in **Tables 2.3** and **2.4.**

Table 2.3 : The list of used chemicals. I

Chemical	Supplier
HCl	Merck (Germany)
Ethanol	Merck (Germany)
Rifampicin	Sigma Aldrich (USA)
Ferrous gluconate	Merck (Germany)
Lactic acid	Merck (Germany)
Glucose	Sigma Aldrich (USA)
NaOH	Merck (Germany)
$Na_2S_2O_3$	J.T.Baker (Holland)
Ethyl methane sulfonate	Alpha-Aeasar (Germany)
Oleuropein	-
Acetone	Merck (Germany)
Glycerol	Sigma Aldrich (USA)
API 50CH kit	bioMérieux (France)
Universal peptone M66	Merck (Germany)
Potassium dihydrogen phosphate	Merck (Germany)
Disodium hydrogen phosphate	Merck (Germany)

Table 2.4 : The list of used chemicals. II

Chemical	Supplier
Potassium chloride	Sigma Aldrich (USA)
Sodium chloride	Merck (Germany)
Glacial acetic acid	Merck (Germany)
Polysorbate 80	Merck (Germany)
Rogosa agar	Sigma Aldrich (USA)
MRS broth	BD DifcoTM (USA)
$NaHCO_3$	Sigma Aldrich (USA)
Sodium acetate	Sigma Aldrich (USA)
Pepsin	Sigma Aldrich (USA)
Lipase F-AP15	Amano Enzyme Inc. (Japan)
Pancreatin, Pancrex V	Paines and Birne (Greenford, England)
Porcine bile extract	Sigma Aldrich (USA)
$CaCl_2$	Sigma Aldrich (USA)
H_2SO_4	Sigma Aldrich (USA)
HPLC-grade water	Merck (Germany)
MRS agar	BD DifcoTM (USA)

2.1.5 Laboratory equipment

The list of the laboratory equipment used is given in **Table 2.5**.

Table 2.5 : The list of the laboratory equipment used.

Equipment	Supplier
Autoclave	Jürgen’s (Germany)
Automated turbidometer	Bioscreen C (Finland)
Spectrophotometer	Perkin Elmer Lambda 35(USA)
Centrifuges	Sigma3K30 Sartorius (Germany)
	HETTICH (Germany)
In vitro dynamic gastrointestinal model	TIM-1 (the Netherlands)
Incubator shaker	GFL (Germany)
Lyophilizer	Telstar Lyobeta (Spain)
Magnetic shaker	HEIDOLPH MR 3004G (Germany)
Incubator	Binder BD53 (Germany)
High performance liquid chromatography (HPLC) system	
- Refractive index detector	Shimadzu RID10A(Japan)
- System controller	Shimadzu SCL10A(Japan)
- Liquid chromatography	Shimadzu LC-10AD (Japan)
- Degasser	Shimadzu ss DGU-14A(Japan)
- Column oven	Shimadzu CT0-10AC (Japan)
Vortex	Heidolph (Germany)
Rotary evaporator	BUCHI 011(Germany)
pH-meter	Thermoscientific Orionstar A221(USA)
Balances	Precisa 205 Precisa 6200(Switzerland)
Light microscope	LEITZ (Germany)
Water bath-shaker	GFL 1086 (Germany)

2.1.6 Softwares and websites

The list of the softwares and websites used is given in **Table 2.6**.

Table 2.6 : The list of the softwares and websites used.

Description	Web address
FDA MPN Tables	http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm109656.htm
Maker web based genome annotation software	http://weatherby.genetics.utah.edu/cgi-bin/mwas/maker.cgi
Blast nucleotide and protein Search tool.	https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch
NCBİ conserved domain search.	http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi HETTICH (Germany)
BioCyc Pathway/Genome Database Collection	http://biocyc.org
Protein databank in Europe	http://www.ebi.ac.uk/pdbe
Codon ussage database	http://www.kazusa.or.jp/codon
Protein structure database	http://www.proteinmodelportal.org/.

2.2 Methods

2.2.1 Methods related to the estimation of the resistance against olive polyphenols and oleuropein

2.2.1.1 Cultivation of lyophilized cultures

Lyophilized cultures of *L. plantarum* were firstly inoculated in sterilized MRS broth and incubated for 3 days, until they reached high cell densities. These vitalized cultures were spread on MRS agar and incubated in anaerobic jars for 48 hours in an incubator set to 35°C. Anaerobic conditions were provided by the activation of Anaerocult®A (Sigma Aldrich, USA). Single colonies were spread again on MRS agar plates and incubated at the same conditions (Borcaklı and Kocer, 2001).

2.2.1.2 Investigation of purity

Sterile physiological water was dropped on microscopy slides; appearing colonies were transferred with an inoculating loop from MRS agar to microscopy slides and dissolved in the previously dropped water. Dissolved colonies were spread on the slides and heat-fixed. After fixation, slides were Gram-stained by the following procedure:

Slides were first stained with crystal violet for two minutes, after which the crystal violet solution was completely washed away and the iodine solution was added for one minute, it was then poured off and the slides were dried. The slides were then washed with ethanol for 15 seconds and counterstained with 1% (w/v) aqueous solution of safranine for 15 seconds. These were then washed and dried prior to microscopic investigation. Stained cultures were visually inspected for their purity under 1000x total magnification (Borcaklı and Kocer, 2001).

2.2.1.3 Prescreening cultures for oleuropein resistance

0.055 g of analytical grade oleuropein was dissolved in 20 ml sterile MRS broth to obtain a solution containing 2.78 g/l oleuropein. This solution was sterilized by filtration through a 0.2 µm filter. Obtained sterile oleuropein solution was serially diluted by a factor of 2 for 10 times. 180 µl of the corresponding dilutions and 20 µl of the microbial culture that was adjusted to 0.5 McFarland units were pipetted to each well of a microplate. The microplate was placed in Bioscreen C® automatic microbiological growth curve analyzer and incubated at 35°C for 20.25 h. The optical density (OD) of the culture was measured every 15 min at 580 nm wavelength. Low amplitude shaking was employed for 10 s before each measurement. Collected data was transferred to Microsoft Office Excel software and the growth curves of the cultures in the presence of 2.5 g/l to 0.005 g/l oleuropein were obtained. The oleuropein concentration of each dilution is shown in **Table 2.7.**

Table 2.7 : The oleuropein concentration of each dilution used in prescreening for oleuropein resistance

Dilution no	Oleuropein concentration
1	2.500 g/l
2	1.250 g/l
3	0.625 g/l
4	0.313 g/l
5	0.156 g/l
6	0.078 g/l
7	0.039 g/l
8	0.020 g/l
9	0.010 g/l
10	0.005 g/l

2.2.1.4 Confirmation of strain identity by mini-API system

Selected two pure potential *L. plantarum* cultures were identified by using Biomérieux® Mini-API 50 CH biochemical lactic acid bacteria identification kit, as described by the supplier.

L. plantarum cultures were transferred to physiological water by a sterile cotton swab until a turbidity of 2 McFarland units was reached. These cultures were inoculated to all wells in the API 50CH kit. These inoculated wells were consequently covered with mineral oil to avoid evaporation and provide anaerobic conditions and incubated for at least 48 h at 35°C, until conclusive results were obtained.

Results were evaluated visually according to the colour changes in each well. Identification of the microorganism was done by entering the results into the API evaluation system.

2.2.1.5 Cultivation of *L. plantarum* DSMZ 10492 culture

DSM 10492 culture arrived in the laboratory in lyophilized form inside a glass ampoule. The culture was vitalized and stored, as described by DSMZ. Briefly, the top of this glass ampoule was heated in fire. Glass was cracked by dropping a few drops of water on the heated glass. The top of the cracked ampoule was removed by a forceps. The inner tube was removed and the protective insulation material was removed to take out the inner vial. The cotton plug was removed from the inner vial and the top of this vial was flamed. 0.5 ml MRS broth was added to the lyophilized culture and the cotton plug was replaced. The pellet was hydrated for 30 min. The hydrated strain was transferred to 7 ml MRS broth and on MRS agar. Grown culture was spread on MRS agar and MRS broth. Fresh MRS broth was used for the experiments and stock cultures were protected by both lyophilisation and cryopreservation in 30% (v/v) glycerol at -80°C deepfreeze. Repeated plating of the culture was avoided to protect strain stability.

2.2.1.6 Isolation of olive phenols

Crude olive extracts (OE) were obtained from Uslu olive cultivar recovered from Akhisar/Manisa region. Extraction of crude OE was modified and performed according to the method described in Medina et al. (2009). Olive kernels were

removed from 1800g ripe olives and 1390g of flesh was obtained. This flesh was crushed for 2 min in a blender by addition of an equal amount of water.

The resulting slurry was centrifuged at 3420g (25cm rotor, 3500 rpm) for 15 min and the supernatant was collected. Additional 2 l of water was added to the pellet and the pellet was resuspended in water. After an incubation at room temperature (22°C) for 1 h, the mixture was recentrifuged at 3420g and the supernatant was collected. This treatment was repeated for two additional times.

All the collected supernatants were centrifuged separately at 3420g for 15 min. These formed two phases of oil and water. The oil-containing phases were removed and the resulting aqueous phase was dried by lyophilisation. 70 g of crude OE was obtained after lyophilisation.

The lyophilized powder was further concentrated by acetone water extraction by modifying the method described by de-la Rosa et al. (2010). Briefly, 10 g of powder was mixed with 100 ml 80% (v/v) acetone-water mixture. This mixture was incubated at 40°C for 2 h in a magnetically stirred water bath. Remaining particles were removed by centrifugation at 15000g for 5 min and the supernatant was transferred to a 100 ml volumetric flask. Acetone-water mixture was evaporated in a rotary evaporator until no acetone was left in the water phase. Solid MRS broth was added to the remaining water extract, as described by the producer. The OE containing MRS broth was sterilized by filtration through a 0.2 µm syringe top filter. The lyophilized crude OE was analysed for oleuropein. Oleuropein was analysed by reverse phase HPLC, as previously described by Malik and Bradford (2006).

2.2.1.7 Testing resistance of cultures to OE oleuropein and NaCl

0.5 g of analytical grade oleuropein was dissolved in 5 ml sterile MRS broth to obtain a solution containing 10 g/l oleuropein. This solution was sterilized by filtration thought a 0.2 µm syringe top filter. The filter-sterilized oleuropein solution was serially diluted by a factor of 2.

The following oleuropein solutions were obtained by this procedure:

1. Dilution 10 g/l oleuropein,

2. Dilution 5 g/l oleuropein.

The self-prepared OE in MRS broth was sterilized by a 0.2 µm syringe top filter. Filter-sterilized OE solution was diluted by a factor of 2.

1. Dilution 100% (w/w) OE,

2. Dilution 50% (w/w) OE.

Optical densities of the overnight *L. plantarum* cultures were measured by a spectrophotometer against MRS broth as the blank. The amount of the culture that had to be added into 7ml sterile MRS broth to obtain an OD_{580} of 0.05 was calculated. The calculated amount of the culture was transferred to sterile MRS broth.

Four different experiments were performed to determine the effect of oleuropein, OE, and NaCl on *L. plantarum* growth in 40% diluted MRS:

1) To determine the effect of oleuropein in diluted MRS broth, 100 µl sterile oleuropein-containing MRS broth was added to each culture well. 80 µl of sterile distilled water and 20 µl of bacteria culture were added to this broth:

   1. Dilution 5 g/l oleuropein in 40% diluted MRS broth.

2) To determine the combined effect of NaCl and oleuropein in diluted MRS broth, two different concentrations of 100 µl sterile oleuropein-containing MRS broth was added to each culture well. 80 µl of NaCl solution (20% w/v) and 20 µl of the bacterial culture were added to this broth:

   1. Dilution 5 g/l oleuropein and 8%(w/v) NaCl in 40% diluted MRS broth.

   2. Dilution 2.5 g/l oleuropein and 8%(w/v) NaCl in 40% diluted MRS broth.

3) To determine the effect of OE in diluted MRS broth, two different concentrations of 100 µl sterile OE-containing MRS broth were added to each culture well. 60 µl of sterile distilled water and 20 µl of the bacterial culture were added to this broth:

   1. Dilution 50% (w/w) OE in 40% diluted MRS broth.

   2. Dilution 25% (w/w) OE in 40% diluted MRS broth.

4) To determine the combined effect of NaCl and OE in diluted MRS broth, two different concentrations of 120 µl sterile OE-containing MRS broth was added to each culture well. 60 µl of NaCl solution(20% w/v) and 20 µl of the bacterial culture were added to this broth:

1. Dilution 50% (w/w) OE and 8%(w/v) NaCl in 40% diluted MRS broth.

2. Dilution 25% (w/w) OE and 8% NaCl in 40%(w/v) diluted MRS broth.

Two experiments were performed as control, to determine the effect of NaCl on 40% diluted MRS broth:

1) Culture grown without an inhibitory agent in 40% diluted MRS broth. 100 µl of sterile MRS broth was transferred to each well, 80 µl of sterile water and 20 µl of the bacterial culture were added to this broth:

1. Dilution Bacterial cultures were grown in 40% diluted MRS broth.

2) Culture grown only with 8% (w/v) NaCl in 40% diluted MRS broth. 100 µl of sterile MRS broth was transferred to each well, 80 µl NaCl solution (20% w/v) and 20 µl of the bacterial culture were added to this broth.

1. Dilution Bacterial cultures were grown in 40% diluted MRS broth

The microplates were placed in Bioscreen C® automatic microbiological growth curve analyzer and incubated at 35°C for 19 h. Culture OD_{580} values were measured every 15 min at 580 nm. Low amplitude shaking was employed for 15 sec before each measurement. Collected data were transferred to Microsoft Office Excel software and the growth curves of each culture at each dilution were obtained.

2.2.1.8 Testing resistance of cultures to analytical grade oleuropein and NaCl

0.5 g of analytical grade oleuropein was dissolved in 5 ml sterile MRS broth to obtain a solution containing 10 g/l oleuropein. This solution was sterilized by filtration through a 0.2 µm filter.

Optical densities of the overnight *L. plantarum* cultures were measured by a spectrophotometer against MRS broth as the blank. The amount of the culture that has to be added into 30 ml sterile MRS broth to obtain an OD_{580} of 0.05 was calculated. The calculated amount of the culture was transferred to sterile MRS broth.

Four different experiments were performed in triplicate to determine the effect of oleuropein and NaCl on the growth of *L. plantarum* T129 and DSMZ 10492 strains in 40% diluted MRS:

1) To provide a control, strains were grown in 40% diluted MRS broth. This set contained 6ml MRS broth, 4.8 ml H_2O and 1.2 ml culture at an OD_{580} of 0.05.

2) To provide a control for 8% (w/v) NaCl stress, strains were grown in 40% diluted MRS broth containing 8% (w/v) NaCl. This set contained 6 ml MRS broth, 4.8 ml 20% (w/v) NaCl solution and 1.2 ml culture at an OD_{580} of 0.05.

3) To determine the effect of oleuropein, strains were grown in 40% diluted MRS broth containing 5 g/l oleuropein. This set contained 6 ml 10 g/l oleuropein-containing MRS broth, 4.8 ml H_2O and 1.2 ml culture at OD_{580} of 0.05.

4) To determine the combined effect of NaCl and oleuropein, strains were grown in 40% diluted MRS broth containing 5 g/l oleuropein and 8% (w/v) NaCl. This set contained 6 ml 10 g/l oleuropein-containing MRS broth, 4.8 ml 20% (w/v) NaCl solution and 1.2 ml culture at an OD_{580} of 0.05.

The 50 ml-culture tubes were placed in water bath at 35°C and incubated by shaking at 100 rpm. The initial cell dry weight (CDW), initial plate counts and initial OD_{580} values were recorded. OD_{580} values were followed at the 2nd ,4th ,6th ,8th , 10th and 24th hour of the experiment. CDW measurements were done at the 8th and 24th h. Plate counts were made at the end of 24 h of cultivation.

CDW measurements

Microfuge tubes (2ml-size) were dried overnight at 85°C and desiccated for 5 h prior to determining their initial weights. Two ml of bacterial culture was transferred in a previously prepared 2ml-microfuge tube. Cultures were harvested by centrifugation at 14000g for 5 min. The pellets were washed twice with sterile distilled water and dried at the same conditions. Dried pellets were weighed using a digital balance at 0.1 mg sensitivity.

Plate counts

Bacterial counts in samples were determined by pour plating serially diluted samples on MRS agar. Cell counts were determined after anaerobic incubation at 35° C for 48 h by counting those plates containing 30-300 CFU.

OD_{580} measurements

OD_{580} measurements of cultures were made in plastic spectrophotometer cuvettes with 1cm light path in a UV/Vis spectrophotometer.

2.2.2 Methods related to the engineering of lactic acid bacteria resistant to NaCl and oleuropein, to be used as starter culture

2.2.2.1 Ethyl methane sulfonate (EMS) mutagenesis

Ethyl methane sulfonate (EMS) was used for mutagenesis and to increase the genetic diversity of the lactic acid bacteria culture. The EMS amount that killed 90% of the inoculum was previously found to be most effective for yeast and bacteria (Lawrence, 1991). The survival rates of *L. plantarum* upon 0, 0.37, 0.72, 0.88 and 1.19 M EMS exposure for 30 min were determined to find the most appropriate mutagenic conditions.

L. plantarum was cultivated overnight at 35°C in MRS broth. The OD_{580} of the resulting culture was determined spectrophotometrically against MRS broth as the blank. The required concentration of the culture to give 2.5 OD_{580} was calculated and this amount of the culture was transferred to 1.5 ml-microfuge tubes. Bacteria were harvested at 14000 rpm for 5 min and the pellet was washed twice with sterile phosphate buffer saline (PBS). Washed cultures were resuspended in 0.5 ml PBS and the predetermined amount of EMS was added. After treatment with EMS for 30 min in a rotary shaker at 150 rpm, EMS was inactivated by the addition of freshly made 0.5 ml of 10% (w/v) $Na_2S_2O_3$. After centrifugation at 14000 rpm for 5 min, the pellet was washed twice with 0.5 ml PBS. The resulting pellet was resuspended in 0.5 ml PBS and most probable number (MPN) analyses were performed. 300 µl of the remaining culture was added to 20 ml MRS Broth for overnight cultivation (Sonderegger and Sauer 2003; Çakar et al., 2005).

Five tube-MPN counts

96-well sterile multiwell plates were used. 5x8 wells were filled with 180 µl of MRS broth. Twenty µl of culture was added to the first row and 20 µl of sample was added to all 5 parallel wells. After careful mixing, 20 µl of sample was pipetted to the second dilution. Eight dilutions were performed in 5 parallels for each sample.

Results were evaluated by using standard MPN tables available online (http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm109656.htm).

OD_{580} measurements

OD_{580} measurements of the cultures were performed at the 14th, 22nd and 36th h of cultivation.

Stock culture preparation

Stock cultures were prepared by the addition of an equal volume of 60% (v/v) sterile glycerol to liquid bacteria culture. All stored bacteria were at their late log phase of growth to have high viability levels.

2.2.2.2 NaCl stress tests

T129 and DSMZ 10492 *L. plantarum* strains subjected to mutagenesis with ethyl methane sulfonate (EMS) at 0.37 M and 0.72 M for 30 min were used. The non-EMS- treated T129 and DSMZ 10492 *L. plantarum* strains were used as control.

L. plantarum cultures were cultivated overnight at 35°C in MRS broth. The cultures were harvested at 5000g for 5 min and washed once with MRS. The OD_{580} of resulting cultures were determined spectrophotometrically against MRS broth as the blank. The required concentrations of the culture to give 0.05 OD_{580} were calculated and this amount of culture was transferred to 10 ml sterile MRS-containing glass tubes.

75 ml of 0, 1, 3, 6, 12, 16 and 20 % (w/v) NaCl solutions were autoclaved in 100 ml borosilicate glass bottles. Five ml of these solutions were transferred to tubes containing 5 ml MRS broth to obtain 50% diluted MRS tubes containing 0, 0.5, 1.5, 3, 6, 8 and 10% (w/v) NaCl. Each tube was inoculated with control and EMS-mutagenized *L. plantarum* strains. 200 µl of each inoculated medium was transferred on a Bioscreen® plate in triplicate to perform OD measurements.

CDW measurements

CDW measurements were done as described previously in Section 2.2.1.8.

OD_{580} measurements

OD_{580} measurements of the cultures were taken every 15 min in triplicate by automated turbidometry.

2.2.2.3 Selection of mutant populations

Mutant populations were selected in successive batch cultures, at gradually increasing NaCl stress levels. 10 ml of 50% diluted MRS broth was inoculated with 100 µl of EMS mutants of *L. plantarum* DSMZ 10492 and *L. plantarum* T129. The inoculated MRS was incubated in a 50 ml culture tube at 35°C and 100 rpm for 24 h. After the incubation period, the OD_{580} of the grown bacterial cultures were determined sprectrophotometrically. The amounts of the inoculum to obtain 0.05 OD_{580} were calculated and the calculated amounts of bacteria were transferred to 10 ml 0.5% (w/v) NaCl-containing MRS and 50% diluted MRS as control. The 10 ml bacterial suspensions were incubated in 50 ml culture tubes at 100 rpm and 35°C for 48 h. NaCl stress level was increased by 0.5% at each successive batch cultivation unless the OD_{580} ratio of stress free and stressed population (the survival ratio) was less than 40%. The same stress conditions were applied again until the desired minimum survival ratio of 40% was obtained.

It was previously observed that autoclaving MRS broth with increasing NaCl concentrations results in precipitate formation. Thus, desired NaCl concentrations were obtained by blending 20% (w/v) NaCl solution and dH_2O with 50% MRS broth.

2.2.2.4 Selection of individual colonies

Individual colonies were selected by spread-plating serial dilutions of finally obtained mutant generation on MRS and randomly picking 12 individual colonies from appropriately diluted plates. Obtained colonies were dissolved in sterile distilled water and transferred to MRS broth. 500 µl of colonies cultivated in MRS broth were transferred to 1.5 ml centrifuge tubes. 500 µl of 30% (v/v) glycerol was added on the colonies. Glycerol-added colonies were stored at -80ºC.

2.2.2.5 Screening NaCl and oleuropein resistance of individual colonies

Isolated mutant individuals were screened for their ability to survive NaCl and oleuropein stress. Ten ml of MRS broth was aseptically transferred to a sterile 50 ml culture tube. MRS broth was inoculated with 100 µl of stock culture which was stored at -80ºC. The inoculated broth was incubated at 35°C and 150 rpm for 24 h. After the incubation period, 100 µl of bacteria were transferred to 10 ml 10% (w/v) NaCl and 10 ml 10% (w/v) NaCl + 4g/l oleuropein-containing 50% diluted MRS. As a control, 100 µl of bacteria were transferred to 10 ml 50% diluted MRS. The 10 ml bacteria suspensions were incubated in 50ml-culture tubes at 150 rpm and 35°C for 72 h.

Bacteria counts were determined by pour plate technique. Bacteria were counted in 3 replicates at 0, 24, 48 and 72 h.

It was previously reported that autoclaving MRS broth with increasing NaCl concentrations results in precipitate formation and oleuropein is heat-sensitive. Thus, desired NaCl concentrations were obtained by blending 20% (w/v) NaCl solution and dH_2O with 50% MRS broth. Oleuropein was added to sterile MRS broth and filter-sterilized.

2.2.2.6 Screening cross-resistance of individual colonies

24 mutant individuals and the two wild-type strains were screened for their ability to survive freeze-drying, lactic acid, acetic acid, alkaline stress, and ferrous gluconate stresses.

Ten ml of MRS broth was aseptically transferred to a sterile 50 ml culture tube. MRS broth was inoculated with 100 µl of stock culture, which was stored at -80º C. The inoculated broth was incubated at 35°C and 150 rpm for 24 h. After the incubation period, 5 µl of each culture were transferred to 2 ml microfuge tubes containing 500 µl of 50% diluted MRS broth adjusted to pH 9 by NaOH addition, 50% diluted MRS broth adjusted to pH 4 by acetic acid addition, 50% diluted MRS broth adjusted to pH 4 by lactic acid addition, or 50% diluted MRS broth containing 0.1% (w/v) ferrous gluconate. These cultures were inoculated at 150 rpm and 35°C for 24 h. Bacteria were counted at 0 and 24 h by pour plate technique. One ml of fresh grown cultures was transferred to sterile empty 1.5 ml-microfuge tubes. The culture was centrifuged at 3000g for 15 min. All supernatants were removed and the cultures were resuspended in 500 µl 5% (w/v) glucose and 2% (v/v) glycerol

solution. Resuspended cultures were transferred to sterile glass vials. Freeze-drying was performed by an increasing temperature gradient from -45ºC to 25ºC in 30 h at 0.9 mbar, followed by drying at 25ºC and 0.15 mbar for 15 h. Freeze-dried pellets in vials were resuspended in 1ml sterile distilled water and counted by pour plate technique on MRS Agar.

2.2.2.7 Physiological analyses of the wild type and the individual mutant strains

Growth physiological analysis was performed for both wild type T129 strain and its two mutants T8 and J1; and the wild type strain DSMZ 10492 and its mutant D2. All bacteria were cultivated in 10 ml 50% diluted MRS broth overnight at 35°C, 150 rpm in 50 ml screw cap polypropylene culture tubes.

Optical densities of the overnight *L. plantarum* cultures were measured by a spectrophotometer against 50 % diluted MRS broth as the blank. The amount of culture that has to be added in to 50 ml sterile MRS broth to obtain an OD_{580} of 0.05 was calculated. The calculated amount of culture was transferred to sterile MRS broth.

Two different experiments were performed in triplicate to determine the effect of 6.5% NaCl (w/v) on both wild type *L. plantarum* strains T129 and DSMZ 10492, and the mutant individuals T8, J1 and D2.

1) To provide a control, strains were grown in 50% diluted MRS broth. This set contained 25 ml MRS broth and 25 ml H_2O
2) To determine the effect of NaCl on growth physiology, strains were grown in 6.5% (w/v) NaCl-containing MRS broth. This set contained 25 ml MRS broth and 25 ml 13% (w/v) NaCl

The 250 ml-flasks were placed in an incubator shaker at 35°C and incubated by shaking at 150 rpm. The initial CDW, initial plate counts and initial OD_{580} were recorded. OD_{580} and CDW were followed every 2 h. Samples were taken for HPLC analysis at the 4th ,8th ,14th , 18th and 24th h of the control experiment in 50% diluted MRS. Additionally, samples were taken for HPLC analysis at the 4th ,8th ,16th , 24th and 30th hour of the experiment performed with 6.5%(w/v) NaCl-containing 50% diluted MRS.

CDW measurements

Microfuge tubes were dried overnight at 85°C and desiccated for 5 h prior to their initial weight determination. One ml of cultures were harvested by centrifugation at 14000g for 5 min. The pellets were washed twice with sterile distilled water and dried at the same conditions. The supernatants of the centrifuged cells were frozen at -18° C and used later for HPLC analysis. Dried pellets were weighed using a digital balance at 0.1 mg sensitivity.

OD_{580} measurements

OD_{580} measurements of cultures were made in plastic spectrophotometric cuvettes with 1cm light path in a UV/Vis spectrophotometer.

High performance liquid chromatography (HPLC) analysis of glucose and lactic acid

Collected samples were thawed and diluted 1:1 with HPLC-grade water. Samples were filtered through 0.2 µm syringe top filters and were filled into HPLC vials.

An Aminex® HPX-87H column was used in HPLC analysis. Isocratic 0.6 ml/min 5 mM sulfuric acid was used as the mobile phase. Eluted analyte was detected by a refractive index (RI) detector.

A stock solution containing 1200 mg glucose and 1000 mg lactic acid was prepared in 100 ml HPLC-grade water. Six standard solutions were prepared by the dilution of this stock solution as given in **Table 2.8.**

Table 2.8 : Preparation of standard solutions for HPLC analysis.

Standard solutions	Stock solution (µl)	HPLC-grade water (µl)	Final volume (µl)
S1	1000	0	1000
S2	500	500	1000
S3	200	800	1000
S4	125	875	1000
S5	100	900	1000
S6	67	933	1000

2.2.2.8 Genetic stability of mutant individuals

Growth physiological analysis was performed for T8 and D2 mutants. Bacteria were cultivated in 10 ml 50% diluted MRS broth at 35°C, 150 rpm in 50 ml screw-cap polypropylene culture tubes. Fresh MRS broth was inoculated every 24 h with grown

mutant culture and mutant cultures were spread as three replicates on MRS agar and 7% (w/v) NaCl-containing MRS agar. The ratio between colony counts on MRS agar and 7% (w/v) NaCl supplemented MRS agar were calculated to determine the genetic stability of NaCl-resistance of selected individuals.

2.2.3 Engineering lactic acid bacteria against upper gastrointestinal stress

2.2.3.1 Preparation of bacterial strains

L. plantarum T129 and DSMZ 10492 were both isolated from olive-processing brines in Turkey and Portugal, respectively. Both bacterial strains were made antibiotic-resistant prior to the Dynamic Gastrointestinal Model (TIM-1) experiments. Rifampicin is an antibiotic that acts by binding RNA polymerase. A random point mutation in DNA coding for RNA polymerase b subunit gives rise to rifampicin resistance. This point mutation naturally occurs at a frequency of 10^{-9} during microbial propagation. Thus, it is possible to select these natural rifampicin resistant mutants (Pijkeren and Britton 2012).

L. plantarum DSMZ 10492 and T129 strains were cultivated in MRS broth. Approximately 100 µl of approximately 10^9 CFU/ml bacteria containing MRS broth was plated on Rogosa agar containing 25 µg/ml rifampicin. After anaerobic incubation of plates at 37°C for 48 h, three colonies were selected from Petri dishes for each strain. Selected colonies were re-plated twice under the same conditions. These rifampicin-resistant colonies (strains) were used in TIM-1 experiments. As TIM-1 is an open system that cannot be sterilized, the use of rifampicin-resistant cultures can provide selection advantage over potential contaminants.

2.2.3.2 EMS mutagenesis

EMS was used for mutagenesis and to increase genetic diversity in lactic acid bacteria. An EMS amount that killed 90 % of the initial inoculum was previously found most effective for screening mutants of yeast and bacteria (Lawrence, 1991). The survival rates of *L. plantarum* T129 upon 0 M, 0.37 M, 0.72 M, 0.88 M, and 1.19 M EMS exposure for 30 min each were experimentally determined to choose the most appropriate mutagenic conditions.

EMS mutagenesis was done as described previously (Sonderegger and Sauer 2003, Çakar et al., 2005). The cell pellet obtained at the end of this protocol was resuspended in PBS and plate counts were performed. 400 µl of the remaining

culture was added to 20 ml MRS Broth containing 25µg/ml rifampicin for overnight cultivation.

Bacterial counts were determined by spread-plating serially diluted samples on 25µg/ml rifampicin-containing RogosaAgar. Cell counts were determined after anaerobic incubation at 37°C for 48 hours.

2.2.3.3 Dynamic gastrointestinal model (TIM-1)

TIM-1 was used, as previously described in detail by Minekus et al. (1995). Briefly, TIM-1 consists of four compartments mimicking the stomach, duodenum, jejunum and ileum. Each compartment is made up of two glass compartments. These glass compartments cover a flexible wall that makes it possible to mimic peristalsis. All compartments are kept at 37°C (body temperature) while gastric acid and gastric enzymes, pancreatic enzymes, bicarbonate and bile salts are delivered to corresponding compartments in physiological quantities. Absorption in the jejunal and ileal compartments are simulated by an integrated dialysis system. An exponential power function is employed to mimic the transit of the chime through the system. This transit is based on *in vivo* data and controlled by the computer, as described by Minekus et al. (1995) and Marteau et al. (1997).

2.2.3.4 Determination of cell survival in TIM-1 System

The survival of strains and mutant mixtures were assessed in samples taken from the gastric and the intestinal residue (after termination of the experiment) and 6 hourly fractions of the ileal efflux. The latter sampling site represents the surviving cells at the end of the small intestine. An outline of this TIM-1 system is given in **Figure 2.1.** Experiments were done in duplicate, except for the mutant mixture. The ileal efflux from the mutant mixture was collected and run through TIM-1 again, to select for the best surviving strain. This selection cycle was repeated 5 times.

The survival of the strains that were fed into the TIM-1 system was determined using the cumulative delivery calculation:

$$\%\ \text{Cumulative delivery} = \frac{\text{B.M}}{A} \text{ x } 100$$

where A is the cfu of the initial bacterial population fed to TIM, B is the cfu/g of the sample collected at given time (e.g. 0-60 min), and M is the mass of the sample (g)

(Marteau et al., 1997; Öztürk et al., 2016). For % cumulative delivery calculations at later time points, the % cumulative delivery values from t = 0 h on were added.

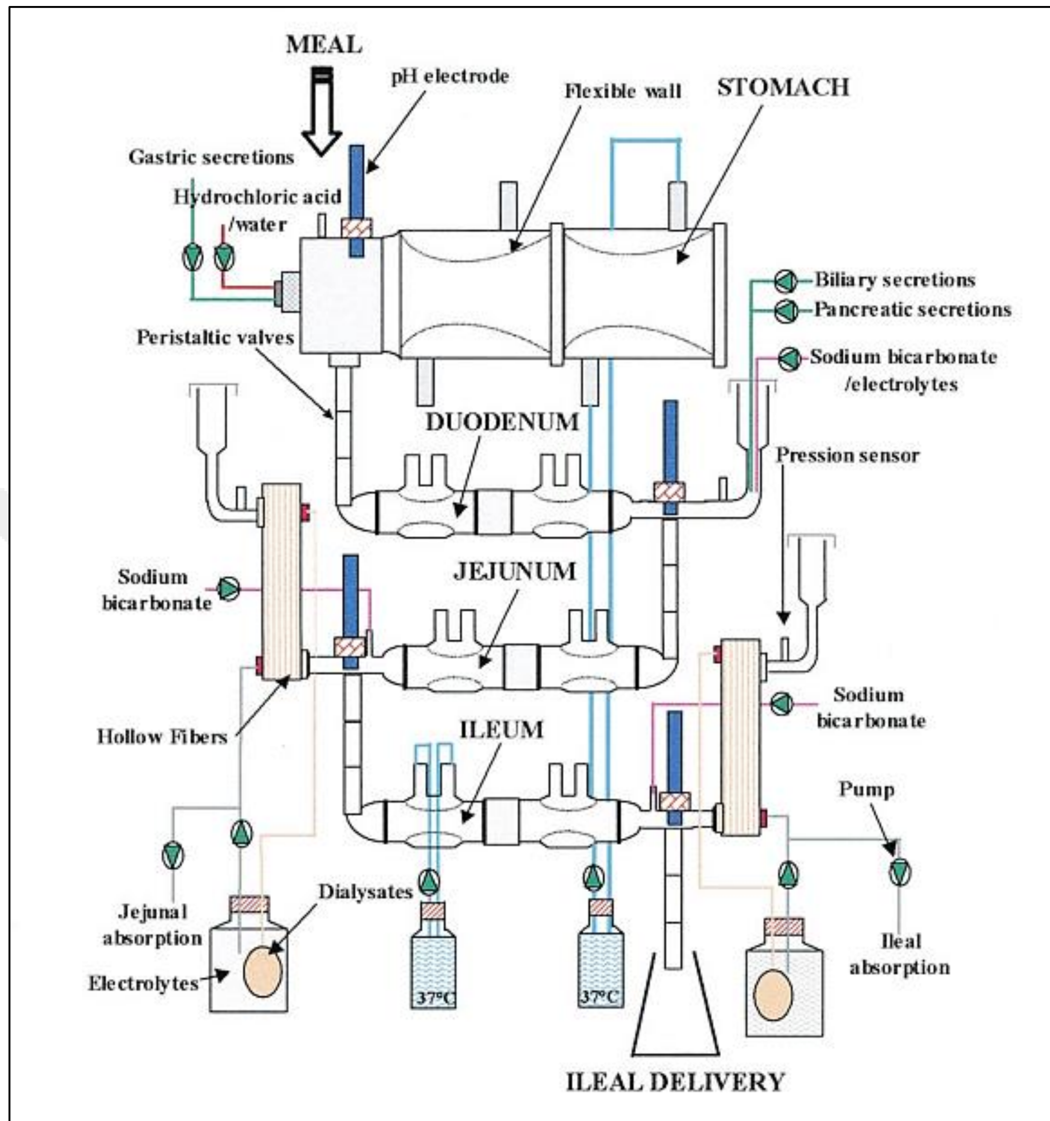

Figure 2.1 : Outline of TIM-1 System (Blanquet et al., 2003).

The model was decontaminated by a cleaning protocol before each run. The system was firstly washed with soap and dirt was removed by a brush. The system was rinsed with water after all dirt was removed and placed in a bleach solution containing approximately 0.5% (w/v) sodium hypochloride for 30 min. Consequently, the system was rinsed again with water and 0.5M HCl. Finally, the system was rinsed with 70% (v/v) ethanol and left to dry.

All secretion tubings in the model were rinsed with 30% (v/v) ethanol and all caps and pH sensors were rinsed with 70% (v/v) ethanol before being attached to the system.

Bacterial cultures (T129 or the mutant mixture) were resuspended in 5ml sterile physiological peptone water. Bacterial suspension was added to a mixture of 150 ml semi skim milk, 70 ml sterile gastric electrolyte, 50 ml sterile distilled water, and 5 ml gastric enzyme solution. The pH of the meal was adjusted to 5.5 and was introduced into the gastric compartment. Gastric electrolyte solution was composed of 6.2 g NaCl, 2.2 g KCl, 0.22 g KCl and 1.2 g $NaHCO_3$ per liter. Gastric enzyme solution was prepared by the addition of 1.5ml 1M sodium acetate at pH 5, 30 mg pepsin and 37.5 ml gastric lipase into 150 ml of gastric electrolyte solution (Minekus et al., 1995). The pH curve in stomach was computer-controlled by addition of 2M HCl and gastric electrolyte solution to mimic the physiological pH conditions of the human digestive system after ingestion of fermented milk products. pH was initially adjusted to 5.5, it then decreased to 4.2 after 30 min, 2.9 after 60 min and was 1.7 after 120 min. The pH of the gastric compartment is shown in **Figure 2.2.** The pH of duodenum, jejenum and ileum was kept at 6.5, 6.8 and 7.2, respectively (Minekus et al., 1995 ; Marteau et al., 1997).

Figure 2.2 : Course of pH in the gastric compartment during the experiments (derived from personal experiments on TIM-1).

Transit of the meal was computer-controlled by regulating the pump valves between compartments. The emptying rates were following an exponential power function. The rate constants were in accordance with the *in vivo* results for the transit of milk, obtained by feeding a non-absorbable meal marker to human volunteers. The emptying half time was 40 and β coefficient was 1 for gastric emptying and the emptying half time was 160 and β coefficient was 1.6 for ileal emptying (Minekus et al., 1995; Marteau et al., 1997).

Duodenal secretion contained 1 mol $NaHCO_3$ or intestinal electrolyte solution (0.25 ml/min), 7% Pancreatin in intestinal electrolyte solution(secretion speed: 0.3 ml/min) and porcine bile extract (0.6 ml/min). Dialysis at the jejunum and ileum was performed against a solution of 5 g/l NaCl, 0.6 g/L KCl, 0.25 g/L $CaCl_2$ and bile salts at only jejenal at physiological concentrations as described by Minekus et al. (1995). The flow rate of the dialysis solution through the dialysis membranes was 10 ml/min. Bile concentration was 4% for the first hour and 2% until the end of the experiment (Minekus et al., 1995; Marteau et al., 1997).

2.2.3.5 Selection of mutant populations

100 µl stock culture was inoculated to 10 ml 25 µg/ml rifampicin-containing MRS broth and was incubated anaerobically for 16 h at 37°C prior to each experiment. The grown bacterial culture was harvested by centrifugation at 5000g for 10 min and added to 150 ml semi skim milk (1.5% fat) containing 280 ml meal, as described above. Before each experiment, the initial microbial counts were determined in the meal.

To select the best surviving mutants by reintroduction of bacterial cultures to TIM-1, 80 ml of ileal efflux samples that were collected between the 3rd and 4th hour were centrifuged for 10 min at 5000g and resuspended in 5 ml physiological salt solution. The salt solution was stored between 4-8°C and used to inoculate the meal of the next day.

The survival rate of bacteria in the ileal efflux was determined by taking samples every hour for 6 hours. The residues in the gastric and intestinal compartments were collected as two separate samples at the end of 360 min. All samples were collected on melting ice and were plated within maximum 60 min after collection (Öztürk et al., 2016).

At the end of the fifth reintroduction experiment; duodenal, jejunal and ileal residues were collected separately and cultivated anaerobically at 37° C for 24 h in order to select superior strains with the ability to grow at these conditions. Mutant colonies were selected either from cultivated jejunal residue or ileal efflux sample taken from the fourth reintroduction experiment between 180 and 240 min after the start of the experiment.

2.2.3.6 Selection of the best surviving mutant strain by tube-based experiments

Tube-based experiments were employed to make an initial selection of better surviving mutant strains. Overnight cultures of bacteria were centrifuged for 5 min at 5000g and resuspended in physiological salt solution.

Two ml-microfuge tubes were filled with 985 µl gastric mixture at pH 3.5 and inoculated with 15 µl fresh prepared bacteria suspension. After 2 h of incubation at 37°C and 150 rpm, 352 µl of pancreatic mixture was added to each microfuge tube. Pancreatine-added microfuge tubes were incubated at 37°C and 150 rpm for 2 more hours and plate-counted. The survival of each culture was determined by calculating the survival ratio. The recipes of gastric and intestinal mixtures are given in **Table 2.9.** Mixtures at neutral pH and no enzymes were used as control conditions.

Table 2.9 : The recipes of gastric and intestinal mixtures.

Gastric mixture	Volume (µl)	Pancreatic mixture	Volume (µl)
Bacterial suspension	15	Intestinal electrolyte	52
Milk	449	Bicarbonate	32
Gastric electrolyte solution	283	Pancreatine	90
Acid	0	Bile	178
Gastric enzyme solution	104	Total	352
Water	150		
Total	1000		

2.2.3.7 Antimicrobial activity of T129 and selected mutants

Antimicrobial activity against enterobacteria was determined by spotting 3 µl of bacteriocin-producing strains (T129, its EMS mutants or *L. lactis* TTC 03.0262 as a positive control) on MRS and M17 Agar. Appearing colonies were inactivated using chloroform vapour after overnight incubation at 37°C. Inactivated colonies were overlaid with soft MRS Agar containing indicator strains. The diameters of the appearing inhibition zones were measured after overnight incubation at 37°C (Öztürk et al., 2016).

2.2.3.8 Determination of the genetic stability of the mutant strain

Selected J1 strain was cultivated in 25µg/ml rifampicin containing MRS broth for 88h (3 successive passages) to test the genetic stability of the mutation.

2.2.3.9 Physiological analysis of the wild types and mutant individuals

The survival rate of bacteria was calculated by the ratio of the cumulative recovered bacteria (in CFU) from the system divided by the number of CFUs fed to the microfuge tubes containing gastric and pancreatic mixtures.

Growth physiological analysis was performed for both wild type T129 and its mutant J1 strain, as described previously in Section 2.2.2.7.

CDW measurements

CDW measurements were done as described previously in Section 2.2.2.7.

OD_{580} measurements of cultivated cells

OD_{580} measurements of the cultures were made in plastic spectrophotometric cuvettes with 1cm light path in a UV/Vis spectrophotometer.

High performance liquid chromatography (HPLC) analysis of glucose and lactic acid

HPLC analyses of glucose and lactic acid were done as described previously in Section 2.2.2.7.

2.2.3.10 Determination of the genetic background of the increased survival in the mutant strain

The DNA of T129 wild type and J1 was extracted with PureLink® Genomic DNA Kits (Invitrogen, USA).

The isolated genomic DNA of the *L. plantarum* T129 and J1 were sequenced by using the NGS instrument HiSeq2500 in high-throughput mode (110 bp x 2) located at GENAM - TUBITAK.(former IGBAM) Generated DNA sequence file (FastQ) pairs contained 17,232,012 and 13,585,558 reads in total for the two strains, respectively. Paired FastQ files for the individual strains were subjected to quality control by using FastQC v0.11.8. Following the quality check, those sequences with low Phred quality score were trimmed by running sickle v1.33.

In order to estimate sequence coverage several methods including the one recommended by the NGS manufacturer Illumina, a standalone script contained in BBMap v38.34 as well as by manually investigating k-mer based output results of the software tool jellyfish v2.2.10..

The first method is based on a simple equation as follows:

$$C = LN / G$$

where C stands for coverage, G is the haploid genome length, L is the read length and N is the number of reads. The latter two methods are both based on calculating k-mer frequencies found in the input FastQ files.

İGBAM assembled genomes based on *L. plantarum* Wcfs1 complete genome and identified single base substitutions.

The obtained genome was annotated by MAKER Web Annotation Service (http://weatherby.genetics.utah.edu/cgi-bin/mwas/maker.cgi). The sequences of the expressed genes that harboured a mutation were exported. The mutated and non-mutated genes were aligned by Blast Nucleotide and Blast Protein search tools available at (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) The domain of the mutated amino acid residues were estimated by NCBI conserved domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.). Smart Blast were used to determine how well the mutated amino acids were preserved among more distantly related species. Codon usage database (http://www.kazusa.or.jp/codon) was employed to estimate the effect of synonymous mutations. BioCyc Pathway/Genome Database Collection (http://biocyc.org/) were used to determine the role of the mutated protein in related pathways. The structure of wild type proteins were retrieved from Protein Databank in Europe (http://www.ebi.ac.uk/pdbe/) and http://www.proteinmodelportal.org/. The protein structure was used to argue the effect of the mutated protein residue on protein structure and stability.

2.2.3.11 Determination of the genetic background of the increased rifampicin resistance in the wild type strain

Rifampicin resistance is generally related to a mutation in *rpoB* gene (Goldstein, 2014). The reference rpoB gene belonging to *L. plantarum* WCFS1 was downloaded from the NCBI gene database. The sequence was extracted from the assembled genomes of the two strains by pairwise alignment through the use of BLAST v2.7.1. +.

3. RESULTS AND DISCUSSION

3.1 Resistance of Strains Against Olive Polyphenols and NaCl

3.1.1 Prescreening of cultures

All selected 20 cultures were well preserved by freeze-drying and grew well in MRS broth. After visual inspection of the samples under the light microscope, a contamination was suspected in two cultures, T147 and T150. Thus, they were not included in further tests. Any of the remaining 18 cultures produced gas and were confirmed not to be *L. brevis*. Growth curves of 18 cultures at serial two-fold dilutions of 2.5 g/l oleuropein (**Table 2.7**) are given in **Figures 3.1-3.18**.

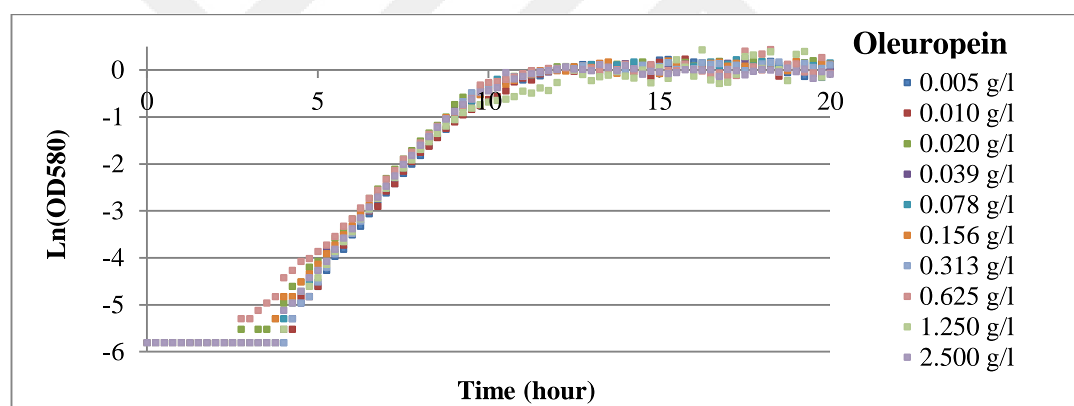

Figure 3.1 : Growth curve of *L. plantarum* T026 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

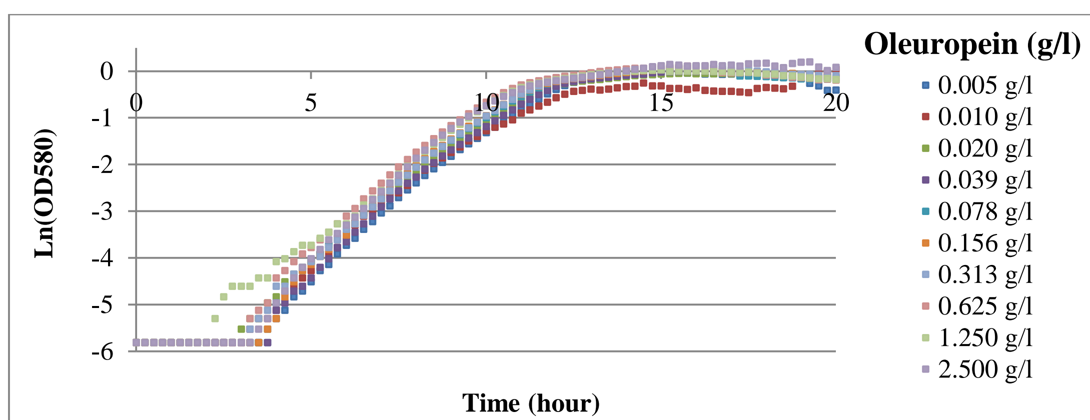

Figure 3.2 : Growth curve of *L. plantarum* T033 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

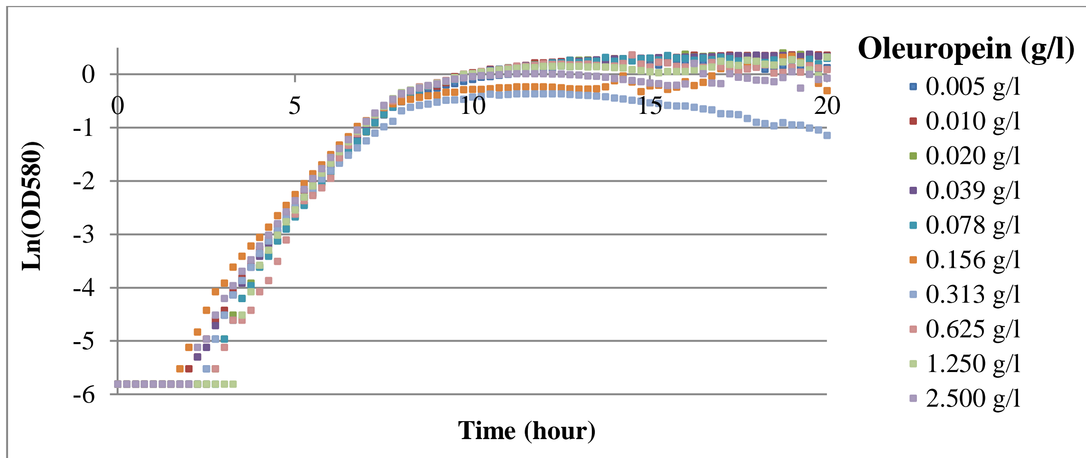

Figure 3.3 : Growth curve of *L. plantarum* T068 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

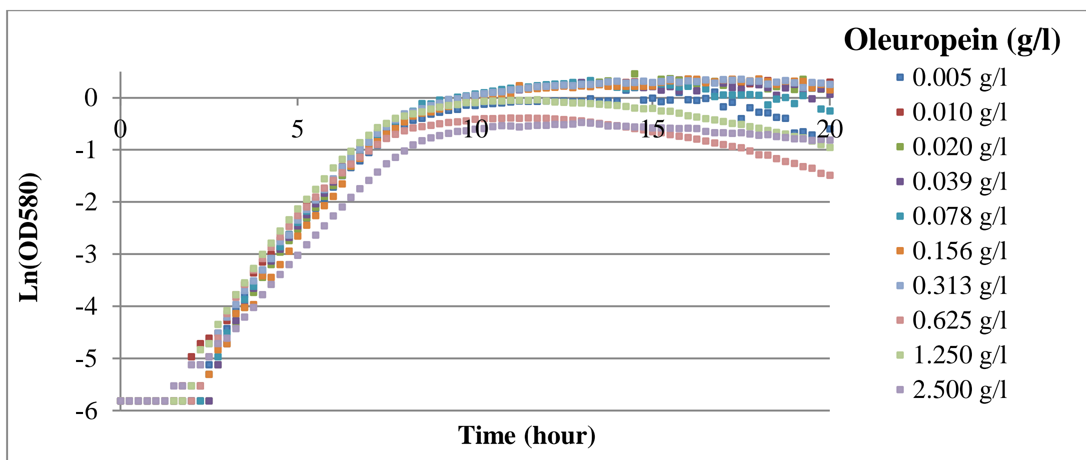

Figure 3.4 : Growth curve of *L. plantarum* T102 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

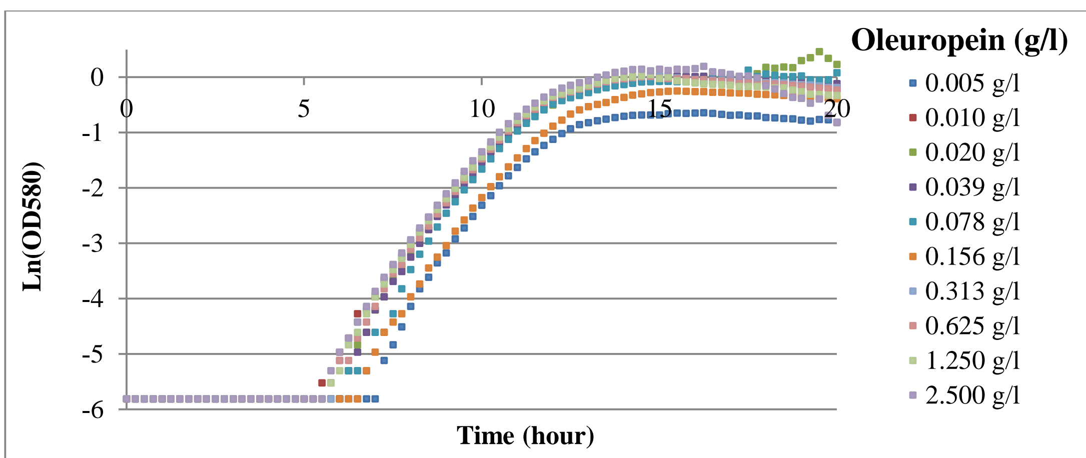

Figure 3.5 : Growth curve of *L. plantarum* T106 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

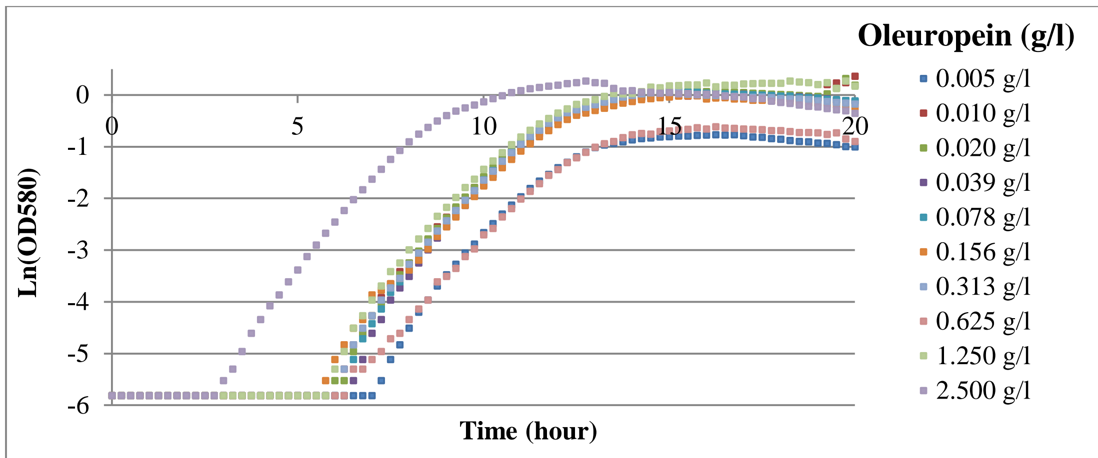

Figure 3.6 : Growth curve of *L. plantarum* T109 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

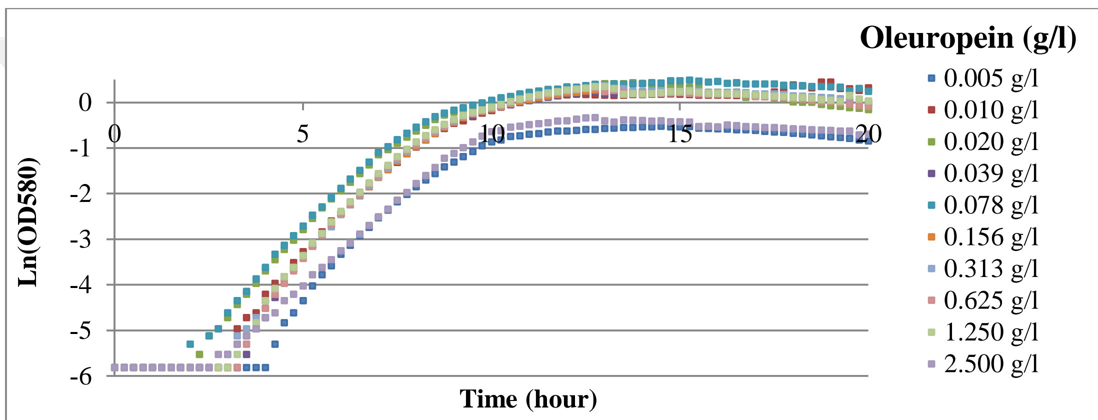

Figure 3.7 : Growth curve of *L. plantarum* T119 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

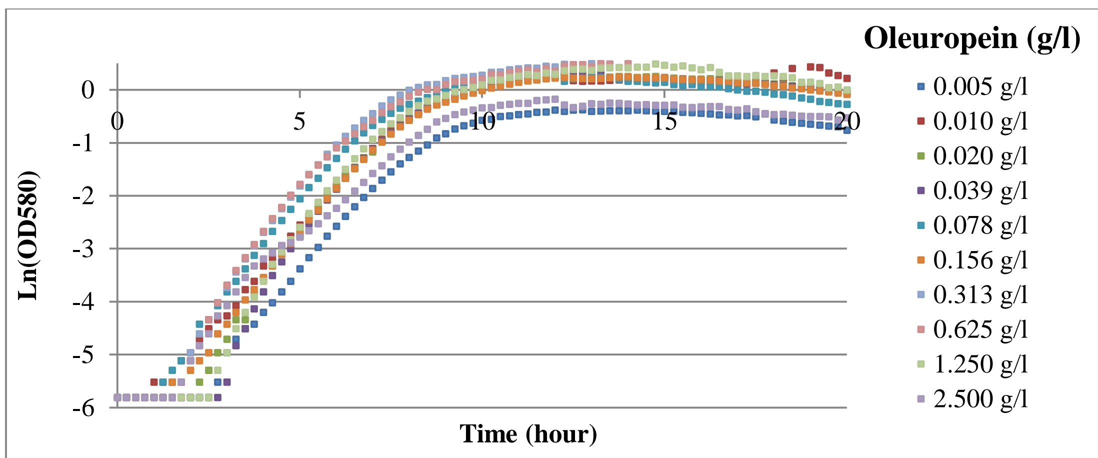

Figure 3.8 : Growth curve of *L. plantarum* T123 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

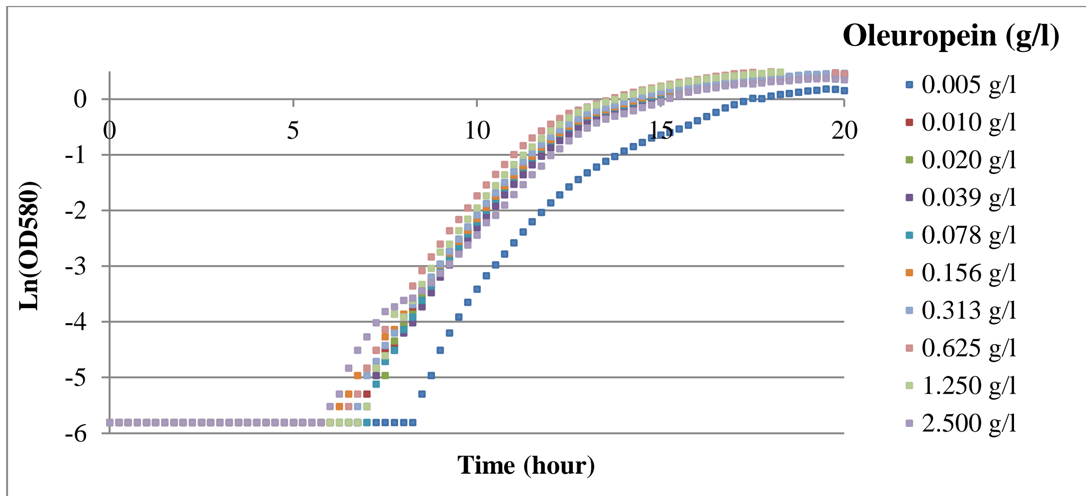

Figure 3.9 : Growth curve of *L. plantarum* T127 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

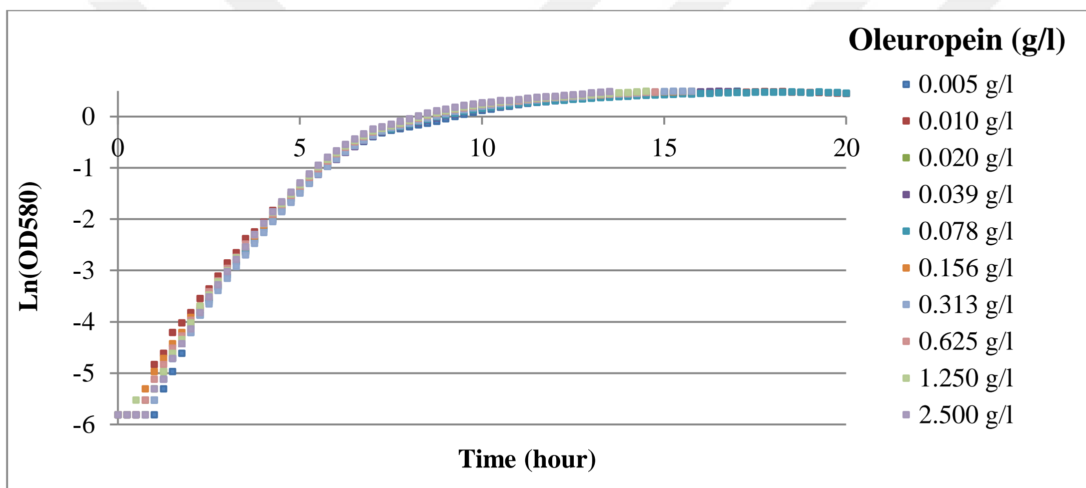

Figure 3.10 : Growth curve of *L. plantarum* T129 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

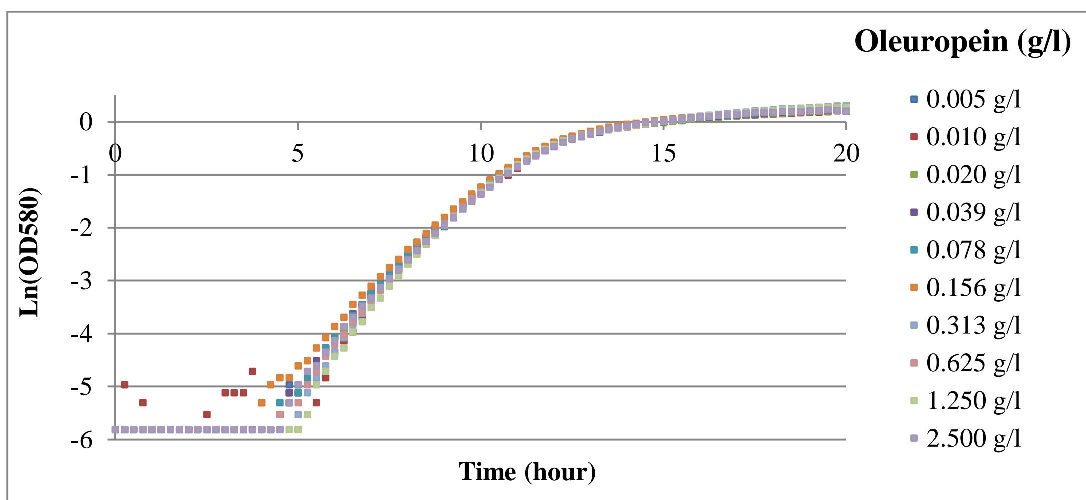

Figure 3.11 : Growth curve of *L. plantarum* T813 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

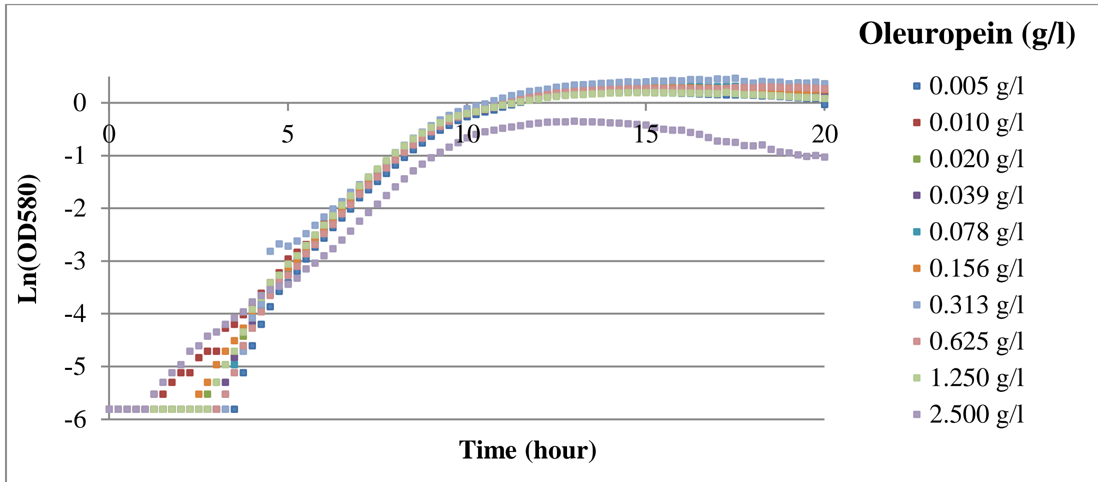

Figure 3.12 : Growth curve of *L. plantarum* T822 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

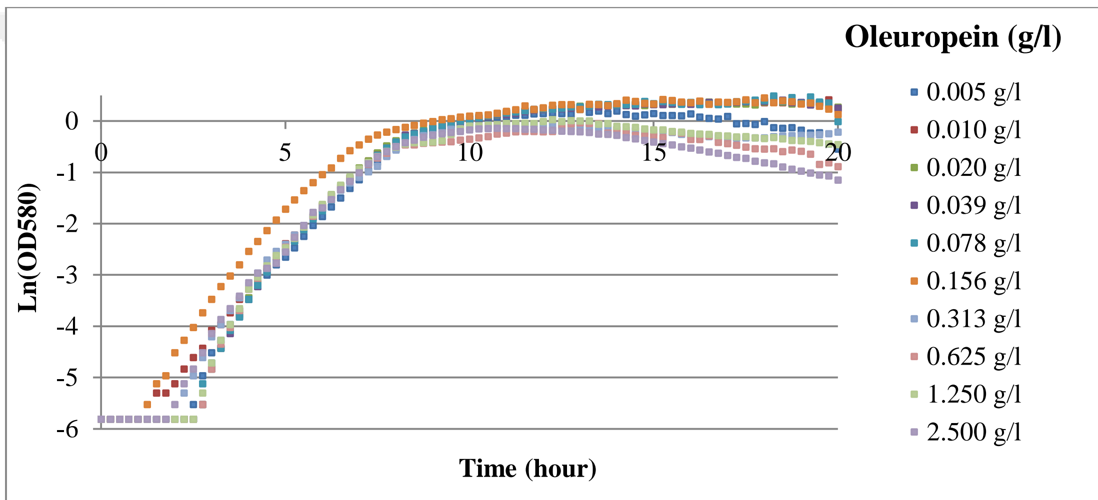

Figure 3.13 : Growth curve of *L. plantarum* T831 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

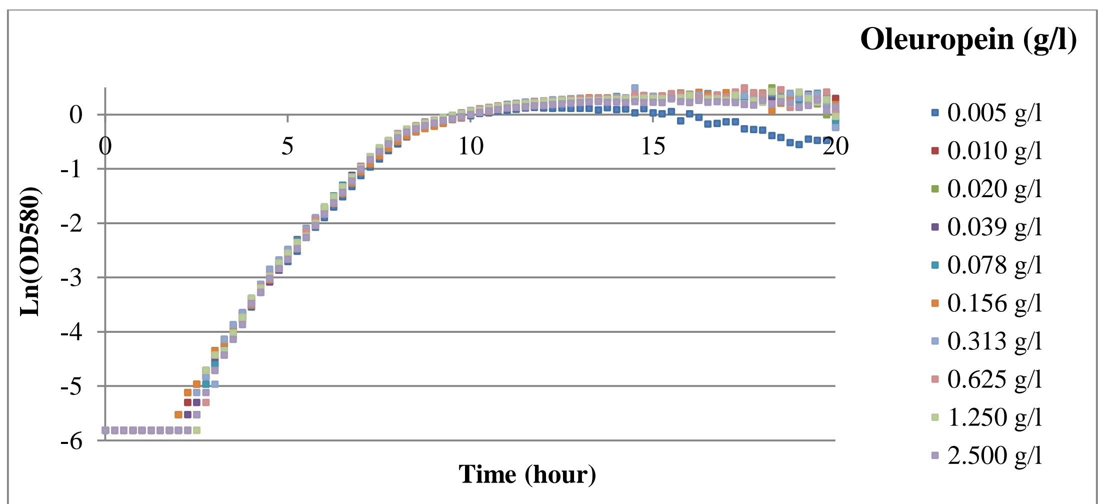

Figure 3.14 : Growth curve of *L. plantarum* T874 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

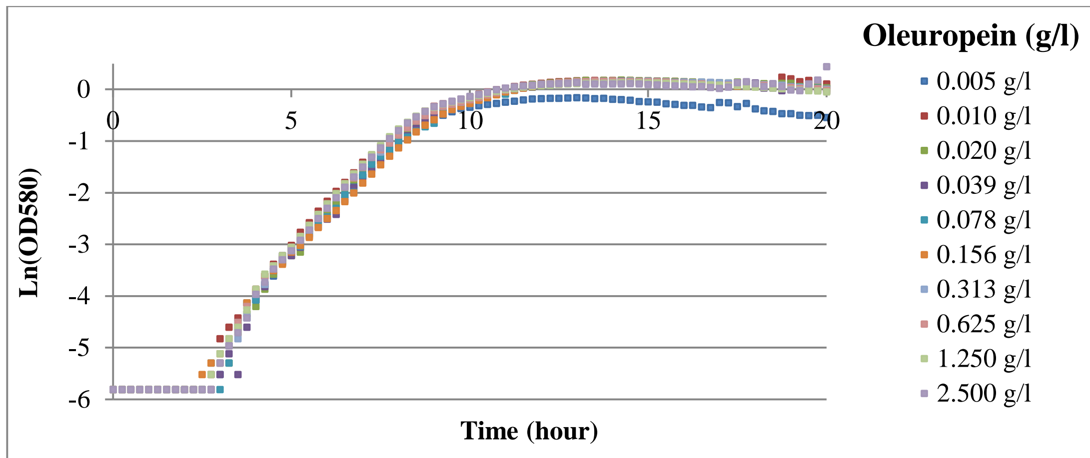

Figure 3.15 : Growth curve of *L. plantarum* T880 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

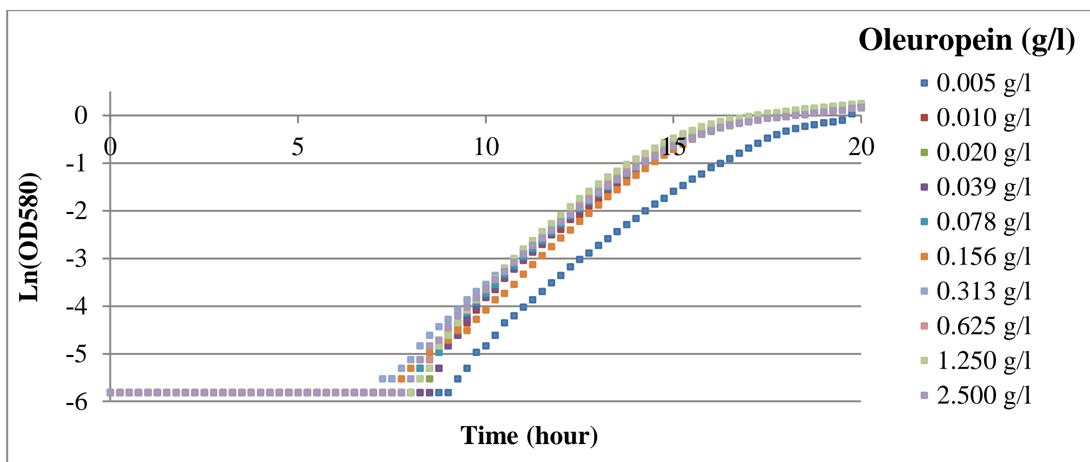

Figure 3.16 : Growth curve of *L. plantarum* T896 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

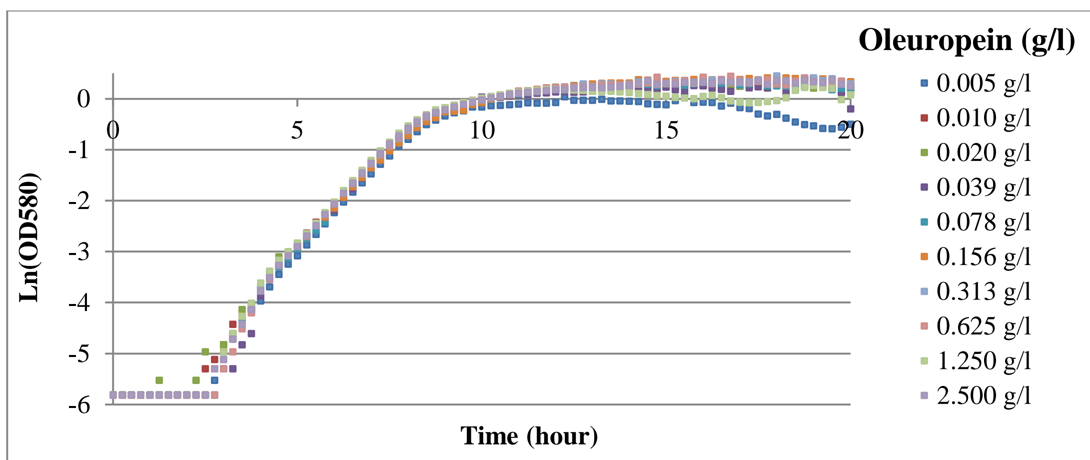

Figure 3.17 : Growth curve of *L. plantarum* T957 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

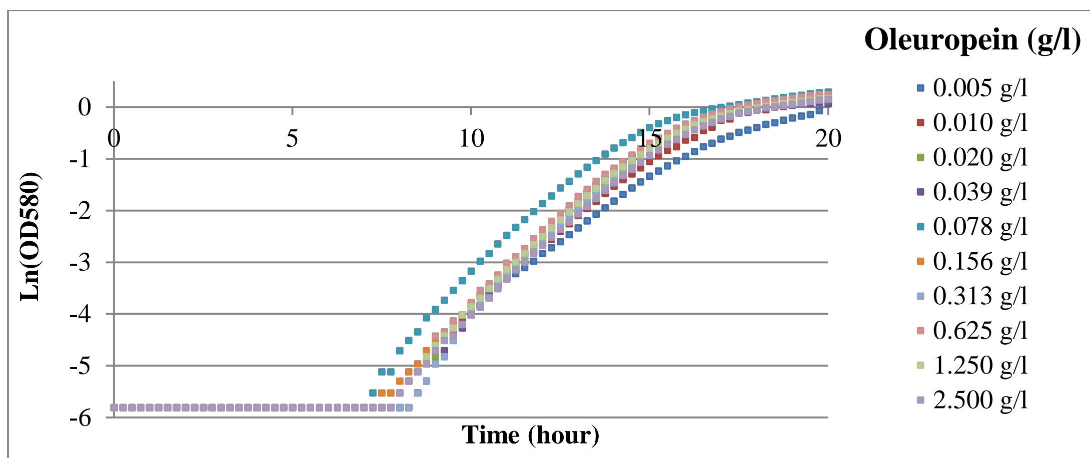

Figure 3.18 : Growth curve of *L. plantarum* T976 at different oleuropein concentrations (0.005 g/l- 2.500 g/l).

Although different *L. plantarum* strains had different lag times and different specific growth rates, oleuropein does not seem to have inhibited the growth of most strains, as they grew similarly at all dilutions. 2.5 g/l oleuropein seems to have increased the lag phase of *L. plantarum* T822, T896, T106, T109, T119 and T127 strains. But their specific growth rates do not seem to be affected by oleuropein addition.

L. plantarum T831, T874, T102, T123, T129 had short lag times, and *L. plantarum* T127, T109, T106, T976, T896, T813 had relatively long lag times. But all cultures reached the stationary phase of growth at 15 hours. T127 and T129 were selected according to the pre-screening results. T129 was selected due to its high oleuropein resistance and known resistance to bile salts and pH 2.5. T127 was selected despite its weaker oleuropein resistance, as it was resistant against bile salts and low pH (2.5) (**Table 2.1**).

3.1.2 Identification by the mini-API system

API tests were made for the selected two *L. plantarum* strains: T127 and T129. T127 and T129 gave an excellent identification result of 99.9% as *L. plantarum.*

The API results were in agreement with previous CO_2 production from glucose tests and microscopic observations of these strains. These strains were used for later studies, after this confirmation. The two selected strains were identified biochemically by API tests as the 16S rDNA region of *L. plantarum* cannot differentiate *L. plantarum* from its related genera, *L. casei and L. paraplantarum* (Torriani et al., 2001).

3.1.3 Effect of oleuropein, OE and NaCl on selected *L. plantarum* strains

In addition to the two selected TÜBİTAK strains, DSMZ 10492 was also included in this study for two main reasons. Firstly, DSMZ 10492 strain was isolated from Portuguese olive processing brine and shared a common origin with TÜBİTAK strains. Secondly, this strain was known for its high oleuropein resistance, and literature information on the survival of this strain in the presence of oleuropein, NaCl and other olive phenols was present (Rozes and Peres 1996). As oleuropein is not the only stress factor in olive fermentation, its combined effect with NaCl and other soluble olive substances was investigated. To get a good representation of soluble olive substances, an OE was prepared. The oleuropein content of this OE was calculated as 4.2 g/kg oleuropein. This value was in good agreement with the actual oleuropein concentrations used in the literature Ruiz-Barba et al. (1991).

3.1.4 Determination of the resistance of selected *L. plantarum* strains against oleuropein, OE and NaCl

3.1.4.1 Effect of oleuropein and NaCl

Growth curve of strains on 40% diluted MRS broth (control), 8% (w/v) NaCl-containing 40% diluted MRS broth, 5 g/l oleuropein-containing 40% diluted MRS broth, 8% (w/v) NaCl and 5 g/l oleuropein-containing 40% diluted MRS broth, 8% (w/v) NaCl and 2.5 g/l oleuropein-containing 30% diluted MRS broth are shown in **Figures 3.19-3.21**. All cultures had similar growth curves. They both grew well in control condition and with 5 g/l oleuropein. But there was almost no growth at 8% (w/v) NaCl, 5 g/l oleuropein + 8% (w/v) NaCl, and 2.5g/l oleuropein+ 8% (w/v) NaCl. It has been observed that the color of the medium changes in the presence of oleuropein and NaCl. This phenomenon caused false increases in OD_{580} value. This is most likely due to the oxidation of oleuropein (Therios, I., 2009).

Ruiz-Barba et.al (1991) described that both combination of 4% (w/v) NaCl and 0.4% (w/v) oleuropein and heat-treated oleuropein showed antimicrobial activity against *L. plantarum*. But alkali-treated oleuropein and/or 4% (w/v) NaCl did not have antimicrobial activities. They also proposed that oleuropein affected the peptidoglycan structure of *L.plantarum*.

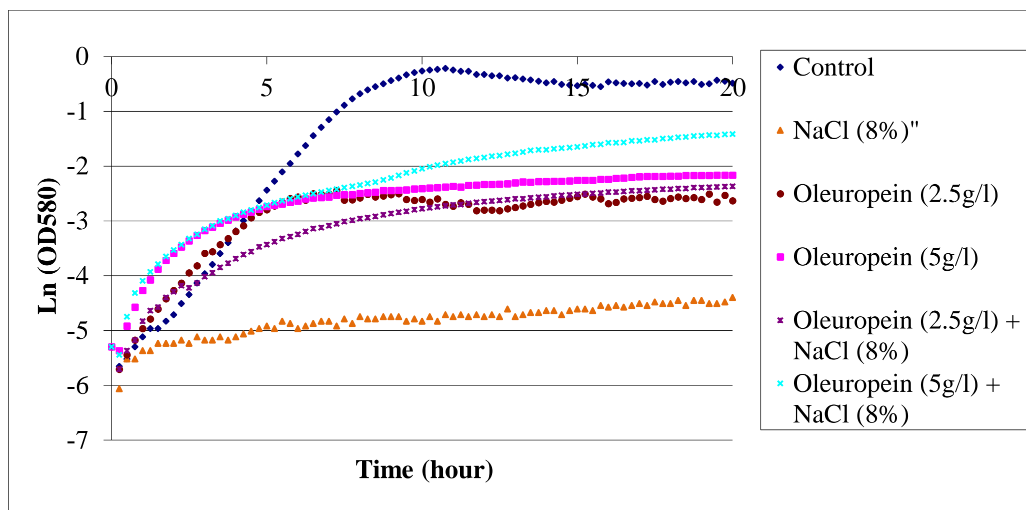

Figure 3.19 : Growth curves of *L. plantarum* DSMZ 10492 strain at different stress conditions.

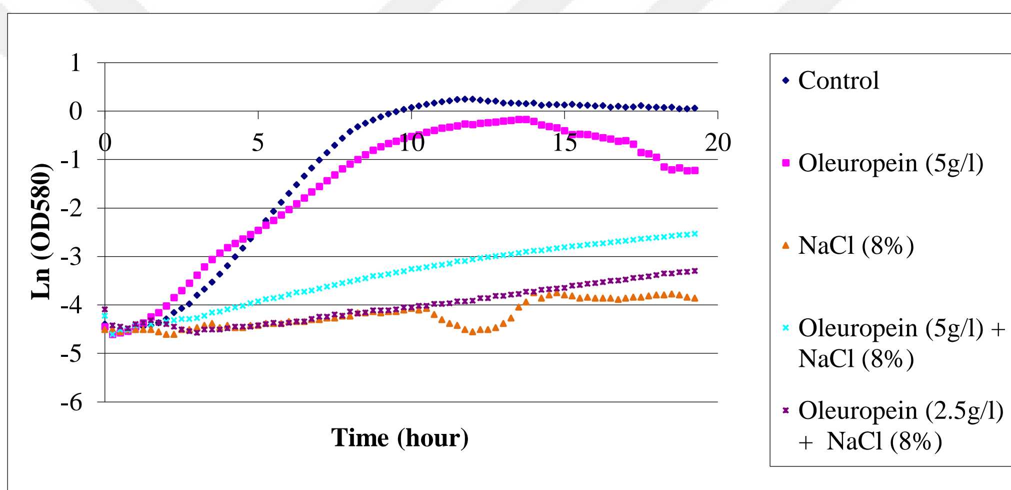

Figure 3.20 : Growth curves of *L. plantarum* T127 strain at different stress conditions.

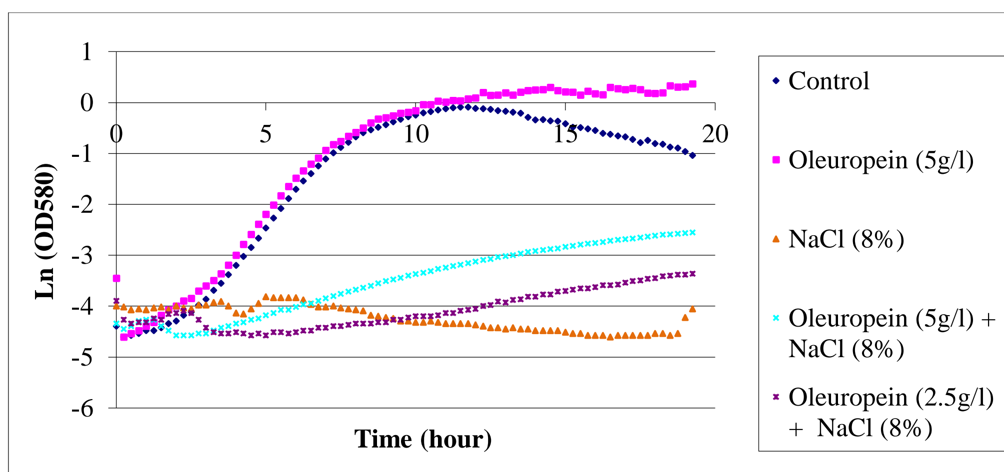

Figure 3.21 : Growth curves of *L. plantarum* T129 strain at different stress conditions.

Rozes and Peres (1996) found that oleuropein was not bactericidal against *L. plantarum* DSM 10492 strain. However, heat-treated oleuropein and NaCl decreased the viability of this strain, where glucose had a protective role against the bactericidal action of NaCl and oleuropein. They did not identify the specific cause of this phenomenon, but described a decrease in malic acid decarboxylation when oleuropein concentrations were increased. They supposed that oleuropein could inhibit malic acid transport by binding membrane proteins and thus its decarboxylation rate. They concluded that the increased osmotic pressure of the NaCl-added medium could be the primary cause of decreased viability of lactic acid bacteria. Therefore, they assumed that increased glucose concentrations could protect the cell against the increasing osmotic pressure of the medium. Furthermore, they described that *L. plantarum* DSMZ 10492 was esculine-positive and had β-glucosidase activity. Thus, it could cleave the glycosidic bond at oleuropein and use this for lactic acid production.

Pieterse, B. (2006) identified that the transcription of osmoprotector proteins did not increase in response to NaCl stress. He identified that the activation of DNA repair mechanisms and modification of the cell wall structure were the main cellular responses against increased NaCl stress.

3.1.4.2 Effect of OE and NaCl

Growth of DSM 10492, T127 and T129 strains on 40% diluted MRS broth (control), 8% (w/v) NaCl-containing 40% diluted MRS broth, 50% (w/w) OE- containing 40% diluted MRS broth, 25% (w/w) OE-containing 40% diluted MRS broth, 8% (w/v) NaCl and 50% (w/w) OE-containing 40% diluted MRS broth, 8% (w/v) NaCl and 25% (w/w) OE-containing 30% diluted MRS broth are shown in **Figures 3.22-3.24**. All strains have similar growth curves and 50% (w/w) OE decreased the growth of both strains. OE (25% w/w) alone had a decreased antimicrobial effect when compared to OE (50%). The combination of 8% (w/v) NaCl and OE inhibited growth of both strains.

The 50% OE (≈ 2.1 g/kg oleuropein) was more effective than 5 g/l oleuropein in inhibiting microbial growth. 25% (≈ 1.05 g/kg oleuropein) OE showed only slightly higher antimicrobial effect than 5g/l oleuropein.

It has previously been described that the color of the medium changes in the presence of oleuropein and NaCl. This phenomenon is also observed in the presence of OE. This phenomenon caused for false increases in OD values (Therios, I., 2009).

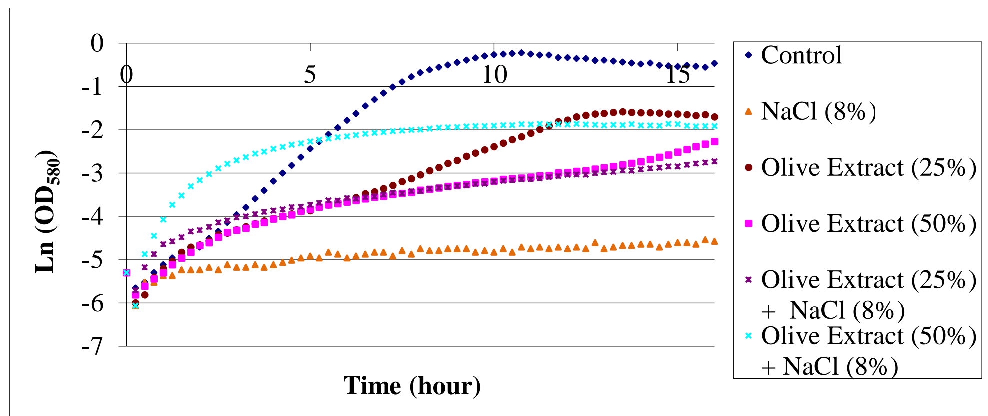

Figure 3.22 : Growth curves of *L. plantarum* DSMZ 10492 strain at different stress conditions.

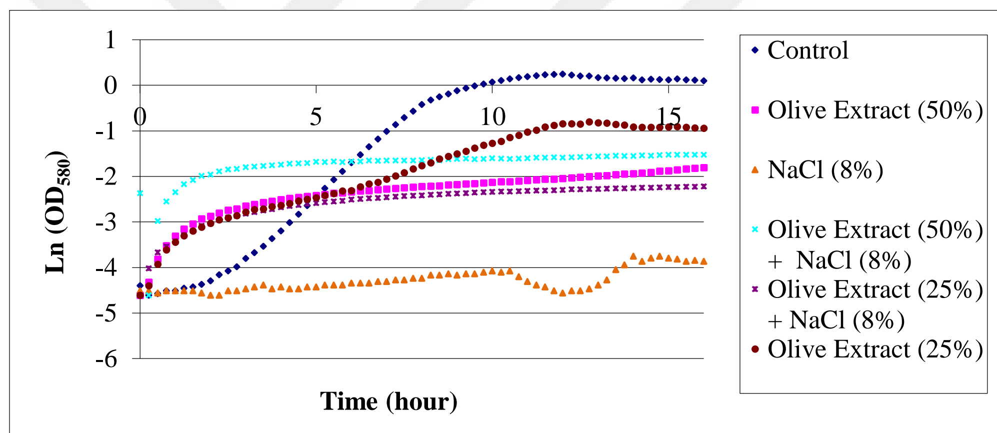

Figure 3.23 : Growth curves of *L. plantarum* T127 strain at different stress conditions.

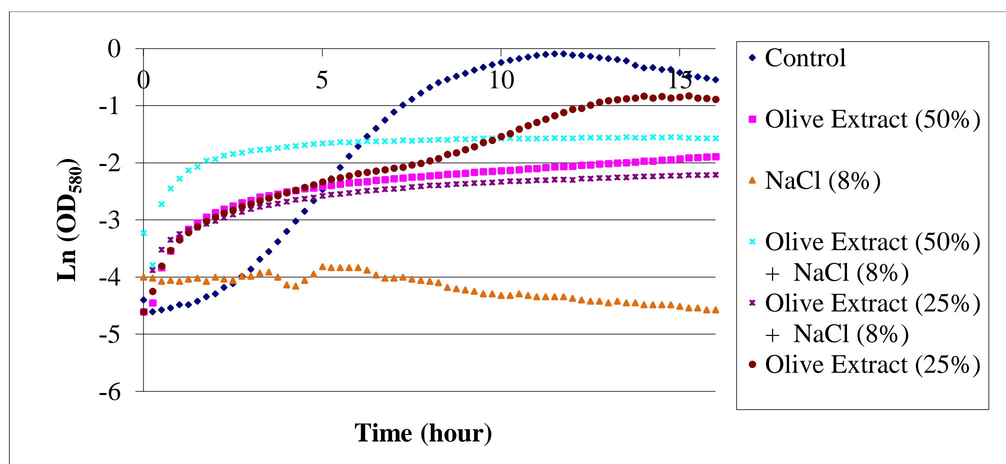

Figure 3.24 : Growth curves of *L. plantarum* T129 strain at different stress conditions.

It also has to be considered that oleuropein is not the only phenolic fraction of olives. Thus, other phenolic compounds can also have antimicrobial activity. Rozes and Peres (1998) showed that tannic, ferulic and caffeic acid also affect the growth of *L. plantarum* DSMZ 10492 strain.

The 50% OE (>2.1 g/kg oleuropein) was more effective than 5 g/l oleuropein by inhibiting microbial growth. 25% (>1.05 g/kg oleuropein) OE showed only a slightly higher antimicrobial effect than 5 g/l oleuropein. These results are in agreement with the literature. They are also in correlation with the results obtained from the DSMZ 10492 type strain.

The OE used to obtain this water extract contained only 4.2g/kg. This value is lower than 5g/l oleuropein. The higher antimicrobial effect of this extract can be due to the presence of other olive phenols or enzymatic hydrolysis products, as described by Medina et.al. (2009). They described the enzymatic degradation of oleuropein after cell disruption. This could also be the case in the present study, as the cells were disrupted at high shear forces generated in the blender and the olives were frozen and thawed prior to disruption procedure. These conditions might have allowed enzymatic degradation of oleuropein.

Medina et al. (2009) proposed that dialdehydic form of decarboxymethyl elenolic acid or its bound form to hydroxytyrosol were the degradation products of oleuropein and they were responsible for higher antimicrobial activity.

3.1.5 Effect of NaCl and oleuropein on T129 and DSM 10492

Experiments showed that NaCl is inhibitory to both strains. But unexpected increases in absorbance values were observed, while following the combined effect of NaCl and oleuropein. T129 and DSMZ 10492 strains were grown in 50 ml culture tubes to elucidate this effect.

The growth of T129 and DSMZ 10492 were followed by OD measurements at 580 nm. Change in OD_{580} of T129 and DSMZ 10492 cultures on 40% diluted MRS broth (control), 8%(w/v) NaCl-containing 40% diluted MRS broth, 5 g/l oleuropein-containing 40% diluted MRS broth, and 8%(w/v) NaCl and 5 g/l oleuropein-containing 40% diluted MRS broth are shown in **Figures 3.25-3.26**.

Cell dry weight measurements were done to confirm the growth of cultures. Samples for CDW measurements were taken at 0, 8 and 24 h in triplicate. CDW measurements are given in **Figures 3.27-3.28**. CDW measurements could not be obtained for NaCl 8% (w/v) and 5 g/l oleuropein + NaCl 8% (w/v) samples as these were below 0.075 g/l. The sensitivity of the balance was measured as ± 0.0003g with empty tubes. This error gave rise to a sensitivity of 0.075g/l CDW.

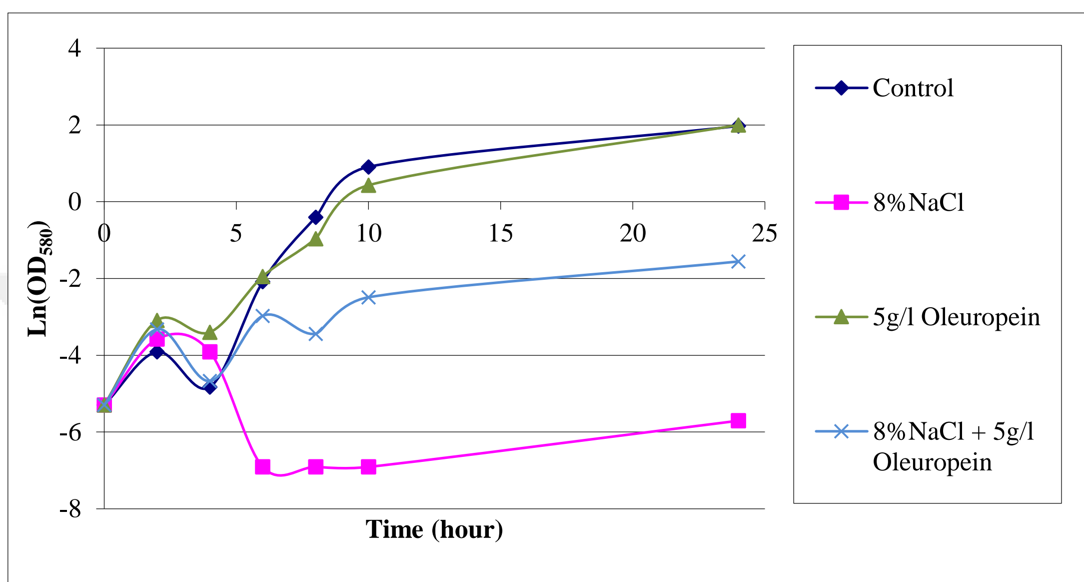

Figure 3.25 : Growth curves of *L. plantarum* DSMZ 10492 strain at different stress conditions.

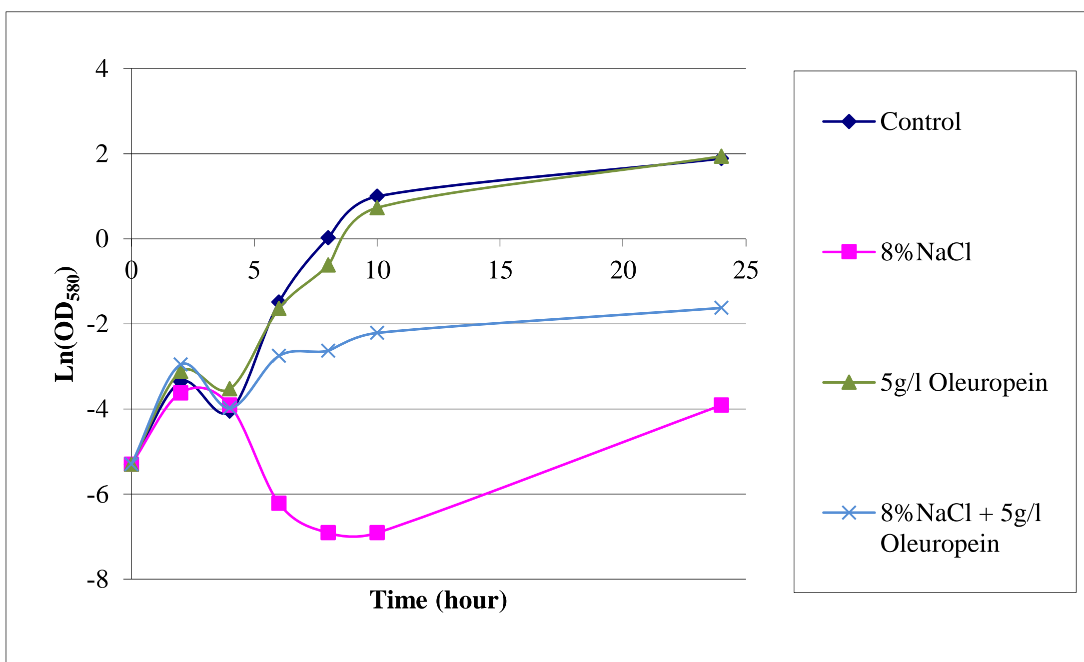

Figure 3.26 : Growth curves of *L. plantarum* T129 strain at different stress conditions.

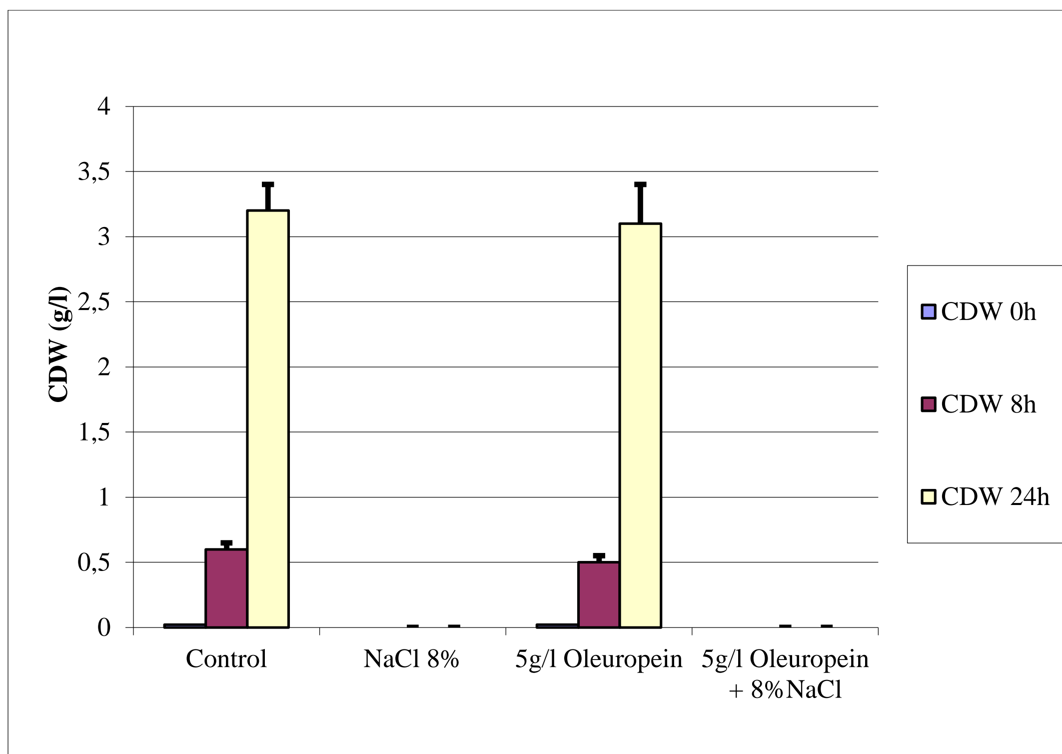

Figure 3.27 : Cell dry weights of *L. plantarum* DSMZ 10492 strain at different stress conditions.

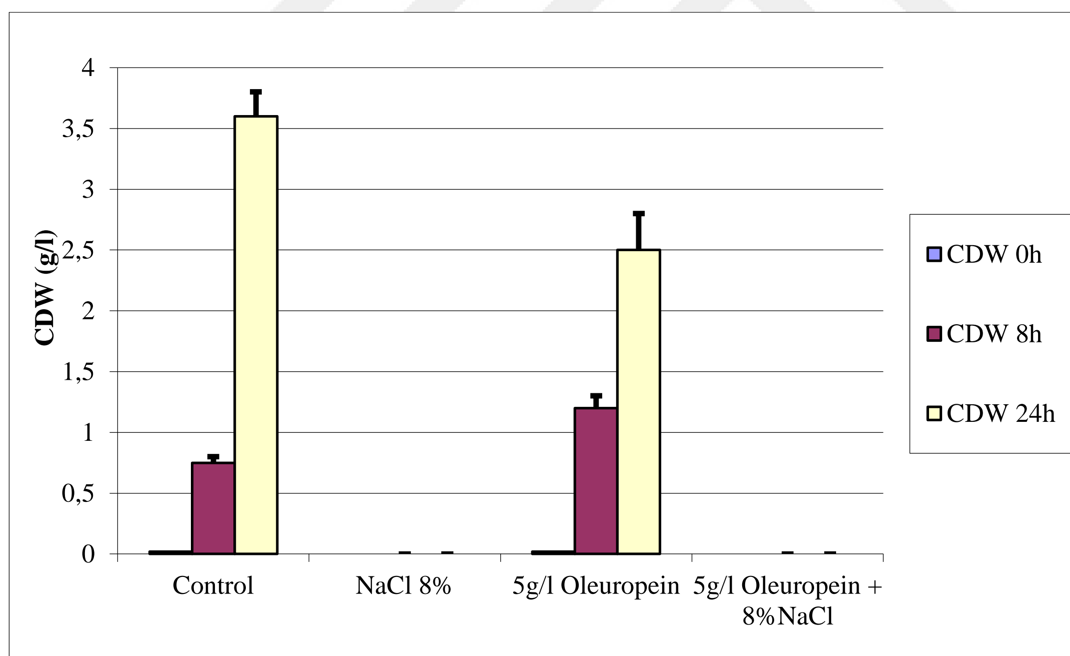

Figure 3.28 : Cell dry weights of *L. plantarum* T129 strain at different stress conditions.

It was not possible to verify the absorbance results with CDW measurements. Plate count measurements were done at the beginning and at the end of 24th hour. The results are given in **Figures 3.29-3.30**.

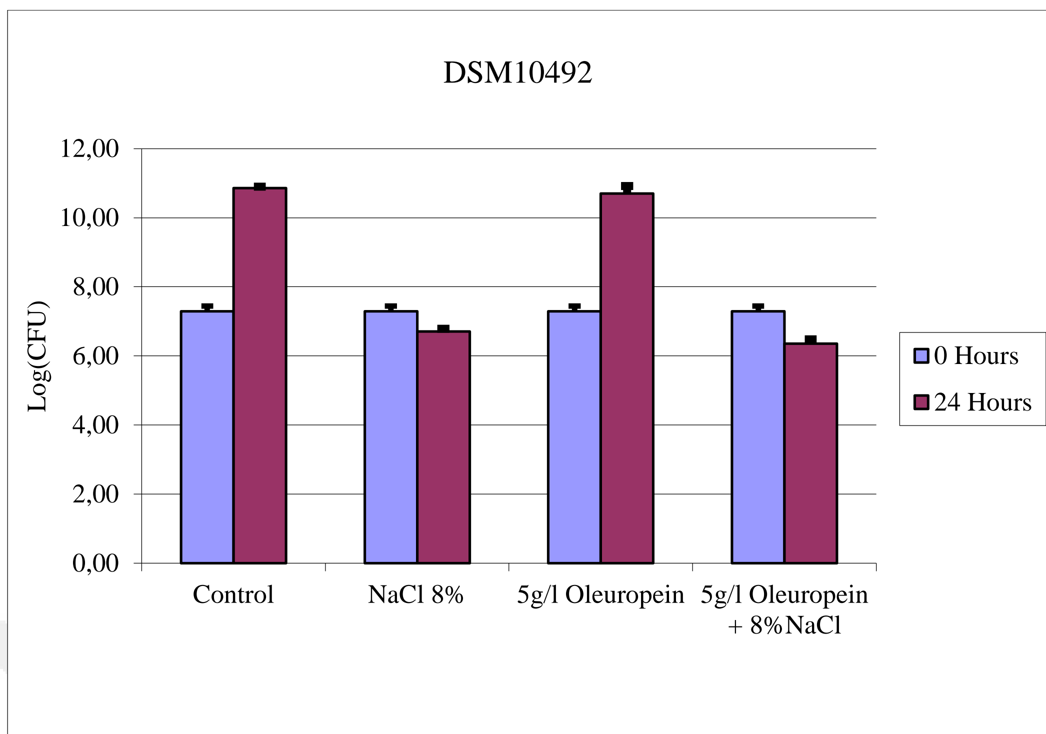

Figure 3.29 : Effect of NaCl and oleuropein on growth of *L. plantarum* DSMZ 10492.

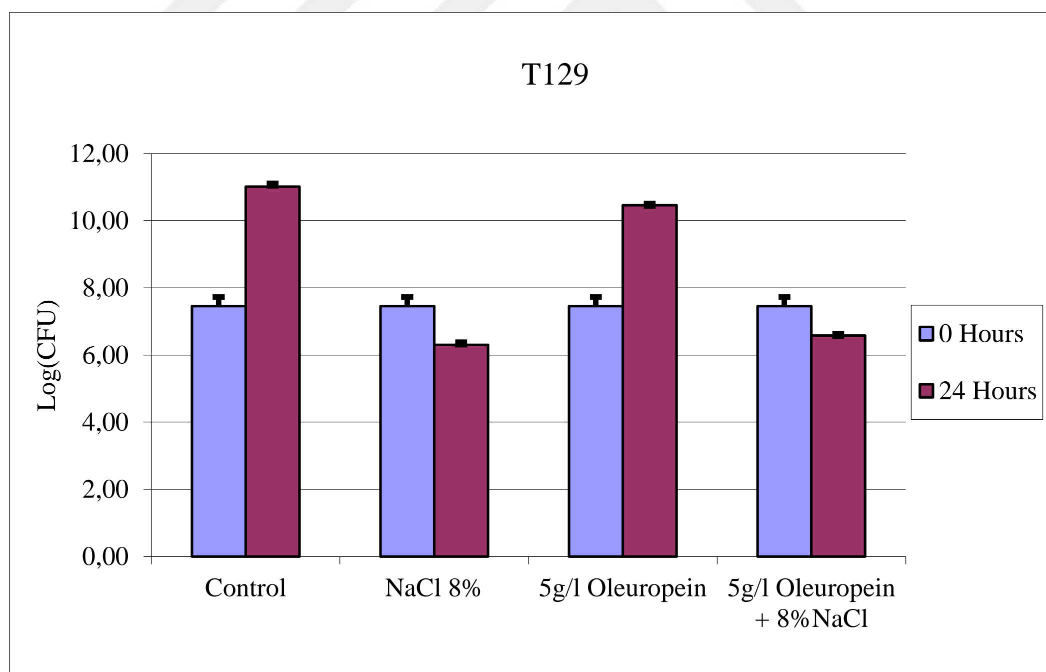

Figure 3.30 : Effect of NaCl and oleuropein on growth of *L. plantarum* T129.

Strains showed a good growth in the presence of oleuropein. Thus, *L. plantarum* strains isolated from olives have a high potential to be resistant against oleuropein. Both DSMZ 10492 and T129 were isolated from olives. This result was also shown by plate counts.

Plate counts revealed that 8% (w/v) NaCl and 8% (w/v) NaCl and 5 g/l oleuropein inhibited the growth of both strains. However, these conditions only caused a decrease of 1 log CFU in 24 h. It was expected that CDW measurements could not be obtained for NaCl 8% (w/v) and 5 g/l oleuropein + NaCl 8% (w/v), as no growth was observed. Although T129 and DSMZ 10492 strains reached similar absorbance and T129 reached higher plate counts, the cell dry weight of DSMZ 10492 was higher. This could be due to the slightly higher cell dry weight of DSMZ 10492 strain. The increase in absorbance of both DSMZ 10492 and T129 strains in the presence of 5 g/l oleuropein and 8% (w/v) NaCl was not due to an increase in cell counts, but due to the previously described change in color. This color change is shown in **Figure 3.31.**

Figure 3.31 : Color change during growth at different stress conditions. From left to right: Control, 5 g/l oleuropein +8% (w/v) NaCl, 5g/l oleuropein, and 8% (w/v) NaCl, respectively.

The color change from yellow to red brown can clearly be seen (**Figure 3.31**). This color change is proportional to oleuropein concentration and seems to be more in the DSMZ strain than in the T129 strain. The increased absorbance during the first 4 hours was most probably due to the colour changing effect that was explained for oleuropein. It was previously described that this color change gives an artificial increase in OD values (Therios, 2009).

The strains showed good growth in the presence of oleuropein. Thus, *L. plantarum* strains isolated from olives have a high potential to be resistant against oleuropein. Both DSMZ 10492 and T129 are isolated from olives. This result was also stated by Plate counts. Experiments showed that both NaCl and NaCl and oleuropein inhibited the growth of these strains. But they only decreased the cell counts by one log. These results are in agreement with previous studies (Rozes and Peres, 1996).

The aim of this thesis was to develop polyphenol-resistant lactic acid bacteria by evolutionary engineering. The primary hypothesis was that lactic acid bacterial starter cultures for olive fermentation were not robust enough due to the presence of olive polyphenols, and mainly oleuropein, in fermentation brines. This hypothesis was revised according to the experimental results of this study and the scientific literature. The new hypothesis was that NaCl-resistant mutant strains would also resist combined polyphenol and NaCl stress. Thus, in the present study, NaCl-resistant lactic acid bacteria were obtained by evolutionary engineering.

3.2 Development of NaCl-resistant *L. plantarum* Strains by Evolutionary Engineering

3.2.1 Ethyl methane sulfonate (EMS) mutagenesis

Guerola and Cerda-Olmedo (1975) described that EMS mutagenesis results in approximately random mutations in the bacterial genome. They also concluded that mutant generations with higher survival rate can be used for the selection of mutants for “biochemical and behavioral” studies with higher confidence than more aggressive mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine. The survival of the cultures after mutagenesis was determined by both MPN counts and OD_{580} measurements. The survival rate of the mutated cultures is important for the success in the selection of suitable mutant populations.

Parkhomchuk et al. (2009) investigated the randomness of EMS-induced mutagenesis by high throughput sequencing. They concluded that EMS mutagenesis is not random due to increased heterogeneity caused by lethal mutations, DNA repair and recombination. These findings support the importance of the survival rate of the mutated strains upon EMS mutation.

3.2.1.1 MPN counts

Growth in multiwell plates was followed visually twice a day for MPN counts. The results were recorded after 72 h of incubation. MPN counts after treatment with different EMS concentrations are given in **Table 3.1**.

EMS affected both L. plantarum strains, as expected. T129 strain was slightly more resistant to EMS mutagenesis than DSMZ 10492 strain when MPN and values were considered. The targeted 10% survival rate was in the survival range of the treatments.

Table 3.1 : MPN counts after treatment with different EMS concentrations.

EMS Concentration (M)	T129 (Cells/ml)	Survival Rate T129 (%)	DSMZ 10492 (Cells/ml)	Survival Rate DSMZ 10492 (%)
Control (0)	$2.4x10^8$	100	$5.4x10^8$	100
0.37	$9.2x10^7$	38.33	$9.2x10^7$	17.04
0.72	$2.4x10^5$	0.10	$2.4x10^5$	0.04
0.88	$3.5x10^4$	0.01	$2.4x10^5$	0.04
1.19	$1.4x10^3$	0.00	$2.4x10^3$	0.00

3.2.1.2 OD_{580} measurements

Optical densities of cultures were measured after 14 h of incubation. The cultures were appropriately diluted and the measurements were done at the confidence interval of 0.1 and 0.6 (OD_{580}). Recorded OD_{580} measurements are given in **Table 3.2.**

Survival rates determined by OD_{580} measurements were higher than these determined by MPN counts. While MPN counts indicated the surviving bacterial counts just after EMS mutagenesis, OD_{580} measurements were affected by the initial cell count and growth rate of the survivors. Populations treated with 0.37 M and 0.72 M EMS were chosen as the most promising initial population candidates for selection by evolutionary engineering, as the targeted 10% survival was in between the survival rates obtained from both conditions.

Table 3.2 : Recorded OD_{580} values of EMS-treated and control cultures at the 14th h of incubation at 35° C.

EMS Concentration (M)	T129 (OD_{580})	Survival Rate T129 (%)	DSMZ 10492 (OD_{580})	Survival Rate DSMZ 10492(%)	Time (h)
Control (0)	9.540	100	8.340	100	14
0.37	7.050	73.7	6.260	75.1	14
0.72	0.210	2.2	0.154	1.9	14
0.88	0.106	1.1	0.092	1.1	14
1.19	0.070	0.7	0.078	0.9	14

3.2.2 NaCl resistance tests

The NaCl resistances of selected strains were studied to determine the NaCl-resistant strains.

3.2.2.1 OD_{580} measurements

Optical densities of cultures were measured at 580 nm wavelength every 15 minutes by automated turbidometry. Recorded OD_{580} measurements are shown in **Figures 3.32-3.37.**

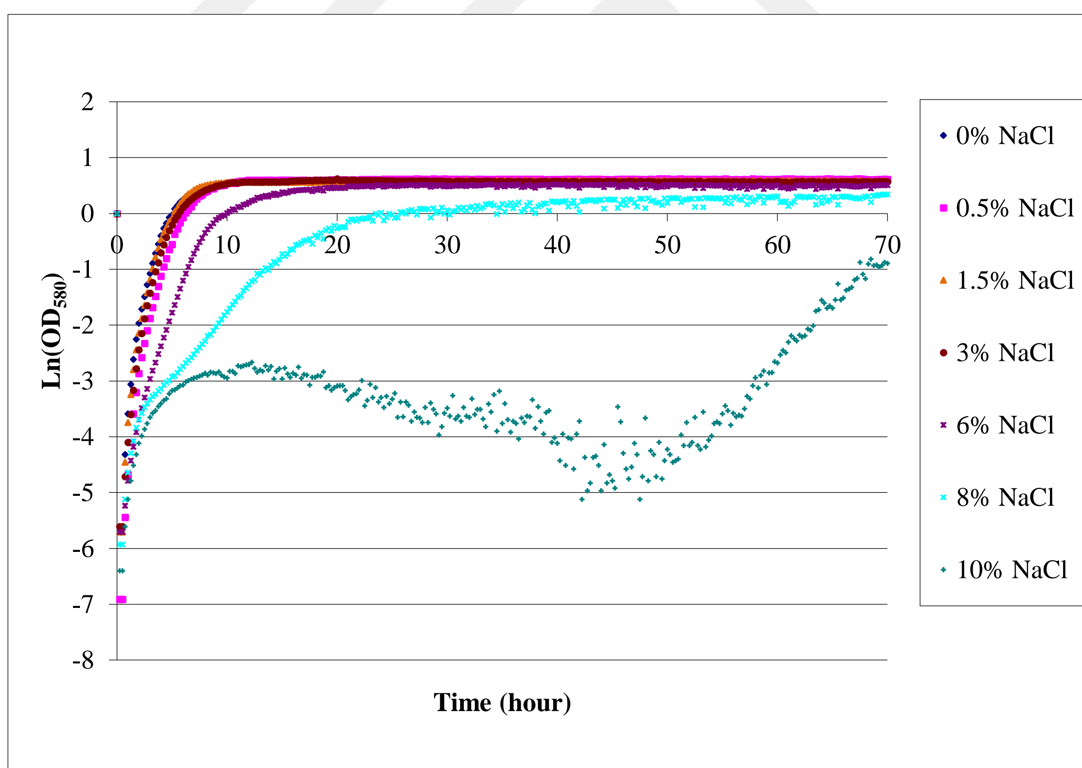

Figure 3.32 : Growth of *L. plantarum* DSMZ 10492 strain at different NaCl (%w/v) concentrations.

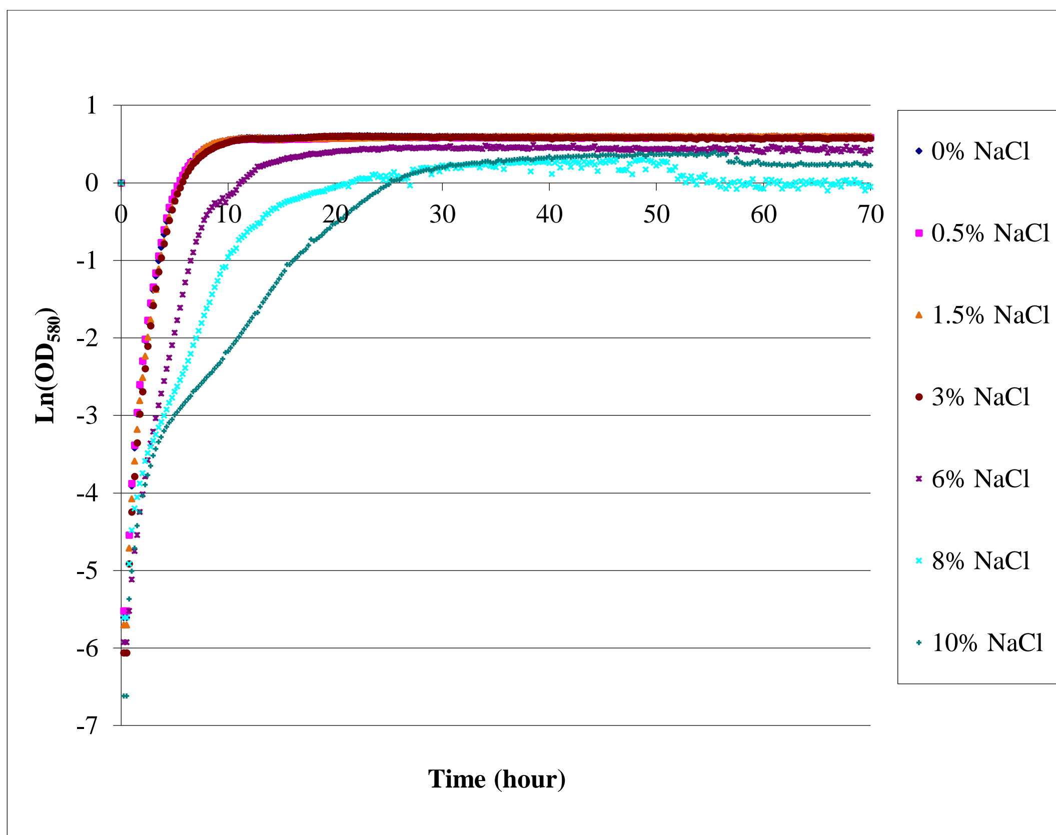

Figure 3.33 : Growth of *L. plantarum* DSMZ 10492 population (0.37 M EMS-treated) at different NaCl (%w/v) concentrations.

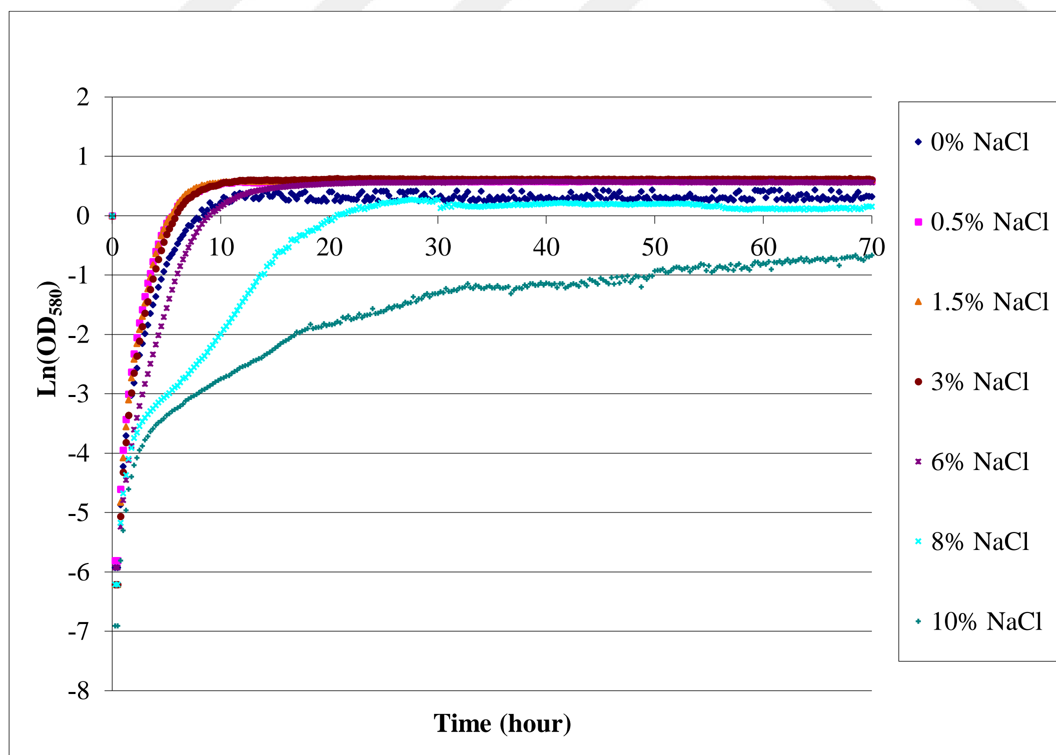

Figure 3.34 : Growth of *L. plantarum* DSMZ 10492 population (0.72 M EMS-treated) at different NaCl (%w/v) concentrations.

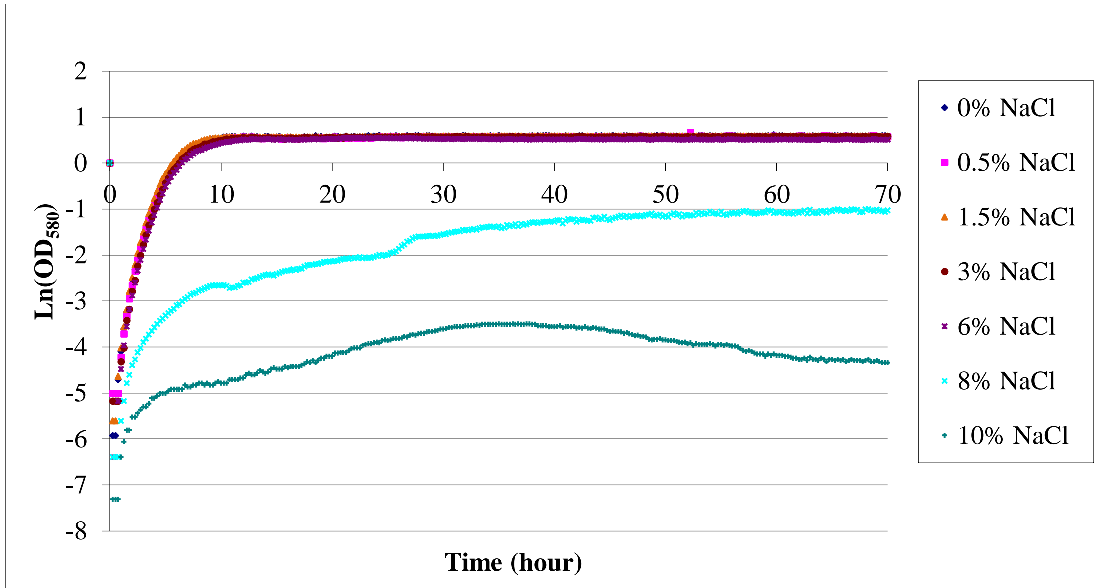

Figure 3.35 : Growth of *L. plantarum* T129 strain at different NaCl (%w/v) concentrations.

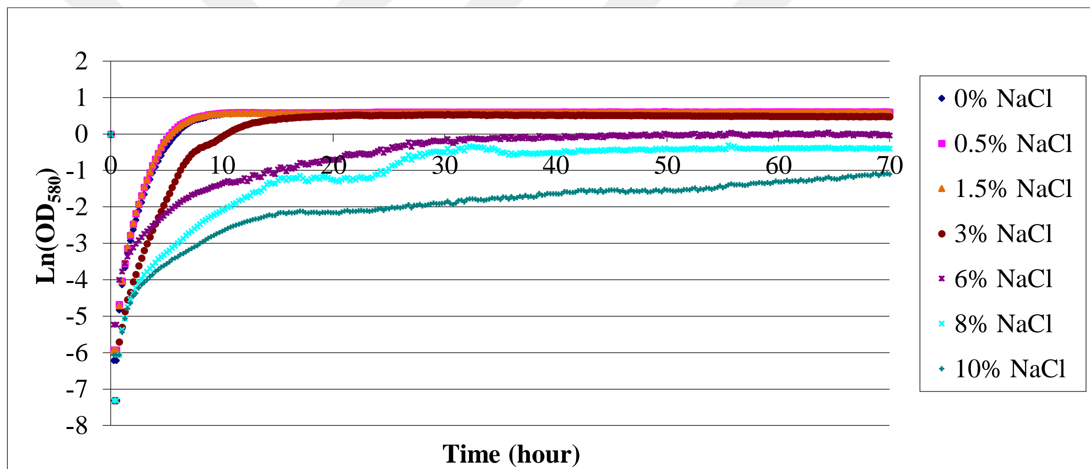

Figure 3.36 : Growth of *L. plantarum* T129 population (0.37 M EMS-treated) at different NaCl (%w/v) concentrations.

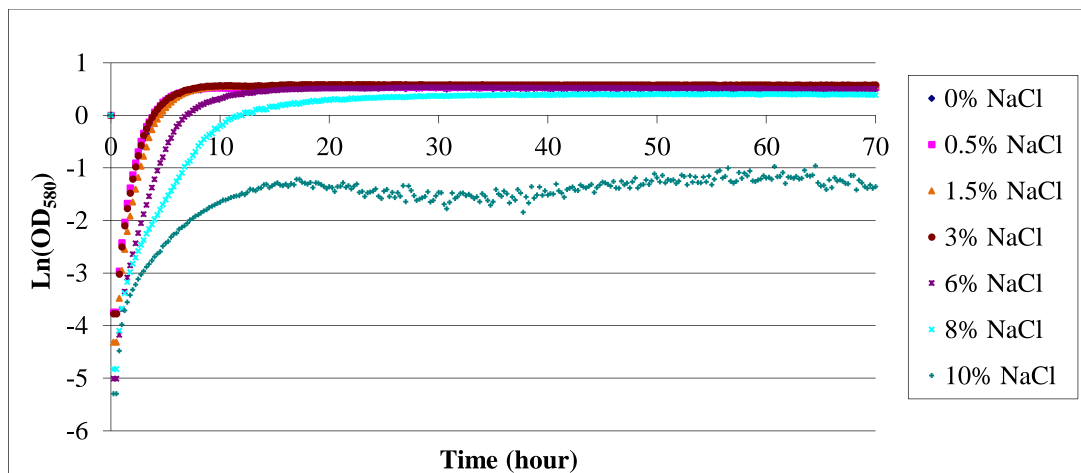

Figure 3.37 : Growth of *L. plantarum* T129 population (0.72 M EMS-treated) at different NaCl (%w/v) concentrations.

Strain growth did not differ at NaCl concentrations below 3% (w/v). The highest NaCl resistance was observed in *L. plantarum* strain DSMZ 10492 that was treated with 0.37 M EMS for 30 min, and *L. plantarum* strain T129, treated with 0.72 M EMS for 30 min.

3.2.3 Selection of mutant populations

Survival data of the mutant populations during selection are given in **Tables 3.3-3.4.** The survival of mutant populations was inversely proportional to the NaCl concentrations as expected. The mutant populations faced a major stress at 8.5%, 9% and 9.5% (w/v) NaCl. Repeated application of similar stress conditions turned out to be a feasible strategy. At 9 % (w/v) NaCl, about 30 % survival was considered to be sufficient to proceed to 9.5% (w/v) NaCl, as there was a clear increase in the survival rate against previously obtained values at the same stress condition.

Selection procedures were finalized at 10% (w/v) NaCl stress level (32nd population). This stress level allows a clear differentiation from the control strain. Additionally, 10% NaCl (w/v) is usually the highest NaCl concentration applied in modern olive-processing plants (Taş et al., 2008).

The NaCl concentration at which the selection experiments started and the increase in NaCl concentration between each step has shaped the evolution of mutant populations. Microbial research showed that adaptive evolution is an effective way to accumulate highly beneficiary mutations among the mutant populations (Barrick and Lenski, 2013). The genetic diversity of these populations is generally inversely proportional to the selection speed and selective power. Previous experiments showed that wild type individuals were able to grow at NaCl concentrations up to 6% (w/v) NaCl without a major inhibition in growth (**Figures 3.32** and **3.35**). The populations were not stressed to adapt specifically to NaCl until this concentration. This low selective stress caused an increase in genetic diversity. The population optimized its genetic make up to grow faster and outcompete the slower growing mutants at this stage. This condition changed at the point where generations faced 6.5% (w/v) NaCl. (Barrick and Lenski, 2013)

Table 3.3 : Survival data for the selection experiment with the T129 strain.

Population Number	NaCl (%)(w/v)	Initial $OD_{(580)}$	Control $OD_{(580)}$	Final $OD_{(580)}$	Survival Rate
1	0	N.a.(frozen stock)	2.203	2.203	100%
2	0.5	0.050	4.275	4.508	105%
3	1.0	0.050	4.831	4.712	98%
4	1.5	0.050	5.224	5.097	98%
5	2.0	0.050	4.479	4.793	107%
6	2.5	0.050	5.114	4.799	94%
7	3.0	0.050	4.897	4.578	93%
8	3.5	0.050	6.324	5.682	90%
9	4.0	0.050	7.259	7.084	98%
10	4.5	0.050	6.964	5.508	79%
11	5.0	0.050	5.090	4.313	85%
12	5.5	0.050	4.701	3.181	68%
13	6.0	0.050	6.161	4.457	72%
14	6.5	0.050	4.722	2.504	53%
15	6.5	0.050	5.605	2.627	47%
16	6.5	0.050	6.252	3.688	59%
17	7.0	0.050	4.908	2.329	47%
18	7.5	0.050	5.337	1.671	31%
19	7.5	0.050	5.776	2.224	39%
20	7.5	0.050	5.346	1.510	28%
21	7.5	0.050	4.513	1.210	27%
22*	7.5	0.050	6.236	2.476	40%
23	8.0	0.050	5.537	2.716	49%
24	8.5	0.050	4.892	2.199	45%
25	8.5	0.050	4.366	2.591	59%
26	9.0	0.050	6.574	1.7008	26%
27	9.0	0.050	7.306	1.91	26%
28	9.0	0.050	5.666	1.9455	34%
29	9.5	0.050	5.1525	1.5915	31%
30	9.5	0.050	5.117	1.401	27%
31	9.5	0.050	3.645	1.1856	33%
32	10.0	0.050	5.127	2.128	42%

*Starting from the population 22, cultures were incubated for 48h, instead of 24h.

Table 3.4 : Survival data for the selection experiment with the DSMZ 10492 strain.

Generation	NaCl (%)(w/v)	initial $OD_{(580)}$	Control $OD_{(580)}$	Final $OD_{(580)}$	Survival ratio
1	0	N.a.(frozen stock)	4.656	4.656	100%
2	0.5	0.050	3.947	3.881	98%
3	1.0	0.050	5.181	5.198	100%
4	1.5	0.050	5.378	5.101	95%
5	2.0	0.050	4.716	4.164	88%
6	2.5	0.050	5.112	4.419	86%
7	3.0	0.050	5.156	4.902	95%
8	3.5	0.050	5.761	4.919	85%
9	4.0	0.050	6.947	6.566	95%
10	4.5	0.050	7.582	6.268	83%
11	5.0	0.050	5.756	1.508	26%
12	5.5	0.050	5.259	3.843	73%
13	6.0	0.050	7.082	5.004	71%
14	6.5	0.050	5.371	2.340	44%
15	6.5	0.050	5.788	2.294	40%
16	6.5	0.050	6.503	4.466	69%
17	7.0	0.050	5.114	2.506	49%
18	7.5	0.050	5.585	1.610	29%
19	7.5	0.050	6.136	2.049	33%
20	7.5	0.050	5.476	1.409	26%
21	7.5	0.050	4.941	0.964	20%
22*	7.5	0.050	6.382	2.418	38%
23	8.0	0.050	5.079	2.459	48%
24	8.5	0.050	5.330	1.231	23%
25	8.5	0.050	4.492	2.329	52%
26	9.0	0.050	4.862	1.011	21%
27	9.0	0.050	5.681	0.882	16%
28	9.0	0.050	5.499	1.736	32%
29	9.5	0.050	3.266	0.622	19%
30	9.5	0.050	4.871	0.864	18%
31	9.5	0.050	5.353	2.020	38%
32	10.0	0.050	4.281	1.131	26%

*Starting from the population 22, cultures were incubated for 48h, instead of 24h.

A growth delay at 6.5%(w/v) NaCl and a sharp decrease in survival rate was significant during selection. This stress level was therefore repeated for the next two populations (population 15 and 16). 7.5% (w/v) NaCl was the next bottleneck for the mutant populations. The stress level stronger than at 6.5% (w/v) NaCl. At 7.5% (w/v) NaCl stress level, it was realized that 24 hours did not seem to be long enough for the growth of the mutant populations, because of the increased stress level. Thus, the incubation time was increased from 24 h to 48 h. Increasing the incubation time was useful and it increased survival rates. The mutant populations were further forced to adapt to increased NaCl concentrations. Finally, mutant populations that grew at 10% (w/v) NaCl-containing MRS were obtained.

Although the internationally accepted salt concentration in olive processing brine is 8% (w/v) NaCl, NaCl concentrations up to 12-14 (w/v) % are utilized during the traditional table olives debittering practices in Turkey. The utilization of 12-14% (w/v) NaCl is undesired and results in highly salted table olives (Özay et al., 1994). Thus, it would be desirable to obtain strains that grow well between 8 and 12% (w/v) NaCl. Taş et al. (2008) stated this information in the literature and indicated that 10% (w/v) NaCl-containing brine is standard for Turkish olive processing, and in general 10% (w/v) NaCl is not exceeded (Taş et al., 2008).

3.2.4 Screening NaCl and oleuropein resistance of individual colonies

Twelve colonies were randomly chosen from the last populations of each selection, using appropriate dilution plates. It was expected that a strain with high NaCl resistance would also survive in the presence of high NaCl and oleuropein concentrations. The strains were therefore subjected to 10% (w/v) NaCl and 10% (w/v) NaCl + 4 g/l oleuropein stress during this experiment. The bacteria were counted at 0, 24, 48 and 72 h by pour plate method, in triplicate. The cell counts obtained at different conditions at 0, 24th , 48th and 72nd hour are given in **Tables 3.5- 3.12.**

Table 3.5 : Cell counts (cfu/ml) of T129 and its mutants at the beginning on incubation (0 h).

	Control medium (cfu/ml)		NaCl (10% (w/v)) (cfu/ml)		Oleuropein (4 g/l) + NaCl (10% w/v) (cfu/ml)	
T129	1.70×10^7	$\pm 2.71 \times 10^6$	1.70×10^7	$\pm 2.71 \times 10^6$	1.70×10^7	$\pm 2.71 \times 10^6$
T 1	1.72×10^7	$\pm 3.84 \times 10^6$	1.72×10^7	$\pm 3.84 \times 10^6$	1.72×10^7	$\pm 3.84 \times 10^6$
T 2	6.03×10^7	$\pm 8.02 \times 10^6$	6.03×10^7	$\pm 8.02 \times 10^6$	6.03×10^7	$\pm 8.02 \times 10^6$
T 3	1.03×10^7	$\pm 6.56 \times 10^5$	1.03×10^7	$\pm 6.56 \times 10^5$	1.03×10^7	$\pm 6.56 \times 10^5$
T 4	3.03×10^7	$\pm 4.23 \times 10^6$	3.03×10^7	$\pm 4.23 \times 10^6$	3.03×10^7	$\pm 4.23 \times 10^6$
T 5	4.40×10^7	$\pm 3.00 \times 10^6$	4.40×10^7	$\pm 3.00 \times 10^6$	4.40×10^7	$\pm 3.00 \times 10^6$
T 6	6.13×10^7	$\pm 2.15 \times 10^7$	6.13×10^7	$\pm 2.15 \times 10^7$	6.13×10^7	$\pm 2.15 \times 10^7$
T 7	4.87×10^7	$\pm 6.03 \times 10^6$	4.87×10^7	$\pm 6.03 \times 10^6$	4.87×10^7	$\pm 6.03 \times 10^6$
T 8	4.67×10^7	$\pm 1.18 \times 10^7$	4.67×10^7	$\pm 1.18 \times 10^7$	4.67×10^7	$\pm 1.18 \times 10^7$
T 9	4.07×10^7	$\pm 5.51 \times 10^6$	4.07×10^7	$\pm 5.51 \times 10^6$	4.07×10^7	$\pm 5.51 \times 10^6$
T 10	5.37×10^7	$\pm 1.17 \times 10^7$	5.37×10^7	$\pm 1.17 \times 10^7$	5.37×10^7	$\pm 1.17 \times 10^7$
T 11	4.47×10^7	$\pm 8.39 \times 10^6$	4.47×10^7	$\pm 8.39 \times 10^6$	4.47×10^7	$\pm 8.39 \times 10^6$
T 12	4.53×10^7	$\pm 1.53 \times 10^6$	4.53×10^7	$\pm 1.53 \times 10^6$	4.53×10^7	$\pm 1.53 \times 10^6$

*T1-T12 are the randomly picked individual mutants of the last population of selection.

Table 3.6 : Cell counts (cfu/ml) of T129 and its mutants at the 24th hour of incubation.

	Control medium (cfu/ml)		NaCl (10% (w/v)) (cfu/ml)		Oleuropein (4 g/l) + NaCl (10% w/v) (cfu/ml)	
T129	4.57×10^9	$\pm 2.52 \times 10^8$	9.87×10^6	$\pm 3.86 \times 10^6$	2.06×10^6	$\pm 4.05 \times 10^5$
T 1	1.03×10^{10}	$\pm 8.62 \times 10^8$	2.42×10^6	$\pm 2.53 \times 10^5$	2.67×10^6	$\pm 4.60 \times 10^5$
T 2	4.90×10^9	$\pm 7.00 \times 10^8$	3.43×10^9	$\pm 1.15 \times 10^8$	3.21×10^9	$\pm 4.19 \times 10^8$
T 3	1.01×10^{10}	$\pm 6.43 \times 10^8$	5.70×10^9	$\pm 6.24 \times 10^8$	4.37×10^9	$\pm 9.02 \times 10^8$
T 4	2.88×10^{10}	$\pm 4.73 \times 10^8$	2.97×10^8	$\pm 9.34 \times 10^7$	1.39×10^9	$\pm 1.26 \times 10^9$
T 5	2.68×10^{10}	$\pm 1.76 \times 10^9$	4.10×10^8	$\pm 6.08 \times 10^7$	1.67×10^9	$\pm 2.85 \times 10^8$
T 6	2.64×10^9	$\pm 4.04 \times 10^8$	2.01×10^9	$\pm 8.62 \times 10^7$	2.46×10^9	$\pm 1.16 \times 10^8$
T 7	7.90×10^9	$\pm 1.59 \times 10^9$	1.69×10^9	$\pm 2.78 \times 10^8$	2.18×10^9	$\pm 1.15 \times 10^8$
T 8	5.37×10^9	$\pm 2.08 \times 10^8$	4.20×10^9	$\pm 6.56 \times 10^8$	2.16×10^9	$\pm 5.29 \times 10^7$
T 9	6.43×10^9	$\pm 4.16 \times 10^8$	6.07×10^9	$\pm 6.51 \times 10^8$	2.10×10^9	$\pm 1.67 \times 10^8$
T 10	8.63×10^9	$\pm 7.57 \times 10^8$	2.69×10^9	$\pm 2.19 \times 10^8$	3.77×10^9	$\pm 6.66 \times 10^8$
T 11	5.87×10^9	$\pm 1.34 \times 10^9$	3.83×10^9	$\pm 2.08 \times 10^8$	3.40×10^9	$\pm 1.00 \times 10^8$
T 12	6.97×10^9	$\pm 1.10 \times 10^9$	7.97×10^6	$\pm 6.81 \times 10^5$	3.88×10^2	$\pm 4.18 \times 10^6$

Table 3.7 : Cell counts (cfu/ml) of T129 and its mutants at the 48th hour of incubation.

	Control medium (cfu/ml)		NaCl (10% (w/v)) (cfu/ml)		Oleuropein (4 g/l) + NaCl (10% w/v) (cfu/ml)	
T129	7.90×10^{9}	$\pm 8.54 \times 10^{8}$	7.67×10^{5}	$\pm 5.13 \times 10^{4}$	6.40×10^{3}	$\pm 7.00 \times 10^{2}$
T 1	5.67×10^{9}	$\pm 7.09 \times 10^{8}$	1.33×10^{6}	$\pm 2.55 \times 10^{5}$	1.53×10^{4}	$\pm 2.31 \times 10^{3}$
T 2	7.37×10^{9}	$\pm 8.62 \times 10^{8}$	2.28×10^{9}	$\pm 3.24 \times 10^{8}$	2.20×10^{9}	$\pm 1.81 \times 10^{8}$
T 3	7.37×10^{9}	$\pm 6.51 \times 10^{8}$	2.89×10^{9}	$\pm 1.08 \times 10^{9}$	1.92×10^{9}	$\pm 1.35 \times 10^{8}$
T 4	4.40×10^{9}	$\pm 8.00 \times 10^{8}$	1.37×10^{9}	$\pm 2.52 \times 10^{7}$	9.13×10^{8}	$\pm 2.52 \times 10^{7}$
T 5	4.13×10^{9}	$\pm 1.27 \times 10^{9}$	9.57×10^{8}	$\pm 5.51 \times 10^{7}$	8.80×10^{8}	$\pm 1.37 \times 10^{8}$
T 6	2.05×10^{9}	$\pm 4.30 \times 10^{8}$	3.01×10^{9}	$\pm 5.15 \times 10^{8}$	2.15×10^{9}	$\pm 2.93 \times 10^{8}$
T 7	1.07×10^{10}	$\pm 1.61 \times 10^{9}$	3.22×10^{8}	$\pm 8.55 \times 10^{7}$	2.17×10^{9}	$\pm 2.70 \times 10^{8}$
T 8	5.67×10^{9}	$\pm 4.93 \times 10^{8}$	2.63×10^{9}	$\pm 3.88 \times 10^{8}$	2.59×10^{9}	$\pm 2.89 \times 10^{8}$
T 9	6.30×10^{9}	$\pm 4.58 \times 10^{8}$	2.97×10^{9}	$\pm 3.39 \times 10^{8}$	1.98×10^{9}	$\pm 1.36 \times 10^{8}$
T 10	5.57×10^{9}	$\pm 5.51 \times 10^{8}$	1.76×10^{9}	$\pm 5.69 \times 10^{7}$	1.68×10^{9}	$\pm 2.21 \times 10^{8}$
T 11	6.63×10^{9}	$\pm 1.33 \times 10^{9}$	1.69×10^{9}	$\pm 1.14 \times 10^{8}$	2.42×10^{9}	$\pm 2.07 \times 10^{8}$
T 12	1.36×10^{10}	$\pm 2.11 \times 10^{9}$	4.67×10^{5}	$\pm 1.50 \times 10^{5}$	4.50×10^{3}	$\pm 9.85 \times 10^{2}$

Table 3.8 : Cell counts (cfu/ml) of T129 and its mutants at the 72nd hour of incubation.

	Control medium (cfu/ml)		NaCl (10% (w/v)) (cfu/ml)		Oleuropein (4 g/l) + NaCl (10% w/v) (cfu/ml)	
T129	6.37×10^{9}	$\pm 1.69 \times 10^{9}$	3.93×10^{4}	$\pm 1.53 \times 10^{3}$	No growth	
T 1	5.13×10^{9}	$\pm 8.14 \times 10^{8}$	4.13×10^{5}	$\pm 8.02 \times 10^{4}$	8.80×10^{1}	$\pm 2.31 \times 10^{1}$
T 2	2.82×10^{9}	$\pm 1.55 \times 10^{8}$	8.07×10^{8}	$\pm 9.07 \times 10^{7}$	1.24×10^{9}	$\pm 8.89 \times 10^{7}$
T 3	1.78×10^{9}	$\pm 1.21 \times 10^{8}$	3.60×10^{8}	$\pm 5.20 \times 10^{7}$	1.54×10^{9}	$\pm 2.10 \times 10^{8}$
T 4	1.78×10^{9}	$\pm 8.54 \times 10^{7}$	4.40×10^{7}	$\pm 1.13 \times 10^{7}$	3.33×10^{8}	$\pm 6.81 \times 10^{7}$
T 5	1.42×10^{7}	$\pm 2.45 \times 10^{6}$	2.50×10^{7}	$\pm 2.00 \times 10^{6}$	6.37×10^{8}	$\pm 2.08 \times 10^{7}$
T 6	2.10×10^{7}	$\pm 3.84 \times 10^{6}$	5.47×10^{8}	$\pm 8.08 \times 10^{7}$	6.37×10^{8}	$\pm 5.51 \times 10^{7}$
T 7	5.80×10^{9}	$\pm 4.36 \times 10^{8}$	3.38×10^{8}	$\pm 1.10 \times 10^{8}$	1.77×10^{9}	$\pm 1.68 \times 10^{8}$
T 8	2.32×10^{9}	$\pm 1.44 \times 10^{8}$	5.46×10^{9}	$\pm 3.84 \times 10^{9}$	5.80×10^{8}	$\pm 8.72 \times 10^{7}$
T 9	2.41×10^{9}	$\pm 2.52 \times 10^{7}$	1.59×10^{9}	$\pm 1.80 \times 10^{8}$	1.29×10^{9}	$\pm 1.18 \times 10^{8}$
T 10	3.38×10^{9}	$\pm 7.98 \times 10^{8}$	9.03×10^{8}	$\pm 2.06 \times 10^{8}$	1.20×10^{8}	$\pm 2.68 \times 10^{7}$
T 11	2.70×10^{9}	$\pm 2.55 \times 10^{8}$	9.97×10^{8}	$\pm 1.91 \times 10^{8}$	2.42×10^{9}	$\pm 1.33 \times 10^{8}$
T 12	6.80×10^{9}	$\pm 1.42 \times 10^{9}$	2.20×10^{4}	$\pm 1.57 \times 10^{3}$	No growth	

Table 3.9 : Cell counts (cfu/ml) of DSMZ 10492 and its mutants at the beginning on incubation (0 h).

	Control medium (cfu/ml)		NaCl (10% (w/v)) (cfu/ml)		Oleuropein (4 g/l) + NaCl (10% w/v) (cfu/ml)	
DSMZ 10494	1.70×10^7	$\pm 2.86 \times 10^6$	1.70×10^7	$\pm 2.86 \times 10^6$	1.70×10^7	$\pm 2.86 \times 10^6$
D 1	1.04×10^7	$\pm 3.48 \times 10^6$	1.04×10^7	$\pm 3.48 \times 10^6$	1.04×10^7	$\pm 3.48 \times 10^6$
D 2	3.80×10^7	$\pm 1.70 \times 10^7$	3.80×10^7	$\pm 1.70 \times 10^7$	3.80×10^7	$\pm 1.70 \times 10^7$
D 3	1.18×10^7	$\pm 2.23 \times 10^6$	1.18×10^7	$\pm 2.23 \times 10^6$	1.18×10^7	$\pm 2.23 \times 10^6$
D 4	7.80×10^6	$\pm 1.76 \times 10^6$	7.80×10^6	$\pm 1.76 \times 10^6$	7.80×10^6	$\pm 1.76 \times 10^6$
D 5	9.23×10^6	$\pm 2.05 \times 10^6$	9.23×10^6	$\pm 2.05 \times 10^6$	9.23×10^6	$\pm 2.05 \times 10^6$
D 6	4.40×10^7	$\pm 3.61 \times 10^6$	4.40×10^7	$\pm 3.61 \times 10^6$	4.40×10^7	$\pm 3.61 \times 10^6$
D 7	1.34×10^7	$\pm 5.78 \times 10^6$	1.34×10^7	$\pm 5.78 \times 10^6$	1.34×10^7	$\pm 5.78 \times 10^6$
D 8	3.97×10^7	$\pm 6.43 \times 10^6$	3.97×10^7	$\pm 6.43 \times 10^6$	3.97×10^7	$\pm 6.43 \times 10^6$
D 9	3.20×10^7	$\pm 1.24 \times 10^7$	3.20×10^7	$\pm 1.24 \times 10^7$	3.20×10^7	$\pm 1.24 \times 10^7$
D 10	5.33×10^7	$\pm 7.51 \times 10^6$	5.33×10^7	$\pm 7.51 \times 10^6$	5.33×10^7	$\pm 7.51 \times 10^6$
D 11	6.47×10^7	$\pm 1.89 \times 10^7$	6.47×10^7	$\pm 1.89 \times 10^7$	6.47×10^7	$\pm 1.89 \times 10^7$
D 12	5.77×10^7	$\pm 5.03 \times 10^6$	5.77×10^7	$\pm 5.03 \times 10^6$	5.77×10^7	$\pm 5.03 \times 10^6$

*D1-D12 are the randomly picked individual mutants of the last population of selection.

Table 3.10 : Cell counts (cfu/ml) of DSMZ 10492 and its mutants at the 24th hour of incubation.

	Control medium (cfu/ml)		NaCl (10% (w/v)) (cfu/ml)		Oleuropein (4 g/l) + NaCl (10% w/v) (cfu/ml)	
DSMZ 10492	3.90×10^9	$\pm 2.00 \times 10^8$	1.10×10^7	$\pm 4.25 \times 10^6$	1.18×10^7	$\pm 1.92 \times 10^6$
D 1	1.94×10^9	$\pm 1.10 \times 10^9$	7.47×10^8	$\pm 2.29 \times 10^8$	1.38×10^9	$\pm 5.13 \times 10^7$
D 2	4.67×10^9	$\pm 5.13 \times 10^8$	5.00×10^8	$\pm 6.24 \times 10^7$	1.75×10^9	$\pm 2.60 \times 10^8$
D 3	1.88×10^9	$\pm 1.48 \times 10^8$	1.36×10^9	$\pm 2.97 \times 10^8$	1.50×10^9	$\pm 8.54 \times 10^7$
D 4	2.64×10^9	$\pm 3.04 \times 10^8$	5.17×10^8	$\pm 1.43 \times 10^8$	1.55×10^9	$\pm 1.40 \times 10^8$
D 5	2.85×10^9	$\pm 6.06 \times 10^8$	3.57×10^8	$\pm 1.00 \times 10^8$	1.93×10^9	$\pm 7.55 \times 10^7$
D 6	6.53×10^9	$\pm 4.93 \times 10^8$	1.34×10^9	$\pm 1.16 \times 10^8$	4.90×10^3	$\pm 6.08 \times 10^2$
D 7	3.28×10^9	$\pm 3.74 \times 10^9$	3.67×10^8	$\pm 1.15 \times 10^7$	2.44×10^9	$\pm 8.54 \times 10^7$
D 8	1.16×10^9	$\pm 3.19 \times 10^8$	1.43×10^9	$\pm 2.20 \times 10^8$	7.07×10^3	$\pm 6.66 \times 10^2$
D 9	3.93×10^9	$\pm 6.43 \times 10^8$	1.31×10^9	$\pm 2.52 \times 10^7$	1.91×10^3	$\pm 2.68 \times 10^2$
D 10	5.03×10^9	$\pm 3.51 \times 10^9$	8.73×10^8	$\pm 5.03 \times 10^7$	3.47×10^7	$\pm 5.69 \times 10^6$
D 11	2.81×10^9	$\pm 1.02 \times 10^9$	1.62×10^9	$\pm 2.40 \times 10^8$	1.51×10^7	$\pm 2.10 \times 10^2$
D 12	6.27×10^9	$\pm 4.24 \times 10^9$	1.45×10^9	$\pm 2.27 \times 10^8$	2.81×10^3	$\pm 6.18 \times 10^2$

Table 3.11 : Cell counts (cfu/ml) of DSMZ 10492 and its mutants at the 48th hour of incubation.

	Control medium (cfu/ml)		NaCl (10% (w/v)) (cfu/ml)		Oleuropein (4 g/l) + NaCl (10% w/v) (cfu/ml)	
DSMZ 10492	3.50×10^{9}	$\pm 7.81 \times 10^{8}$	2.21×10^{6}	$\pm 8.89 \times 10^{4}$	4.87×10^{5}	$\pm 1.81 \times 10^{5}$
D 1	2.55×10^{9}	$\pm 2.97 \times 10^{8}$	1.02×10^{9}	$\pm 9.54 \times 10^{7}$	1.29×10^{9}	$\pm 1.85 \times 10^{8}$
D 2	2.93×10^{9}	$\pm 2.42 \times 10^{8}$	1.07×10^{9}	$\pm 1.27 \times 10^{8}$	6.10×10^{8}	$\pm 5.57 \times 10^{7}$
D 3	1.92×10^{9}	$\pm 2.44 \times 10^{8}$	1.20×10^{9}	$\pm 1.51 \times 10^{8}$	1.22×10^{9}	$\pm 6.11 \times 10^{7}$
D 4	2.89×10^{9}	$\pm 4.47 \times 10^{8}$	9.37×10^{8}	$\pm 1.51 \times 10^{8}$	1.01×10^{9}	$\pm 3.00 \times 10^{7}$
D 5	2.30×10^{9}	$\pm 4.11 \times 10^{8}$	5.30×10^{8}	$\pm 9.54 \times 10^{7}$	1.50×10^{9}	$\pm 1.00 \times 10^{8}$
D 6	1.13×10^{9}	$\pm 1.16 \times 10^{8}$	6.33×10^{7}	$\pm 2.85 \times 10^{7}$	2.40×10^{2}	$\pm 1.76 \times 10^{2}$
D 7	3.27×10^{9}	$\pm 1.11 \times 10^{9}$	7.53×10^{8}	$\pm 5.13 \times 10^{7}$	9.47×10^{8}	$\pm 1.99 \times 10^{8}$
D 8	1.03×10^{9}	$\pm 1.71 \times 10^{8}$	5.27×10^{7}	$\pm 1.53 \times 10^{7}$	1.16×10^{3}	$\pm 2.37 \times 10^{2}$
D 9	4.73×10^{8}	$\pm 1.88 \times 10^{8}$	1.61×10^{7}	$\pm 6.52 \times 10^{6}$	2.30×10^{2}	$\pm 1.74 \times 10^{2}$
D 10	1.80×10^{9}	$\pm 5.41 \times 10^{8}$	9.37×10^{8}	$\pm 1.93 \times 10^{8}$	1.96×10^{8}	$\pm 6.81 \times 10^{6}$
D 11	1.03×10^{9}	$\pm 2.84 \times 10^{8}$	1.24×10^{8}	$\pm 3.86 \times 10^{7}$	2.63×10^{7}	$\pm 1.19 \times 10^{7}$
D 12	7.47×10^{8}	$\pm 3.00 \times 10^{8}$	1.92×10^{8}	$\pm 6.27 \times 10^{7}$	3.70×10^{2}	$\pm 1.51 \times 10^{2}$

Table 3.12 : Cell counts (cfu/ml) of DSMZ 10492 and its mutants at the 72nd hour of incubation.

	Control medium (cfu/ml)		NaCl (10% (w/v)) (cfu/ml)		Oleuropein (4 g/l) + NaCl (10% w/v) (cfu/ml)	
DSMZ 10492	3.37×10^{9}	$\pm 3.06 \times 10^{8}$	4.17×10^{4}	$\pm 4.51 \times 10^{3}$	No growth	
D 1	2.17×10^{9}	$\pm 1.86 \times 10^{8}$	7.23×10^{8}	$\pm 1.85 \times 10^{8}$	4.40×10^{8}	$\pm 1.42 \times 10^{8}$
D 2	3.73×10^{9}	$\pm 1.53 \times 10^{8}$	3.73×10^{8}	$\pm 4.73 \times 10^{7}$	4.93×10^{8}	$\pm 3.51 \times 10^{7}$
D 3	8.77×10^{8}	$\pm 7.57 \times 10^{7}$	5.40×10^{8}	$\pm 1.25 \times 10^{8}$	5.03×10^{8}	$\pm 6.66 \times 10^{7}$
D 4	9.73×10^{8}	$\pm 1.67 \times 10^{8}$	5.17×10^{8}	$\pm 1.18 \times 10^{8}$	5.60×10^{8}	$\pm 1.01 \times 10^{8}$
D 5	1.51×10^{9}	$\pm 1.56 \times 10^{8}$	1.14×10^{9}	$\pm 3.36 \times 10^{8}$	3.90×10^{8}	$\pm 6.24 \times 10^{7}$
D 6	1.18×10^{7}	$\pm 1.14 \times 10^{6}$	No growth		No growth	
D 7	1.13×10^{9}	$\pm 1.42 \times 10^{8}$	2.41×10^{8}	$\pm 2.40 \times 10^{7}$	5.80×10^{8}	$\pm 9.64 \times 10^{7}$
D 8	6.77×10^{6}	$\pm 2.22 \times 10^{6}$	3.41×10^{2}	$\pm 1.20 \times 10^{2}$	No growth	
D 9	3.07×10^{8}	$\pm 8.39 \times 10^{7}$	No growth		No growth	
D 10	9.57×10^{7}	$\pm 1.71 \times 10^{7}$	3.46×10^{2}	$\pm 6.47 \times 10^{1}$	3.37×10^{7}	$\pm 1.53 \times 10^{6}$
D 11	3.70×10^{8}	$\pm 5.00 \times 10^{7}$	No growth		No growth	
D 12	1.55×10^{8}	$\pm 4.41 \times 10^{7}$	No growth		No growth	

The survival rates of the isolated individuals were determined, based on cell counts at the 72nd h of incubation where a significant loss in viability was observed (**Tables 3.8** and **3.12**). These survival rates are given in **Tables 3.13** and **3.14**.

Mutant individuals that grew at 10% (w/v) NaCl-containing MRS were obtained. At this NaCl concentration, no growth was observed for the wild type strains. Although the wild type strains survived at these stress condition for some days, their cell counts decreased dramatically after 72 h. Oleuropein and NaCl apparently had a synergistic antibacterial effect on the wild type. No growth was observed in the presence of 10% (w/v) NaCl and oleuropein after 72 h for both wild type strains (**Table 3.8** and **3.12**).

Oleuropein had a mixed effect on mutant individuals. Some were not affected by oleuropein or even showed slightly better growth in the presence of oleuropein though some mutant strains were almost completely inhibited when oleuropein was added to NaCl. This indicates a more complex relationship between oleuropein and NaCl stress response of lactic acid bacteria. This effect can further be investigated by the comparison of genomic differences among oleuropein-resistant and oleuropein-sensitive mutant individuals derived from the DSMZ 10492 strain.

Table 3.13 : Survival rates of T129 and its mutants upon 72h exposure to NaCl and NaCl + oleuropein.

T129-72nd hour	Control medium (cfu/ml)	NaCl (cfu/ml)	NaCl + Oleuropein (cfu/ml)	Survival ratio (NaCl)	Survival ratio (NaCl + Oleuropein)
T129	6.37 x10^{9}	3.93 x10^{4}	0	6.17 x10^{-6}	0
T 1	5.13 x10^{9}	4.13 x10^{5}	8.80 x10^{1}	8.05 x10^{-5}	1.72 x10^{-8}
T 2	2.82 x10^{9}	8.07 x10^{8}	1.24 x10^{9}	2.86 x10^{-1}	4.40 x10^{-1}
T 3	1.78 x10^{9}	3.60 x10^{8}	1.54 x10^{9}	2.02 x10^{-1}	8.65 x10^{-1}
T 4	1.78 x10^{9}	4.40 x10^{7}	3.33 x10^{8}	2.47 x10^{-2}	1.87 x10^{-1}
T 5	1.42 x10^{7}	2.50 x10^{7}	6.37 x10^{8}	1.76	4.49 x10^{1}
T 6	2.10 x10^{7}	5.47 x10^{8}	6.37 x10^{8}	2.60 x10^{1}	3.03 x10^{1}
T 7	5.80 x10^{9}	3.38 x10^{8}	1.77 x10^{9}	5.83 x10^{-2}	3.05 x10^{-1}
T 8	2.32 x10^{9}	5.46 x10^{9}	5.80 x10^{8}	2.35	2.50 x10^{-1}
T 9	2.41 x10^{9}	1.59 x10^{9}	1.29 x10^{9}	6.6 x10^{-1}	5.35 x10^{-1}
T 10	3.38 x10^{9}	9.03 x10^{8}	1.20 x10^{8}	2.67 x10^{-1}	3.55 x10^{-2}
T 11	2.70 x10^{9}	9.97 x10^{8}	2.42 x10^{9}	3.69 x10^{-1}	8.96 x10^{-1}
T 12	6.80 x10^{9}	2.20 x10^{4}	0	3.24 x10^{-6}	0

Table 3.14 : Survival rates of DSMZ 10492 and its mutants upon 72h exposure to NaCl and NaCl + oleuropein.

DSMZ 10492-72nd hour	Control medium (cfu/ml)	NaCl (cfu/ml)	NaCl + Oleuropein (cfu/ml)	Survival ratio (NaCl)	Survival ratio (NaCl + Oleuropein)
DSMZ 10492	3.37 x10^{9}	4.17 x10^{4}	0	1.24x10^{-5}	0
D 1	2.17 x10^{9}	7.23 x10^{8}	4.40 x10^{8}	3.33 x10^{-1}	2.03 x10^{-1}
D 2	3.73 x10^{9}	3.73 x10^{8}	4.93 x10^{8}	1.00 x10^{-1}	1.32 x10^{-1}
D 3	8.77 x10^{8}	5.40 x10^{8}	5.03 x10^{8}	6.16 x10^{-1}	5.74 x10^{-1}
D 4	9.73 x10^{8}	5.17 x10^{8}	5.60 x10^{8}	5.31 x10^{-1}	5.76 x10^{-1}
D 5	1.51 x10^{9}	1.14 x10^{9}	3.90 x10^{8}	7.55 x10^{-1}	2.58 x10^{-1}
D 6	1.18 x10^{7}	0	0	0	0
D 7	1.13 x10^{9}	2.41 x10^{8}	5.80 x10^{8}	2.13 x10^{-1}	5.13 x10^{-1}
D 8	6.77 x10^{6}	3.41 x10^{2}	0	5.04 x10^{-5}	0
D 9	3.07 x10^{8}	0	0	0	0
D 10	9.57 x10^{7}	3.46 x10^{2}	3.37 x10^{7}	3.62 x10^{-6}	3.52 x10^{-1}
D 11	3.70 x10^{8}	0	0	0	0
D 12	1.55 x10^{8}	0	0	0	0

Zago et al. (2013) indicated that resistance to NaCl concentrations between 6-10% (w/v) is one of the most important characteristics of the strains that are intended to be used as starter cultures for olive fermentation. Most of the mutant individuals derived from the two strains, T129 and DSMZ 10492, showed high resistance to 10% (w/v) NaCl and 0.4% (w/v) oleuropein. Thus, these strains have the potential to be used as starter cultures for olive fermentation.

3.2.5 Screening cross-resistance of individual colonies against other stress types

NaCl and oleuropein are not the only stress factors in olive fermentation. Alkali stress is mainly caused by alkali-treatment of olives for debittering purposes and insufficient washing of alkali-treated olives. Acid stress is mainly caused by acid addition to olive fermentations to prevent spoilage. Ferrous gluconate is applied as a colorant to stain black olives (Sánchez Gómez et al., 2006; Corsetti et al., 2012). Thus, it is desirable to have "robust" multistress-resistant strains in industrial olive fermentations. For this purpose, the cross-resistance of the mutant individuals obtained by NaCl selection was determined against a variety of stress types that are common in olive fermentations. The results are shown in **Tables 3.15** and **3.16.**

Table 3.15 : Cell counts (cfu/ml) of T129 and its mutants at control and various stress conditions, at the 24th hour of incubation.

	Control condition (no stress) (cfu/ml)	Freeze drying (cfu/ml)	pH 9 (NaOH) (cfu/ml)	pH 4 (Acetic Acid) (cfu/ml)	pH 4 (Lactic Acid) (cfu/ml)	Ferrous Gluconate (0.1% w/v) (cfu/ml)
T129	2.19×10^9	1.62×10^9	4.40×10^9	4.00×10^7	4.00×10^7	3.60×10^9
T 1	1.79×10^{10}	1.10×10^9	3.60×10^9	7.30×10^7	1.06×10^8	2.54×10^9
T 2	3.70×10^9	4.50×10^8	8.00×10^9	4.00×10^7	4.20×10^7	4.70×10^9
T 3	6.20×10^9	1.49×10^9	3.20×10^9	6.60×10^7	4.30×10^7	2.38×10^9
T 4	9.50×10^9	3.50×10^8	3.10×10^9	3.30×10^7	3.40×10^7	2.18×10^9
T 5	8.70×10^9	1.02×10^9	8.20×10^9	4.20×10^7	3.10×10^7	6.50×10^8
T 6	7.60×10^9	6.30×10^9	2.42×10^9	0.00×10^7	3.50×10^7	1.48×10^9
T 7	1.14×10^{10}	1.06×10^{10}	2.15×10^9	5.90×10^7	3.60×10^7	2.80×10^9
T 8	1.55×10^9	1.29×10^9	5.80×10^9	7.60×10^7	6.30×10^7	3.10×10^9
T 9	7.20×10^9	1.25×10^9	7.30×10^9	7.90×10^7	4.30×10^7	3.20×10^9
T 10	1.86×10^9	1.80×10^9	8.60×10^9	4.40×10^7	5.60×10^7	1.03×10^9
T 11	2.16×10^9	1.43×10^9	5.80×10^9	4.50×10^7	5.40×10^7	3.20×10^9
T 12	5.10×10^9	3.20×10^9	4.50×10^9	5.80×10^7	5.40×10^7	1.02×10^9

Table 3.16 : Cell counts (cfu/ml) of DSMZ 10492 and its mutants at control and various stress conditions, at the 24th hour of incubation.

	Control condition (no stress) (cfu/ml)	Freeze drying (cfu/ml)	pH 9 (NaOH) (cfu/ml)	pH 4 (Acetic Acid) (cfu/ml)	pH 4 (Lactic Acid) (cfu/ml)	Ferrous Gluconate (0.1% w/v) (cfu/ml)
DSMZ 10492	1.87×10^{9}	8.20×10^{8}	2.40×10^{9}	9.20×10^{6}	1.16×10^{7}	3.50×10^{9}
D 1	2.71×10^{9}	1.80×10^{9}	3.40×10^{6}	9.00×10^{6}	1.52×10^{6}	1.52×10^{10}
D 2	5.89×10^{9}	5.60×10^{9}	4.60×10^{9}	5.60×10^{7}	5.10×10^{7}	4.10×10^{9}
D 3	3.70×10^{9}	3.40×10^{9}	2.76×10^{8}	9.70×10^{6}	3.30×10^{7}	1.28×10^{9}
D 4	2.15×10^{9}	3.80×10^{8}	3.50×10^{7}	5.30×10^{6}	7.10×10^{7}	1.43×10^{9}
D 5	2.97×10^{9}	2.81×10^{9}	7.40×10^{4}	3.20×10^{6}	1.13×10^{7}	2.64×10^{9}
D 6	6.90×10^{8}	4.20×10^{8}	6.30×10^{3}	5.70×10^{6}	1.04×10^{7}	2.24×10^{9}
D 7	6.40×10^{8}	2.20×10^{8}	1.52×10^{7}	7.90×10^{6}	5.20×10^{6}	1.59×10^{9}
D 8	1.55×10^{9}	1.14×10^{9}	8.60×10^{3}	1.58×10^{7}	1.17×10^{7}	3.10×10^{9}
D 9	1.26×10^{10}	1.36×10^{9}	9.10×10^{3}	9.90×10^{6}	8.90×10^{6}	3.70×10^{9}
D 10	8.30×10^{9}	1.08×10^{8}	2.88×10^{4}	5.30×10^{6}	1.32×10^{6}	2.65×10^{9}
D 11	9.30×10^{9}	4.30×10^{8}	9.80×10^{3}	1.11×10^{6}	1.36×10^{7}	3.00×10^{9}
D 12	7.90×10^{8}	7.80×10^{8}	1.15×10^{4}	3.20×10^{7}	8.10×10^{6}	2.19×10^{9}

The survival rates of the mutant individuals were also compared with those of the wild type strains (T129 and DSMZ 10492). The results are shown in **Tables 3.17** and **3.18.**

Table 3.17 : Survival rates of T129-derived mutants at tested stress conditions, as percentage of the wild type strain, T129.

	Freeze drying	pH 9 (NaOH)	pH 4 Acetic acid	pH 4 Lactic acid	Ferrous Gluconate (0.1%)
Wild type	100.00%	100.00%	100.00%	100.00%	100.00%
T 1	8.31%	10.01%	22.33%	32.42%	8.63%
T 2	16.44%	107.62%	59.19%	62.15%	77.27%
T 3	32.49%	25.69%	58.28%	64.46%	23.35%
T 4	4.98%	16.24%	19.02%	19.59%	13.96%
T 5	15.85%	46.91%	26.43%	19.51%	4.55%
T 6	112.06%	15.85%	0.00%	25.21%	11.85%
T 7	125.70%	9.39%	28.34%	17.29%	14.94%
T 8	112.51%	186.25%	268.45%	222.53%	121.67%
T 9	23.47%	50.46%	60.07%	32.70%	27.04%
T 10	130.82%	230.13%	129.52%	164.84%	33.69%
T 11	89.50%	133.65%	114.06%	136.88%	90.12%
T 12	84.82%	43.92%	62.26%	57.97%	12.17%

Table 3.18 : Survival rates of DSMZ 10492-derived mutants at tested stress conditions, as percentage of the wild type strain, DSMZ10492.

	Freeze drying	pH 9 (NaOH)	pH 4 Acetic acid	pH 4 Lactic acid	Ferrous Gluconate (0.1%)
DSMZ 10492	100.00%	100.00%	100.00%	100.00%	100.00%
D 1	151.47%	0.10%	67.50%	9.04%	299.67%
D 2	216.82%	60.85%	193.25%	139.58%	37.19%
D 3	209.56%	5.81%	53.29%	318.06%	18.48%
D 4	40.31%	1.27%	50.11%	532.36%	35.54%
D 5	215.76%	0.00%	21.90%	61.33%	47.49%
D 6	138.81%	0.00%	167.91%	242.98%	173.45%
D 7	78.39%	1.85%	250.90%	130.98%	132.74%
D 8	167.73%	0.00%	207.19%	121.69%	106.86%
D 9	24.61%	0.00%	15.97%	11.39%	15.69%
D 10	2.97%	0.00%	12.98%	2.56%	17.06%
D 11	10.54%	0.00%	2.43%	23.57%	17.24%
D 12	225.16%	0.00%	823.34%	165.29%	148.11%

Among the mutants derived from T129 strain, T8 was the most robust mutant which had higher cross-resistance than the wild type T129 against all four stress types tested (**Table 3.17**). Similarly, among the DSMZ 10492-derived mutants, D2 was the most robust mutant, regarding its cross-resistance (**Table 3.18**). As both mutants are already resistant to NaCl and oleuropein, and as they are more robust than the other mutants tested, they were chosen as the most promising mutants for further analysis.

Lyophilisation (freeze drying) is a commonly applied industrial process in the production of dry starter cultures. Bacterial viability decreases due to dehydration stress and ice crystal formation during freeze drying (Strasser et al., 2009). Thus, lyophilisation is also a major stress factor. In this study, the resistance of the mutant strains against lyophilisation greatly varied. This heterogeneity may result from the genetic complexity of lyophilisation resistance. Survival rates during freeze drying could further be increased by optimization of the freeze drying process.

During natural black olive fermentation, it is not uncommon to decrease the brine pH below 4.5 to inhibit the growth of spoilage bacteria. pH is most commonly controlled by the addition of acetic acid. The pH further decreases during the process and reaches commonly between 3.9-4.1 at the end of the olive fermentation (Sánchez Gómez et al., 2006). Thus, an acid stress level of pH4 was selected in this study.

TUBİTAK wild type strain T129 was generally more resistant to acid stress than the DSMZ 10492 strain. All strains were vulnerable to acid stress at pH4 irrespectively of the acidifying agent. Some strains slightly increased their cell number and began to grow at these conditions, whereas the viability of some strains decreased at both conditions.

As a result of the cross-resistance tests, no correlation could be made between NaCl and oleuropein resistance and the other tested stress types such as acid, alkaline or ferrous gluconate stress. Hypotheses such as NaCl resistance and acid or alkali resistance may have different molecular mechanisms can be made.

The results also revealed that ferrous gluconate concentrations of 0.1% (w/v) had almost no inhibitory effect on all strains (**Tables 3.15** and **3.16**). 0.1% (w/v) ferrous gluconate is the same concentration used in industry to fix the color of black olives. (Sánchez Gómez et al., 2006). Thus, it can be assumed that all mutant individuals would protect their survival after colour fixation.

Spanish style olives are debittered chemically by diluted lye treatment at concentrations between 2-5% (w/v). Debittered olives are subsequently washed several times with tap water and brined at brines containing approximately 10% (w/v) NaCl (Sánchez Gómez et al., 2006). pH of washed olives can remain high after washing step. Sánchez et al. (2001) determined that inoculation of Spanish olives above pH 9 improved their microbiological quality. Thus, cross resistance to pH 9 was investigated. Although all TUBİTAK strains were resistant against alkali pH 9, only four mutant individuals of DSMZ 10492 retained their ability to survive at pH 9 (**Table 3.16**). High pH caused an increased lag phase in all strains tested, thus survival rates were moderate. However, it could be expected that mutant individuals which showed slight growth at 24 h would continue to grow at extended incubation times. This phenomenon was explained by Sánchez et al. (2001) who had observed a 1-2 log decrease in starter culture concentration during the beginning of fermentation.

It should be noted that not all of these stress conditions are observed during every kind of olive fermentation process. However, they are observed depending on the selected olive processing method. Where ferrous gluconate is only applied in black olive fermentation, alkaline stress is observed only if the olives are debittered by

NaOH. The cross-resistance experiments were replicated for the most robust mutant strains, TUBİTAK strain 8 and DSMZ strain 2. The results are shown in **Tables 3.19** and **3.20**, respectively.

Table 3.19 : Cross-resistance of the wild type strain T129 and its selected robust mutant Strain 8 against various stress conditions

Stress conditions	Survival rate (% Control)	
	T129	T8
Control	100	100
Lyophilisation	82.01	41.89
pH9 (NaOH)	185.71	420.57
pH4 (acetic acid)	2.37	5.47
pH4 (lactic acid)	2.12	3.32
ferrous gluconate (%0.1)	207.41	184.57

High survival rates especially against NaOH at pH 9 and ferrous gluconate were observed in both T129 and its mutant T8. This strain could be especially used after alkali treatment of olives (Sánchez et al., 2001). The high resistance to ferrous gluconate could make this strain applicable in black olive fermentations, as this chemical can be used there to darken the colour of the processed olives (Sánchez Gómez et al., 2006).

Table 3.20 : Cross-resistance of DSMZ 10492 wild type strain and its selected robust mutant Strain D2 against various stress conditions

Stress conditions	Survival ratio (% of initial)	
	DSMZ 10492	D2
Control	100	100
Lyophilisation	39.12	63.81
pH9 (NaOH)	123.46	98.89
pH4 (acetic acid)	0.36	0.87
pH4 (lactic acid)	0.44	0.70
ferrous gluconate (%0.1)	126.34	60.16

High survival rates especially against NaOH at pH 9 and ferrous gluconate were observed in both DSMZ 10492 and its mutant D2. This strain could also be used

after alkali treatment of olives (Sánchez et al., 2001). The high resistance to ferrous gluconate could also make this strain suitable for black olive fermentations (Sánchez Gómez et al., 2006), as the other mutant strain T8.

The resistance of D2 against lyophilisation or freeze-drying is moderate, but it should be considered that lyophilisation was performed without further optimization. The optimization of the lyophilisation cycle and the use of an improved cryoprotectant would increase the survival rate (Strasser et al., 2009).

The strain D2 has moderate levels of resistance against lactic acid and acetic acid at pH 4. Although this could be an advantage when excessive acid is used prior to olive fermentation, it could also have beneficial effects when too low pH values are not desired, as these strains would only poorly grow at low pH and prevent further decrease in olive pH (Sánchez Gómez et al., 2006).

3.2.6 Growth physiological analyses of wild type strains and their selected robust mutant individuals

The growth of the wild type T129 strain and its mutant T8, and the wild type DSMZ 10492 and its mutant D2 were followed in shake flasks containing 6.5% (w/v) NaCl-containing 50% diluted MRS medium (stress condition) or 50% diluted MRS medium (control condition). Their growth was monitored by OD and cell dry weight (CDW) measurements. Their substrate utilization and lactic acid production rates were determined based on HPLC measurements.

3.2.6.1 OD_{580} measurements of cultivated cells

The OD_{580} measurement results of T129 and its mutant T8 in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth are given in **Table 3.21.**

Table 3.21 : OD_{580} of T129 and its mutant T8 in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth

Sampling time (hour)	50% diluted MRS		50% diluted MRS + 6.5% (w/v) NaCl	
	T129	T8	T129	T8
2	0.002±0.002	0.023±0.02	0.0864±0.01	0.071±0.013
4	0.012±0.002	0.028±0.011	0.0528±0.013	0.097±0.006
6	0.106±0.01	0.132±0.027	0.074±0.016	0.154±0.006
8	0.346±0.007	0.473±0.01	0.076±0.01	0.212±0.039
10	1.074±0.038	0.693±0.067	0.141±0.013	0.272±0.023
12	1.838±0.106	0.881±0.041	0.191±0.033	0.573±0.016
14	3.124±0.005	1.123±0.075	0.351±0.07	0.795±0.016
16	3.616±0.055	1.252±0.04	0.452±0.027	1.039±0.142
18	4.435±0.059	1.469±0.107	0.557±0.044	1.243±0.045
20	4.590±0.061	1.636±0.02	0.748±0.064	1.428±0.119
22	4.956±0.132	1.929±0.04	0.968±0.043	1.531±0.062
24	4.900±0.073	1.901±0.056	1.174±0.05	1.773±0.098
26	-	-	1.37±0.046	1.887±0.064
28	-	-	1.397±0.011	1.874±0.029
30	-	-	1.378±0.027	1.939±0.016

The growth curves of T129 and its mutant T8 in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth are shown in **Figures 3.38** and **3.39**, respectively.

The maximum specific growth rates of T129 and its mutant T8 were calculated using the $lnOD_{580}$ vs. time graph. The slope of the trend lines yields the maximum specific growth rate of the strains, during exponential growth phase (Yilmaz, Ü., 2013). The maximum specific growth rate (μ_{max}) of T129 and its mutant T8 were 0.928 and 0.721 h^{-1}, respectively (**Figure 3.38**). The generation time of these strains were calculated by taking the reciprocal of the specific growth rate. Thus, the generation times of T129 and T8 were 1.08 and 1.39 h, respectively. It could clearly be observed that the mutant individual has a reduced growth rate and a lower maximum OD_{580} value. This situation is not uncommon in previous evolutionary engineering studies with yeast (Yilmaz, Ü., 2013).

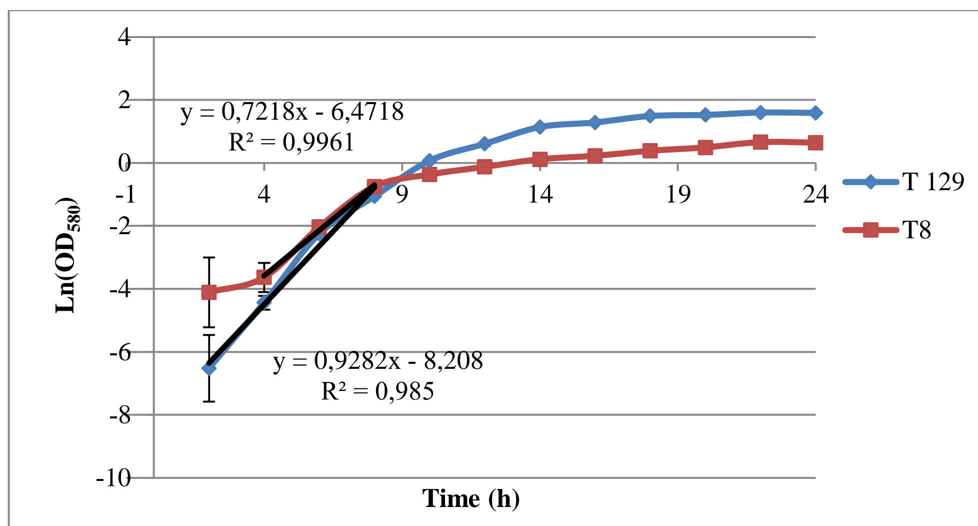

Figure 3.38 : Growth curve of T129 and its mutant T8 in 50% diluted MRS broth.

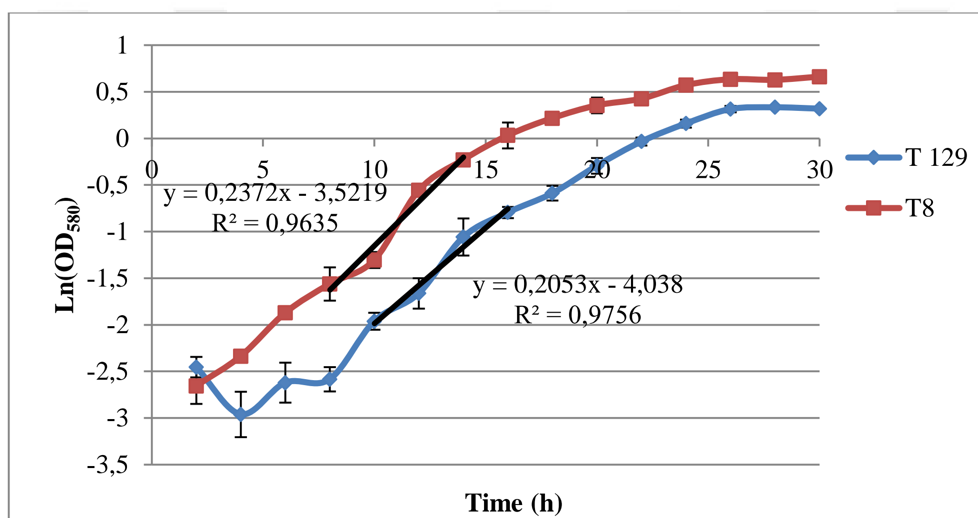

Figure 3.39 : Growth curve of T129 and its mutant T8 in 6.5% (w/v) NaCl-containing 50% diluted MRS broth.

The maximum specific growth rate (μ_{max}) of T129 and its mutant T8 were 0.205 and 0.237 h^{-1} in 6.5% (w/v) NaCl-containing MRS broth. The generation times of these strains were 4.878 and 4.219 h, respectively. The mutant individual T8 had a shorter lag phase than its wild type (T129) and reached higher maximum OD_{580} values during the experiments.

Lactic acid bacteria are frequently subjected to osmotic stress in their natural environmental niches. Numerous studies revealed that the accumulation of compatible solutes was the primary stress response against osmotic stress in lactic acid bacteria. However, it has been revealed that this is an emergency response and does not really overlap with the long term osmotic stress response of lactic acid bacteria. It was in contrary observed that solute transporters were negatively

regulated during continuous osmotic stress. The long term stress response was based on changes in cell wall composition. The changes in cell wall composition could alleviate the effects of the osmotic stress (Pieterse B. 2006). A mutation could have resulted in a shift in cell wall structure of T8. This strain had markedly slower growth rate and glucose uptake rate. This decrease might have resulted from the extra barrier formed due to the changed cell wall structure.

The OD_{580} measurement results of DSMZ 10492 and DSMZ 10492 D2 in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth are given in **Table 3.22**.

Table 3.22 : OD_{580} of DSMZ 10492 and its mutant D2 in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth.

Sampling time (hour)	50% diluted MRS		50% diluted MRS + 6.5% (w/v) NaCl	
	DSMZ 10492	D2	DSMZ 10492	D2
2	0.067±0.058	0.067±0.058	0.1±0	0.1±0
4	0.1±0	0.033±0.058	0.1±0	0.133±0.058
6	0.133±0.058	0.1±0	0.133±0.058	0.267±0.058
8	0.333±0.058	0.433±0.058	0.133±0.058	0.4±0
10	1.033±0.058	1.4±0	0.433±0.058	0.867±0.058
12	2.267±0.208	2.8±0	0.533±0.115	1.333±0.115
14	2.9±0.1	3.7±0.346	0.767±0.058	1.767±0.058
16	3.6±0.1	4.2±0.1	0.933±0.153	1.867±0.058
18	4.467±0.231	4.933±0.208	1.067±0.153	1.8±0.1
20	4.833±0.153	4.9±0.1	1.2±0.1	1.8±0.1
22	4.9±0.1	5±0.173	1.233±0.115	1.633±0.115
24	4.933±0.058	4.867±0.289	1.333±0.058	1.867±0.058
26	-	-	1.433±0.115	1.733±0.058
28	-	-	1.333±0.058	1.733±0.115
30	-	-	1.233±0.058	1.767±0.115

The growth curves of DSMZ 10492 and its mutant D2 in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth are shown in **Figures 3.40** and **3.41**, respectively.

The maximum specific growth rate (μ_{max}) of DSMZ 10492 and its mutant D2 in 50% diluted MRS were 0.641 and 0.652 h^{-1}, respectively (**Figure 3.40**). The generation times of these strains were 1.560 and 1.534 h, in 50% diluted MRS, respectively (**Figure 3.40**). Unlike T8 mutant strain, it was observed that both D2 and its wild type strain had similar specific growth rates in 50% diluted MRS. Thus, it could be inferred that in D2 strain a different survival mechanism than in T8 has been developed by evolutionary engineering.

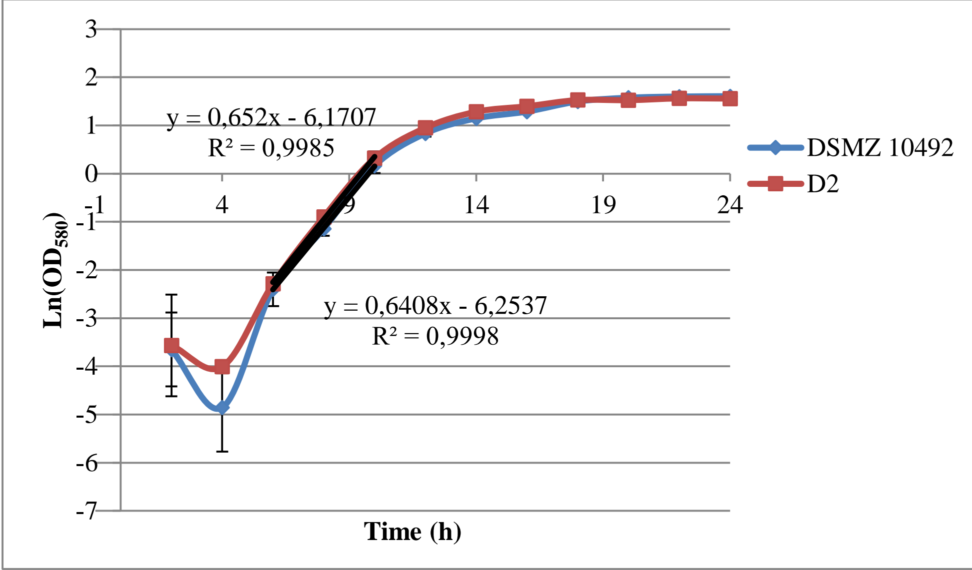

Figure 3.40 : Growth curve of DSMZ 10492 and its mutant D2 in 50% diluted MRS broth.

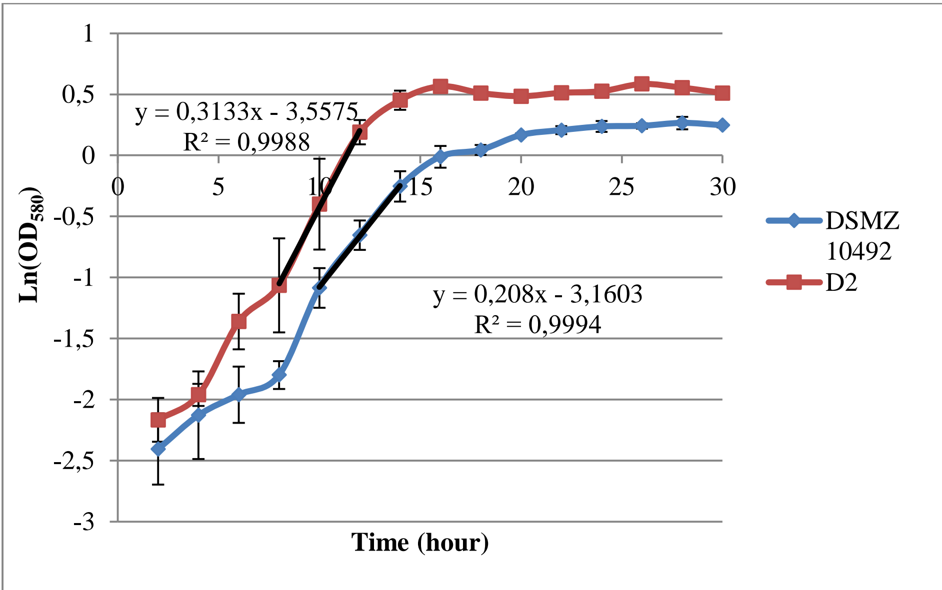

Figure 3.41 : Growth curve of DSMZ 10492 and its mutant D2 in 6.5% (w/v) NaCl-containing 50% diluted MRS broth.

3.2.6.2 CDW measurements of cultivated cells

The CDW measurement results of T129 and its mutant strain T8 in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth are given in **Table 3.23.**

The CDW profiles of T129 and its mutant T8 during growth in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth are shown in **Figures 3.42** and **3.43**, respectively.

Table 3.23 : CDW (g/l) of T129 and its mutant strain T8 in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth.

Sampling time (hour)	50% diluted MRS		50% diluted MRS + 6.5% NaCl	
	T129 CDW (g/l)	T8 CDW (g/l)	T129 CDW (g/l)	T8 CDW (g/l)
2	0.033±0.058	0.1±0	0.067±0.058	0.067±0.058
4	0.067±0.058	0.067±0.058	0.067±0.058	0.133±0.058
6	0.067±0.058	0.133±0.058	0.067±0.058	0.167±0.058
8	0.3±0.1	0.5±0.1	0.033±0.058	0.2±0.1
10	1.067±0.231	0.733±0.115	0.1±0.1	0.367±0.058
12	2±0.1	0.9±0.1	0.267±0.058	0.5±0.1
14	2.933±0.058	1.233±0.058	0.433±0.153	0.633±0.208
16	3.7±0.3	1.333±0.058	0.5±0.1	0.867±0.058
18	4.767±0.252	1.533±0.208	0.6±0.1	1.167±0.058
20	4.7±0.173	1.8±0	0.767±0.058	1.467±0.115
22	4.8±0.265	1.967±0.058	0.967±0.115	1.533±0.058
24	5±0.2	2±0.1	1.333±0.152	1.667±0.057
26	-	-	1.333±0.115	1.833±0.153
28	-	-	1.5±0.1	1.9±0.1
30	-	-	1.4±0.173	1.933±0.058

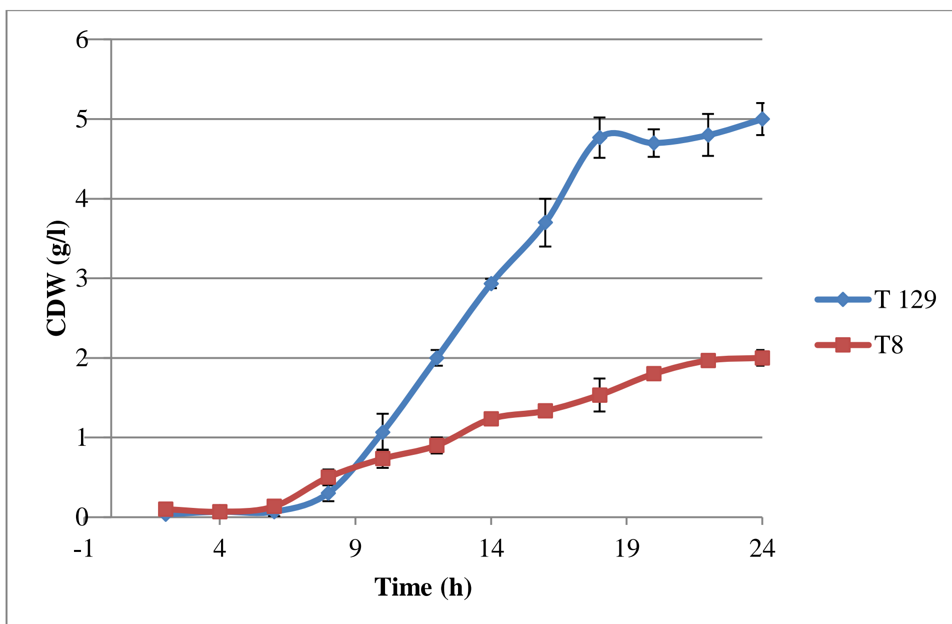

Figure 3.42 : CDW profiles of T129 and its mutant strain T8 during growth in 50% diluted MRS broth.

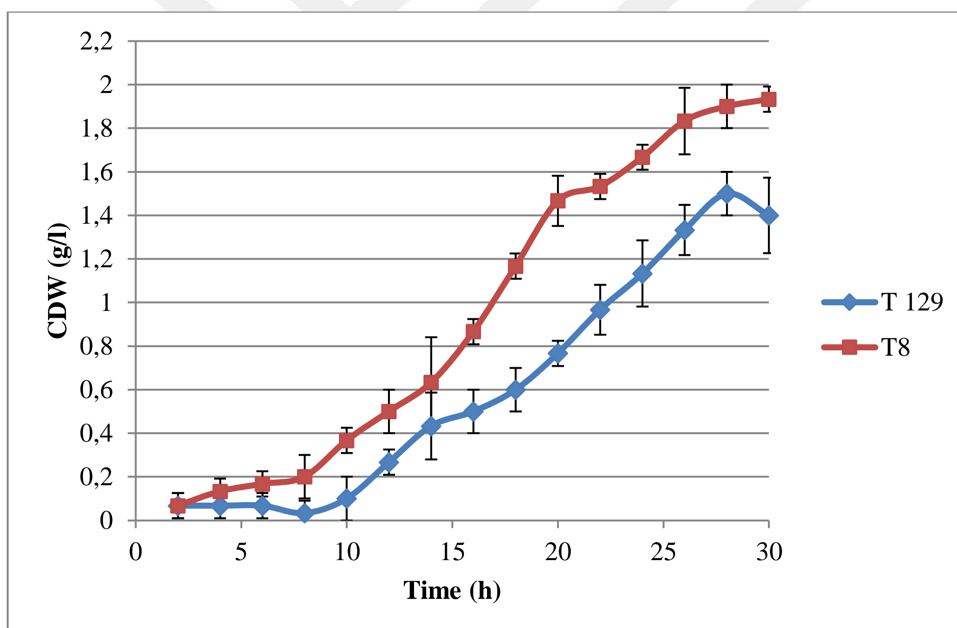

Figure 3.43 : CDW profiles of T129 and its mutant strain T8 during growth in 6.5% (w/v) NaCl-containing 50% diluted MRS broth.

It can be concluded that the CDW data were consistent with the OD_{580} data of T129 and T8 for growth in 50% diluted MRS and 6.5% (w/v) NaCl-containing 50% diluted MRS (**Figures 3.42** and **3.43**).

The CDW data of DSMZ 10492 and its mutant D2 in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth are given in **Table 3.24**.

Table 3.24 : CDW (g/l) of DSMZ10492 and its mutant D2 in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth.

Sampling time (hour)	50% diluted MRS		50% diluted MRS + 6.5% (w/v) NaCl	
	DSMZ10492	D2	DSMZ10492	D2
2	0.067±0.058	0.067±0.058	0.1±0	0.1±0
4	0.1±0	0.033±0.058	0.1±0	0.133±0.058
6	0.133±0.058	0.1±0	0.133±0.058	0.267±0.058
8	0.333±0.058	0.433±0.058	0.133±0.058	0.4±0
10	1.033±0.058	1.4±0	0.433±0.058	0.867±0.058
12	2.267±0.208	2.8±0	0.533±0.115	1.333±0.115
14	2.9±0.1	3.7±0.346	0.767±0.058	1.767±0.058
16	3.6±0.1	4.2±0.1	0.933±0.153	1.867±0.058
18	4.467±0.231	4.933±0.208	1.067±0.153	1.8±0.1
20	4.833±0.153	4.9±0.1	1.2±0.1	1.8±0.1
22	4.9±0.1	5±0.173	1.233±0.115	1.633±0.115
24	4.933±0.058	4.867±0.289	1.333±0.058	1.867±0.058
26	-	-	1.433±0.115	1.733±0.058
28	-	-	1.333±0.058	1.733±0.115
30	-	-	1.233±0.058	1.767±0.115

The CDW profiles of both DSMZ 10492 and its mutant D2 during growth in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing 50% diluted MRS broth are shown in **Figures 3.44** and **3.45**, respectively.

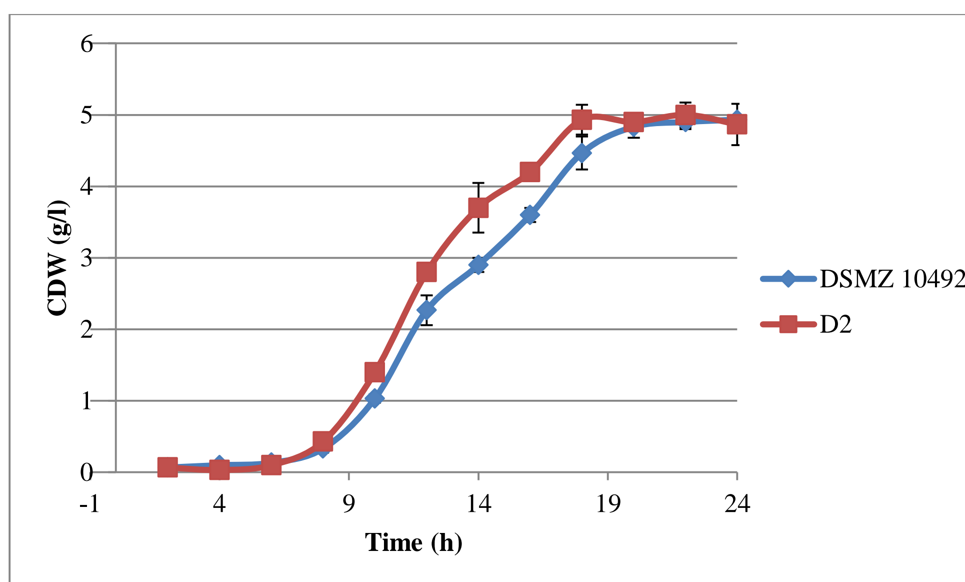

Figure 3.44 : CDW profiles of DSMZ 10492 and its mutant strain D2 during growth in 50% diluted MRS broth.

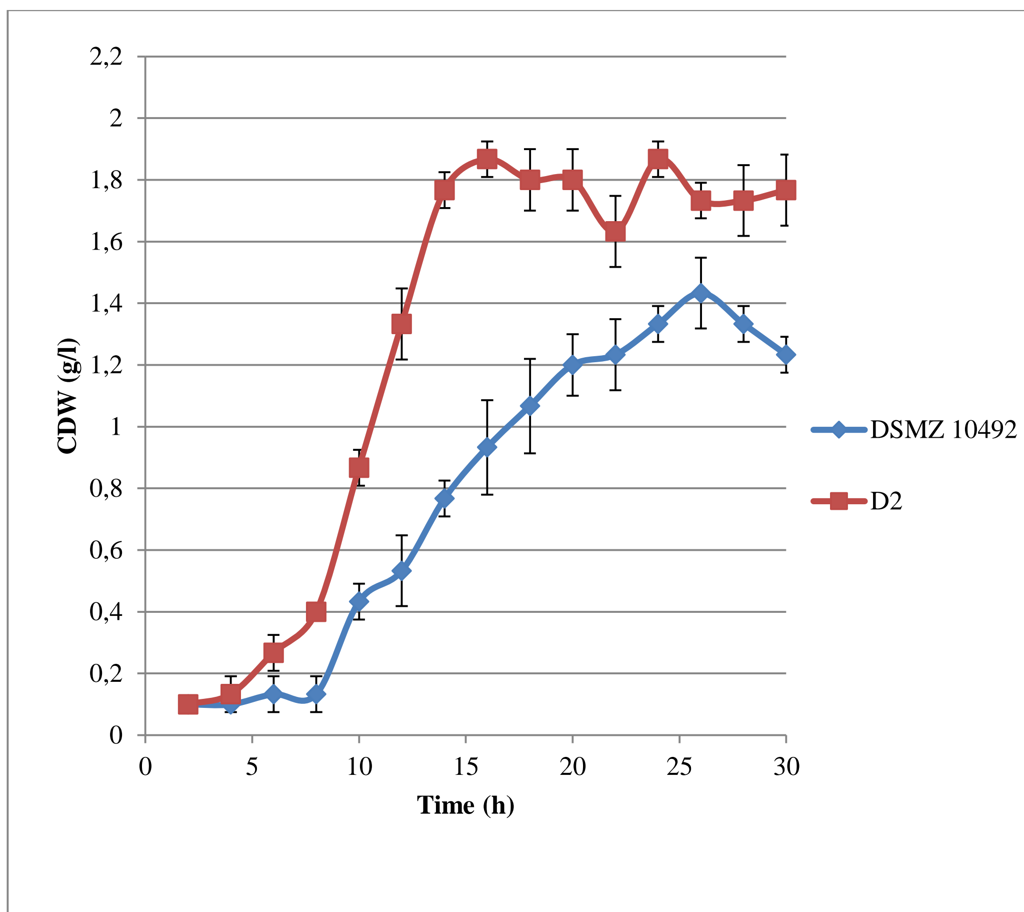

Figure 3.45 : CDW profiles of DSMZ 10492 and its mutant strain D2 during growth in 6.5% (w/v) NaCl-containing 50% diluted MRS broth.

It is known that OD and CDW data are in linear relationship (Passos et al., 1994). As expected, the CDW data were consistent with the OD_{580} data during the growth of DSMZ 10492 and its mutant D2 in 50% diluted MRS broth. It was also clearly observed that the CDW of D2 was significantly higher than that of the wild type strain DSMZ 10492 under NaCl stress conditions (**Figure 3.45**). This may be potentially resulting from an increased solute transport system in D2.

3.2.6.3 Metabolite analyses of cultivated cells by high performance liquid chromatography (HPLC)

Culture supernatants collected during growth physiology experiments were analysed for their glucose and lactate contents by HPLC. Glucose and lactate concentrations of T129 and T8 under control conditions are shown in **Figures 3.46** and **3.47**, respectively.

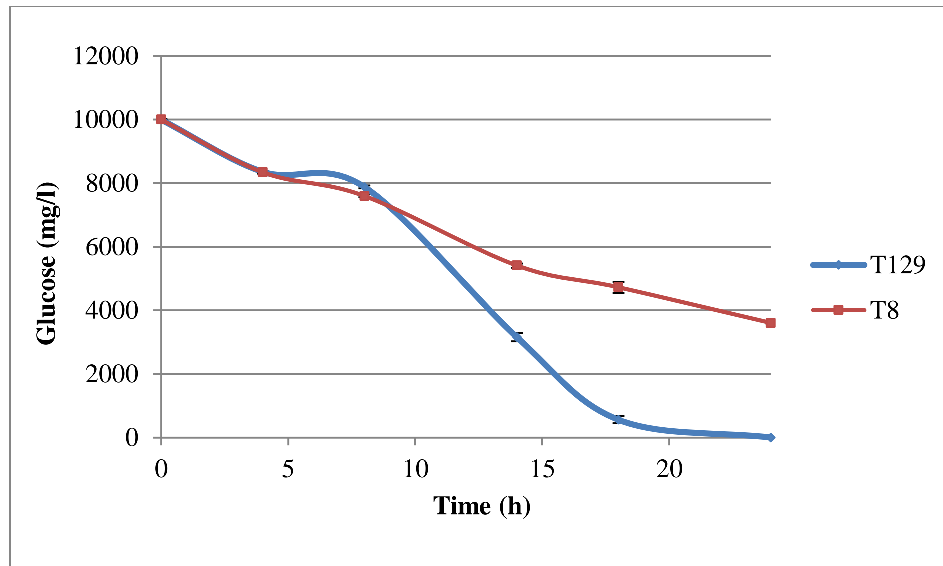

Figure 3.46 : Residual glucose concentrations of T129 and T8 under control conditions.

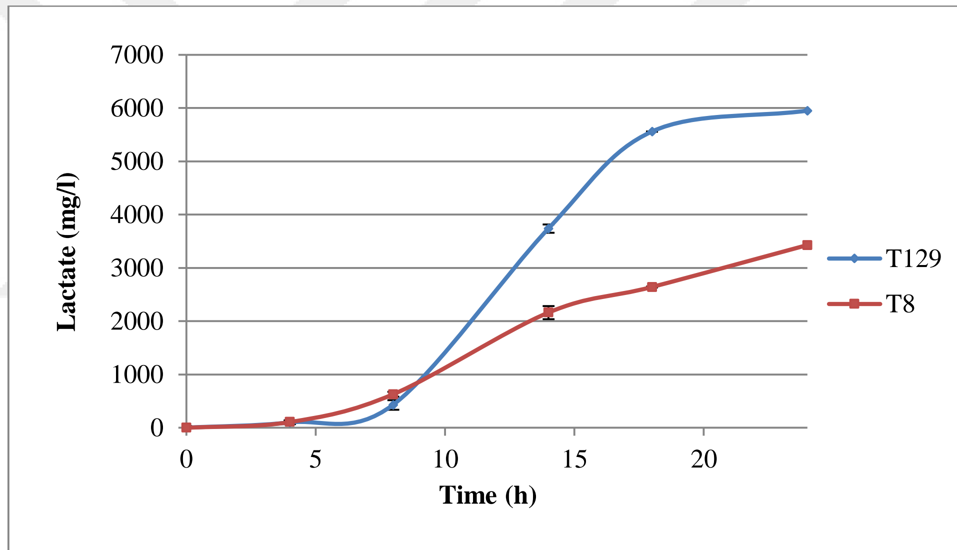

Figure 3.47 : Lactate concentrations of T129 and T8 under control conditions.

Glucose was consumed more slowly by the mutant strain T8 than its wild type under control conditions (50% diluted MRS broth). The lactate production was consistent with the glucose consumption. This was expected as lactic acid is a primary metabolite of *L. plantarum*. The growth of the wild type slowed down as the glucose concentration decreased in the medium. Glucose was totally consumed by the wild type strain T129 at 24 h of cultivation. However, at 24h, there was still 0.4 g/l glucose left in the T8 culture, and lactate production was still continuing, unlike T129 (**Figures 3.46** and **3.47**).

Culture supernatants collected during growth physiology experiments were analysed for their glucose and lactate contents by HPLC. Glucose and lactate concentrations of DSMZ 10492 and its mutant strain D2 under control conditions are shown in **Figures 3.48** and **3.49**, respectively.

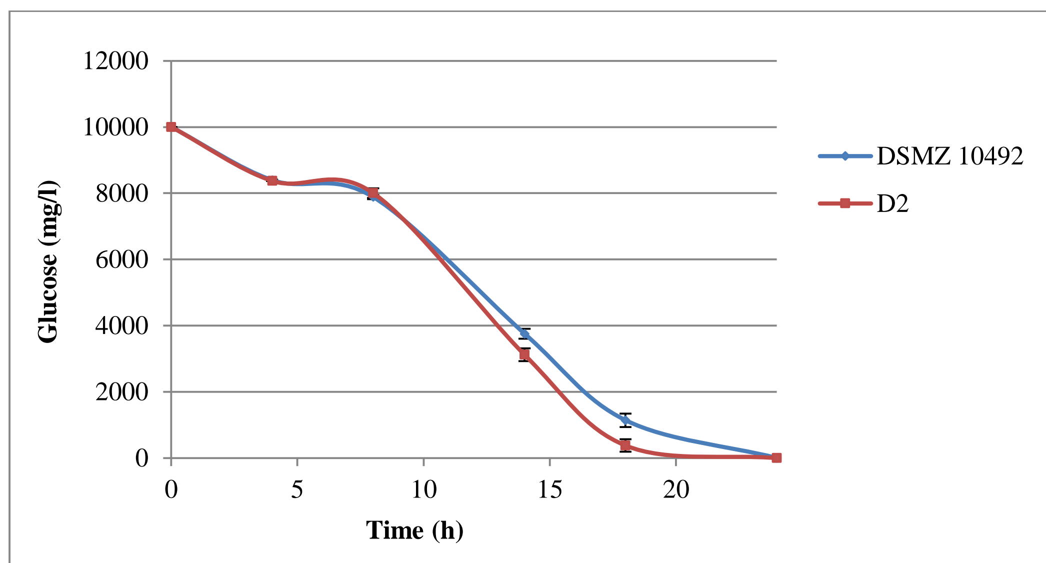

Figure 3.48 : Residual glucose concentrations of DSMZ 10492 and D2 under control conditions.

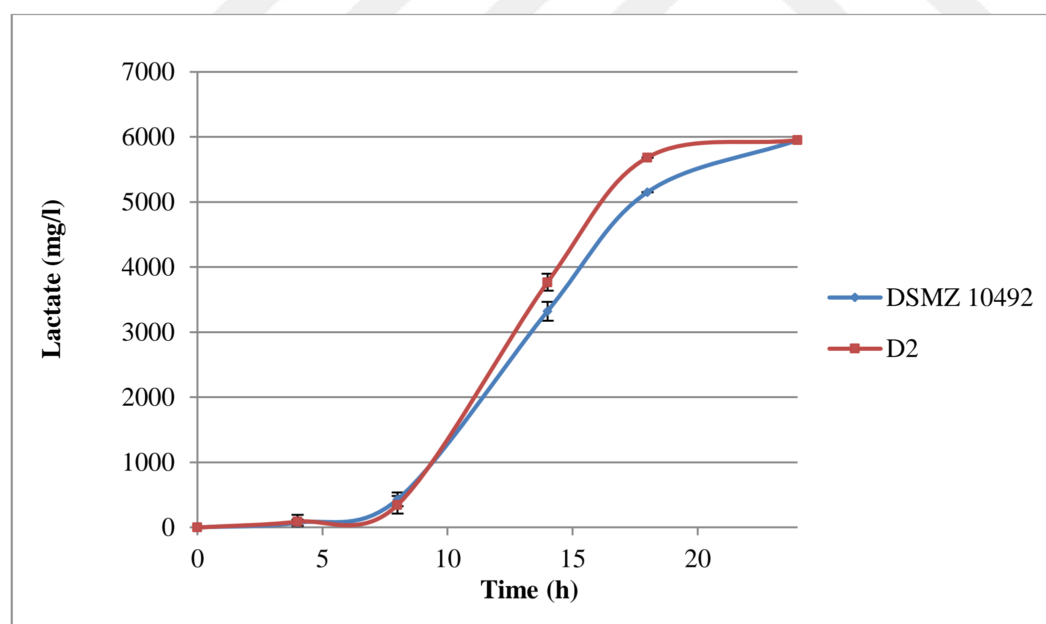

Figure 3.49 : Lactate concentrations of DSMZ 10492 and D2 under control conditions.

The lactate production of DSMZ 10492 and D2 were also consistent with their glucose consumption. This was again expected, as lactic acid is a primary metabolite of *L. plantarum*. Unlike T129 and T8, the glucose consumption and lactate

production profiles of DSMZ and D2 were similar. Glucose was totally consumed by both strains by the 24th hour of cultivation (**Figures 3.48** and **3.49**).

Glucose and lactate concentrations of T129 and T8 during cultivation under stress conditions (6.5% (w/v) NaCl) are shown in **Figures 3.50** and **3.51**, respectively.

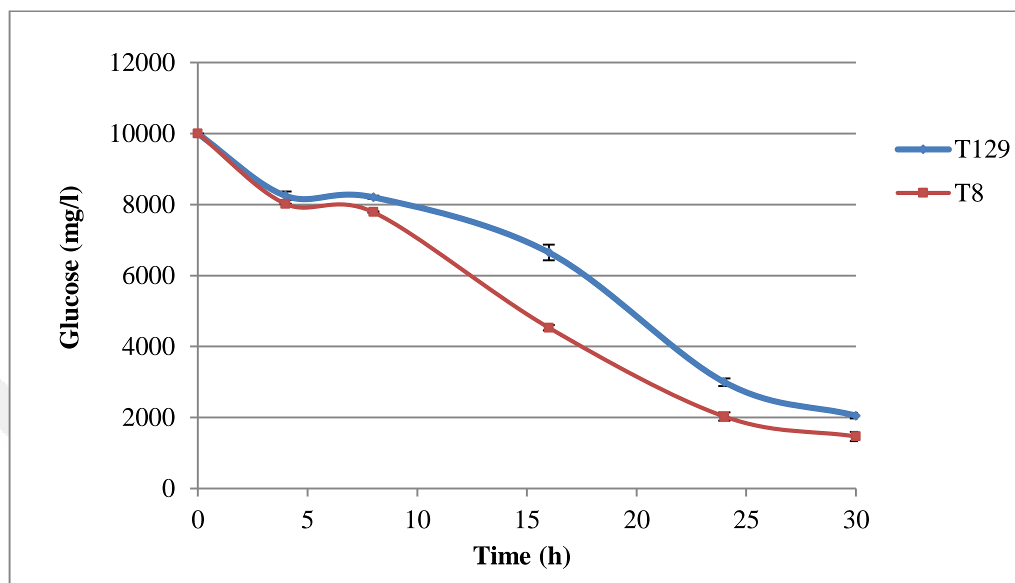

Figure 3.50 : Residual glucose concentrations of T129 and T8 under stress conditions (6.5% (w/v) NaCl).

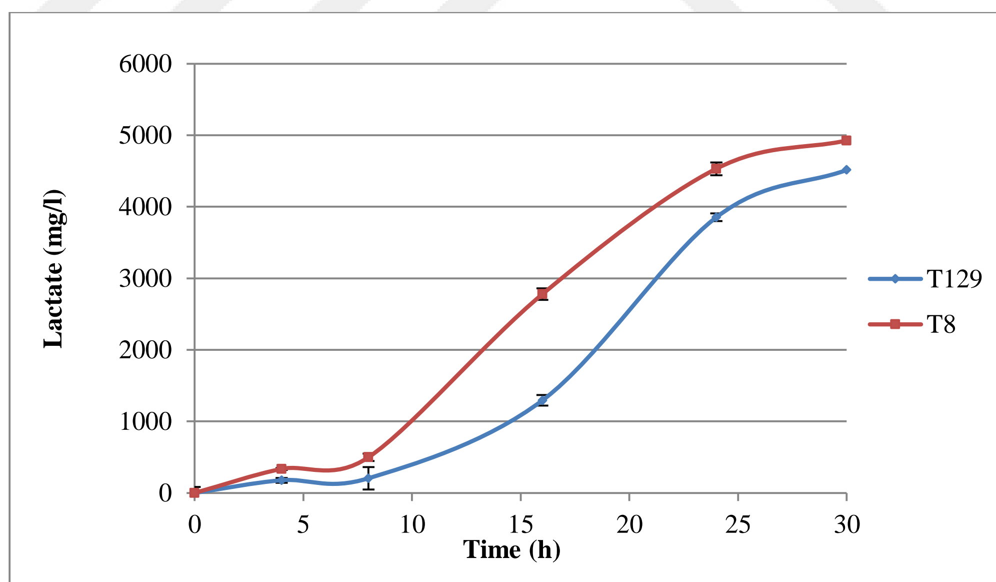

Figure 3.51 : Lactate concentrations of T129 and T8 under stress conditions (6.5% (w/v) NaCl).

It was observed that the presence of NaCl stress had an inhibitory effect on glucose consumption and lactate production in both strains. However, this inhibitory effect was less than T129 in the mutant strain T8, as the mutant has increased resistance to NaCl stress (**Figures 3.50** and **3.51**), respectively.

Glucose and lactate concentrations of DSMZ 10492 and D2 under stress conditions (6.5% (w/v) NaCl) are given in **Figures 3.52** and **3.53**, respectively.

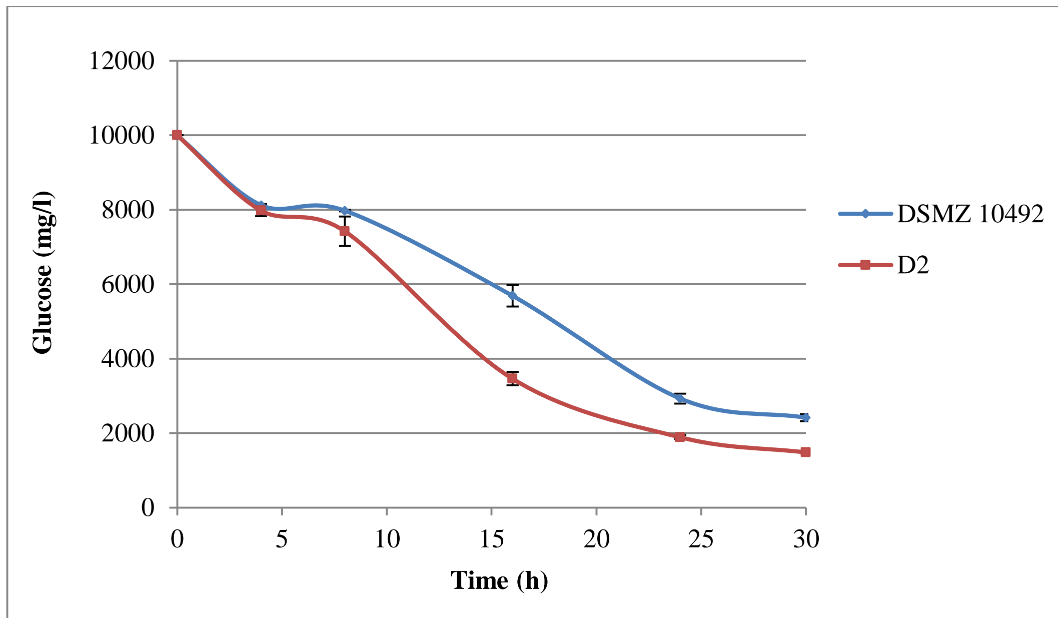

Figure 3.52 : Residual glucose concentrations of DSMZ 10492 and D2 under stress conditions (6.5% (w/v) NaCl).

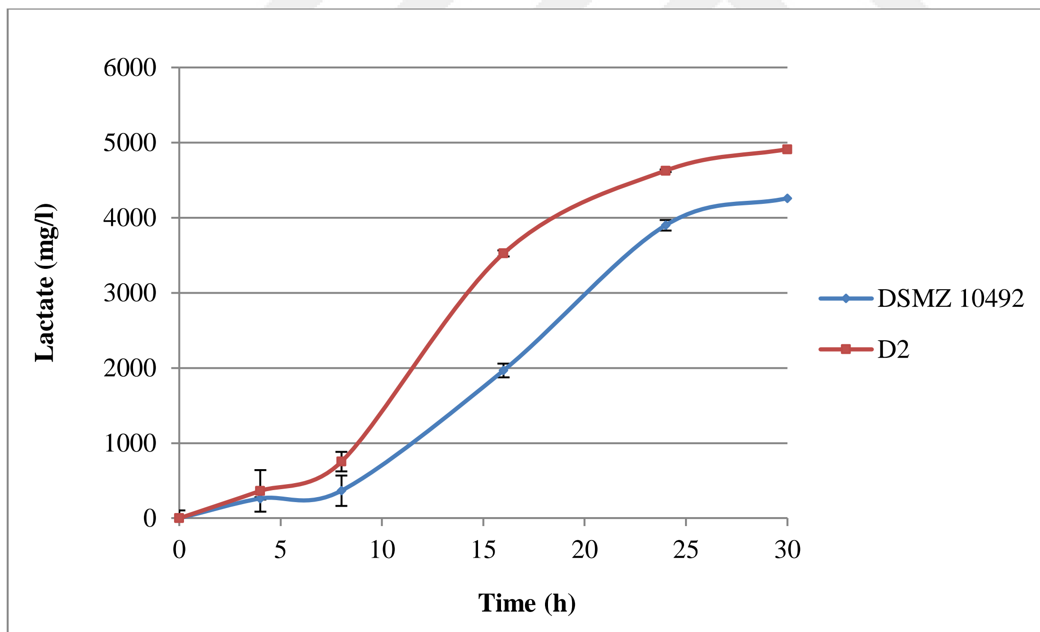

Figure 3.53 : Lactate concentrations of DSMZ 10492 and D2 under stress conditions (6.5% (w/v) NaCl).

Similar to the results obtained with T129 and T8, the NaCl-resistant mutant strain D2 was less inhibited by NaCl stress than its wild type DSMZ 10492, based on its glucose consumption and lactate production results (**Figures 3.52** and **3.53**).

3.2.7 Genetic stability of mutant individuals

The genetic stabilities of the NaCl-resistant mutants were determined by their serial cultivation under non-selective (NaCl stress-free) conditions and plating serial dilutions of each culture passage on MRS and 7% (w/v) NaCl-containing MRS. It was observed that neither T129 nor DSMZ 10492 wild type strains were able able to grow on 7% (w/v) NaCl-containing MRS agar. The NaCl-resistance of the successive cultures (passages) of T8 and D2 is shown in **Figures 3.54** and **3.55**, respectively.

The NaCl resistance of T8 and D2 did not show a decreasing trend during successive cultivations under non-selective (stress-free) conditions (**Figures 3.54** and **3.55**). This implies that the NaCl resistance of the mutant individuals are most likely due to the accumulated mutations and not due to a transient adaptation.

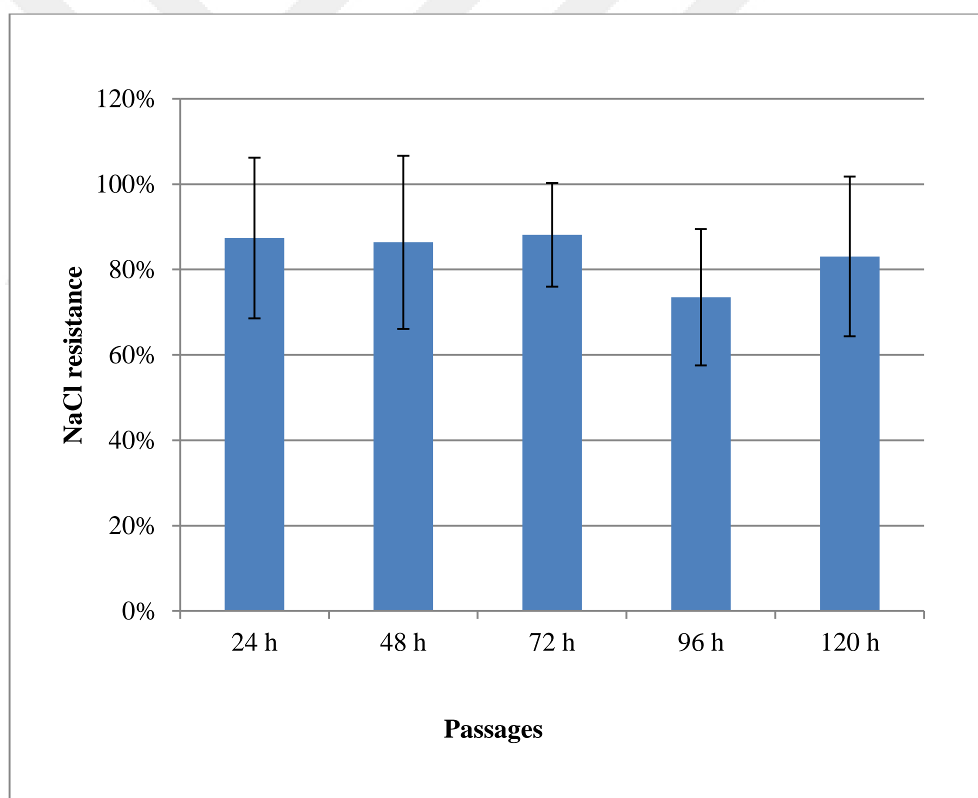

Figure 3.54 : NaCl resistance of successive 24h-cultures (passages) of the mutant strain T8.

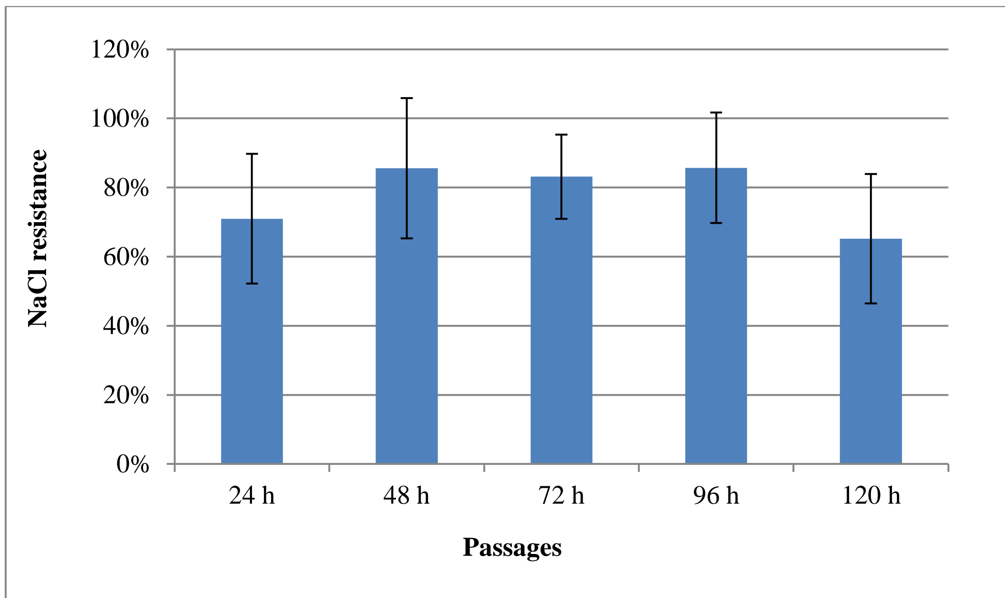

Figure 3.55 : NaCl resistance of successive 24h-cultures (passages) of the mutant strain D2.

3.3 Engineering Lactic Acid Bacteria to be Used as Probiotic Culture

3.3.1 Survival of the wild type strain T129 during the passage through the upper digestive tract (TIM-1)

Cumulative delivery of *L. plantarum* T129, after introduction of the meal is shown in **Figure 3.56.** The survival of *L. plantarum* T129 strain was moderate (59.7%) (**Figure 3.56**). The standard deviation was about 10%. The survival rate decreased after 240 minutes of ingestion under physiological conditions, as observed before (Marteau et al., 1997). But the total cumulative survival continued at a lower rate until the end of the experiment. 4.6 ± 0.6 % of the ingested bacteria were recovered in the intestinal residues at the end of the experiment, after 360 minutes of ingestion.

The survival of *L. plantarum* T129 was determined as 59.7 % (Öztürk et al., 2016). It has been demonstrated that TIM-1 system gives comparable results to *in vivo* survival studies (Marteau et al., 1997). Although it is not possible to make a direct comparison due to different gastric emptying half times and meal composition between this study and that of Marteau and colleagues, this survival rate is definitely higher than those of the common yoghurt bacteria and comparable with the survival of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* (Marteau et al., 1997). The unpublished survival rates for different *L. plantarum* strains at the same conditions were 61.3% for a known probiotic strain and 32.1 % for a *L. plantarum* strain

isolated from a plant source. *L. plantarum* T129 showed a similar survival rate to the probiotic *L. plantarum* strains tested in the TIM-1 *in vitro* model under the same conditions (K.Venema, personal communication).

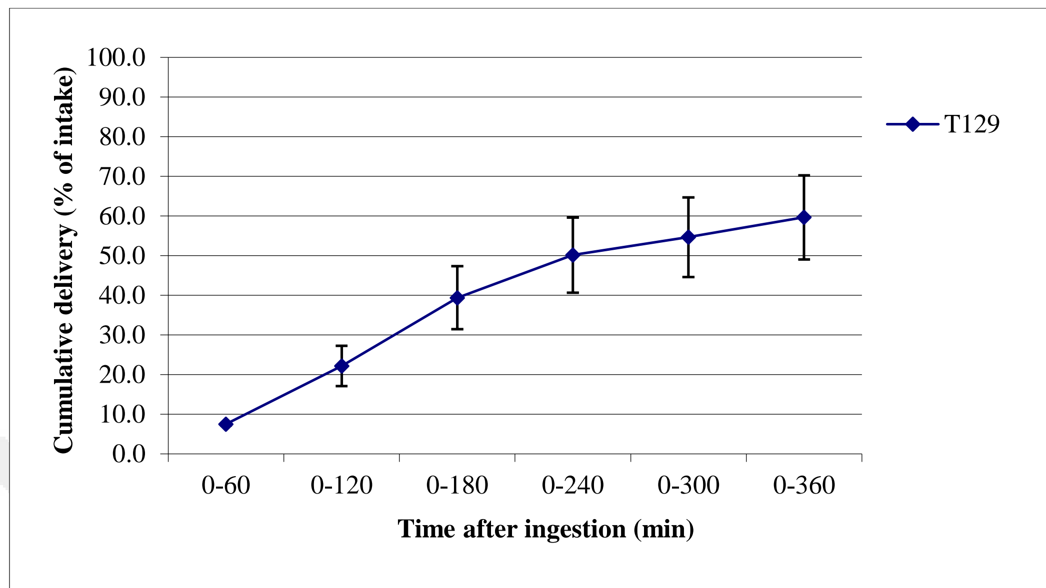

Figure 3.56 : Cumulative delivery of *L. plantarum* T129, after introduction of the meal (Öztürk et al., 2016).

L. plantarum strains showed a survival between 0.003-10% in static experiments where strains were first subjected to hydrochloric acid treatment at pH 2 and consequently treated with bile salts (Haller et al., 2001). 7 ± 2% of *L. plantarum* NCIMB 8826 fed to humans were recovered at ileum (Vesa et al., 2000), and this was 8% in TIM-1 (unpublished results). It is difficult to compare the survival of *L. plantarum* from different studies due to the difference in methods, such as meal matrix, growth conditions, etc. But it was clearly demonstrated that the isolated *L.plantarum* T129 has a survival rate comparable to or perhaps even higher than those of the known probiotic strains.

L. plantarum has a relatively high number of genes and an ability to adapt to diverse environments (de Vries et al., 2006). Although *L. plantarum*-containing health products are mainly being marketed as capsules and beverages, *L. plantarum* is also encountered in fermented plant products, dairy products and meat products (de Vries et al., 2006). This would make a probiotic *L. plantarum* strain easily applicable to a variety of products without altering product characteristics.

L. plantarum has a long history of safe use in human consumption. It has also been shown that no *L. plantarum* was present in blood and heart, 96 h after inter-venous

admission of 10^8 cfu of *L. plantarum* 299v strain to Sprague-Dawley rats. Thus, *L. plantarum* 299v strain did not pose a risk even if it could pass the intestinal barrier (Adawi et al., 2002).

Different strains of *L. plantarum* have been associated with health benefits in adult healthy volunteers. Tested strains were associated with increased short chain fatty acid in faeces, an up to six times reduction in carriage of faecal *enterobacteriacea*, or a reduction in LDL, cholesterol and fibrinogen (Johansson et al., 1998; Kingamkono et al., 1999; Naruszewicz et al., 2002). *L. plantarum* was also associated with positive outcomes in clinical trials, as well. Different *L. plantarum* strains were helpful in reducing symptoms of the Irritable Bowel Syndrome or decreasing the reoccurrence of *Clostridium difficile*-associated diarrhoea (Niedzielin et al., 2001; Wullt et al., 2003). Although *L. plantarum* strains are associated with many positive effects on human health, probiotic traits are strain-specific and need to be determined by double blind placebo controlled cross-over studies. *L. plantarum* T129 has a superior survival rate in TIM-1 and a potential in decreasing counts of enterococci due to its demonstrated *in vitro* antimicrobial activity, likely due to the production of a bacteriocin, although this needs to be confirmed in future studies.

3.3.2 EMS mutagenesis

The rifampicin-resistant variant strain of T129 (T129 Rif^r) was subjected to EMS mutagenesis. EMS mutagenesis was used to increase the genetic diversity of the initial population that will be exposed to TIM-1 selection. The survival rate of the strain upon EMS mutagenesis decreased with increasing EMS concentrations. The survival rates of the strain T129 Rif^r upon exposure to 0, 0.19, 0.37 and 0.72 M EMS for 30 min are given in **Table 3.25.** 0.19 M EMS was insufficient to get the desired survival rates, whereas 0.72 M EMS caused too low survival rates. Thus, the mutant population obtained upon 0.37 M EMS exposure was used in TIM-1 selection.

Table 3.25 : The survival rates of the rifampicin- resistant T129 Rif^r strain upon exposure to 0, 0.19, 0.37 and 0.72 M EMS for 30 min.

	Survival rate (%)			
Strain	0 M EMS	0.19 M EMS	0.37 M EMS	0.72 M EMS
T129 Rif^r	100.00	78.85	78.27	0.06

Ibrahim and O'Sullivan (2000) used chemical mutagenesis for the isolation of food-grade β-galactosidase-overproducing mutants of *Bifidobacterium*, *Lactobacillus* and *Streptococcus thermophiles* strains by MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) or EMS mutagenesis. They demonstrated the possibility to use both chemical mutagenesis strategies with similar effectiveness to increase enzyme activity by 137% for *L. delbrueckii ssp. bulgaricus*; 104% for *S. thermophilus*, 70% for *Bifidobacterium breve*; and 222% for *Bifidobacterium longum* mutants. Genetically altered organisms by mutagenesis are not considered as genetically modified organisms according to Regulation (EC) No 1829/2003 Article 2-5. Mutagenesis is a technique listed in the Annex 1B referred by Article 3-1 in the Directive 2001/18/EC. This study demonstrates the possibility to alter the activity of a single enzyme by chemical mutagenesis. Application of multiple selective stress conditions such as acid and bile salt stress in gastrointestinal tract can give rise to mutants with increased survival rate. Bron et al. (2004) demonstrated that expression of certain genes of *L. plantarum* WCFS1 changed during gastrointestinal passage in mice. Although it is known that EMS mutagenesis is effective in altering enzyme activity and certain gene expression rates are effective at increasing the survival rate of *L. plantarum*, chemical mutagenesis was applied in this thesis study for the first time to select *L. plantarum* mutants resistant to multiple stress conditions during gastrointestinal passage. This was possible by the use of a dynamic *in vitro* model of the upper gastrointestinal tract (TIM-1).

3.3.3 Selection of mutant populations of T129 Rif^r via TIM-1 system

The cumulative survival of each succesive reintroduction of the mutant populations of *L. plantarum* T129 Rif^r is shown in **Figure 3.57**. Repeated reintroduction of the EMS-mutagenized population of *L. plantarum* T129 Rif^r resulted in an increased survival until the fourth reintroduction of the ileal delivery sample taken between 180 and 240 min.

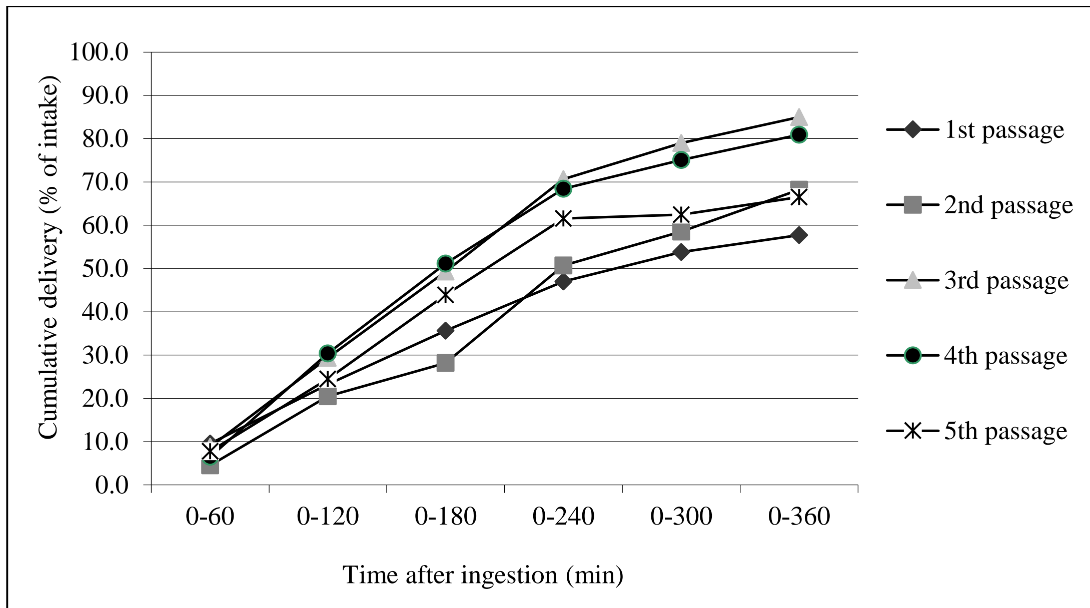

Figure 3.57 : Cumulative survival of repeatedly reintroduced mutant populations of *L. plantarum* T129 Rif^r in the TIM-1 system.

The initial survival rate of the EMS-mutagenized population was in the range of the survival rates obtained for the mother strain (57.7%). Reintroduction of ileal efflux sample taken between 180-240 min gave rise to mutant populations with increased survival. Although the survival rate between the first and second reintroduction was not significantly different (p=0.832), the survival rate significantly increased after the third reintroduction (p=0.003). The survival rate peaked at the third and fourth reintroduction and was 85.0 and 80.9%, respectively. The survival rate at the fifth reintroduction significantly decreased (p=0.020) and was 66.5. Although the survival rate in the cumulative ileal efflux decreased at the end of the experiment, the bacteria in the intestinal residue continued to increase until the fifth reintroduction. The bacteria in the intestinal residue were 6.2% of the intake at the first introduction of the EMS-mutagenized population. The bacteria ratio in the intestinal residue to the intake increased to 11.3, 8.7, 13.4 and 16.3% of the consequent second, third and fourth reintroduction of the ileal efflux to the TIM-1 system.

The number of CFUs in the intestinal residue increased during reintroduction of the mutant populations to the TIM-1 model. This increase indicates an adaptation to the intestinal environment and even slight growth despite the presence of low nutrient concentrations and bile salts. These increases in intestinal residue made it possible to obtain cumulative survival rates over 100%.

The intermediate cultivation of strains gave rise to unusual phenotypes and the domination of clumping strains (data not shown). It has previously been shown that human probiotic strains, *Lactobacillus paracasei* NFBC 338 and *Bifidobacterium* sp. strain UCC 35612 formed clumps which caused difficulties in direct counting and plate counting method (Auty et al., 2001). It is expected that the genetic diversity of the mutant population has rapidly decreased after the first passage through the TIM-1 system, since the selection pressure was high. Thus, every passage caused approximately 1 log decrease in survival. Unsuccessful experiments revealed that cultivation of mutant populations resulted in clumping colonies. Thus, highly surviving mutants were sequentially selected with minimal growth. Mutant populations only found a limited opportunity to grow between serial reintroduction steps into the TIM-1 system. The major opportunity to grow was in the small intestine and in the meal. It was expected that genetic recombination and accumulation of mutations were limited due to the limited availability of resources.

Mouse or human models could not be used due to the need to obtain relatively uncontaminated cultures at high amounts and the need for the continuous reintroduction of ileal efflux. The survival of *L. plantarum* T129 Rif^r increased from 57.9 to 90% at the end of the selection experiment. A similar strategy could also be used to develop potential probiotic strains of other microorganisms or adapt certain microorganisms to certain food matrices that cause problems in probiotic product development.

3.3.4 Isolation of individual colonies

Thirteen colonies were isolated at the end of selection experiments. Ten colonies were isolated from the ileal efflux samples and three colonies were isolated from the incubated residues of jejunum. Isolated colonies did not clump when grown on MRS+rif broth.

3.3.5 Selection of best surviving mutant strain by tube-based experiments

Thirteen mutants of the T129 Rif^r strain were isolated during these studies. Thirteen strains were too much to assay at the TIM-1 system. Thus, a more practical tube-based static protocol was employed. The survival data of the selected mutants are given in **Table 3.26.**

The results of the tube-based experiments deviated from the results previously obtained with the TIM-1 system. The survival of T129 Rif^r was determined as 59.7 % in the TIM-1 system. Although the results were not consistent with the results obtained with TIM-1, they were still useful to make an initial selection of the more promising mutants. I6 was selected as it had a superior survival rate in the tube-based experiments. I8 showed the second highest survival rate among all isolates from the ileal efflux. I1 was selected as it had a survival rate close to that of T129 Rif^r and a low chance to be a related to I6 and I8. J-1 and J-2 had similar survival rates and could be genetically very similar due to the highly selective conditions of the jejunal environment. Thus, only J-1 were selected from these two mutants. J-3 was also selected as it had the highest survival rate among all mutants isolated from the incubated residue of the jejunum.

Table 3.26 : Survival of the mutant strains as percentage of stress-free control in tube-based static experiments.

Strain	Survival %
Control of T129-Rif^r (without stress)	100.00%
T129-Rif^r	72.93%
TIM T129 ileal efflux strain-1 (I1)	26.48%
TIM T129 ileal efflux strain -2 (I2)	10.47%
TIM T129 ileal efflux strain -3 (I3)	0.61%
TIM T129 ileal efflux strain -4 (I4)	17.02%
TIM T129 ileal efflux strain -5 (I5)	16.03%
TIM T129 ileal efflux strain -6 (I6)	471.19%
TIM T129 ileal efflux strain -7 (I7)	14.28%
TIM T129 ileal efflux strain -8 (I8)	84.58%
TIM T129 ileal efflux strain -9 (I9)	31.99%
TIM T129 ileal efflux strain -10 (I10)	56.81%
TIM-1 T129 jejunal efflux strain-1 (J1)	118.46%
TIM-1 T129 jejunal efflux strain -2 (J2)	139.49%
TIM-1 T129 jejunal efflux strain -3 (J3)	171.82%

3.3.6 Antimicrobial activity of the selected individuals

Probiotic bacteria are expected to confer some antimicrobial activity against potential pathogens, such as *Enterococci*, in the human gut (Szachta et al., 2011). The antimicrobial activity of selected strains was assessed by testing their ability to suppress the growth of 10 *enterococcal* indicator strains. The antimicrobial activity is given as the diameter of the inhibition zone in mm.

The antimicrobial activities of five selected mutant individuals are shown in **Table 3.27.**

Table 3.27 : Antimicrobial activities of mutant individuals against indicator *Enterococcus* strains (Öztürk et al., 2016).

Strain	*Enterococcus* Indicator Strain Number									
	259	138	173	251	395	551	632	282	83	74
	Inhibition zone diameter (mm)									
L.lactis TTC 03.0262 (positive control)	3	1.5	4	3	5	7	2	1.5	6.5	1.5
DSM 10492	3	6	9	3	5	0	0	7	0	7
T129	5	6	5	0	2	0	0	10	0	6
I1	7	5	3	3	3	0	0	8	0	3
I6	2	4	0	8	1	0	0	7	0	0
I8	6	4	2	5	4	0	0	8	0	2
J1	6	5	5	10	1	0	0	9	0	2
J3	6	3	5	8	3	0	0	9	0	4

3.3.7 Survival of the selected J1 mutant strain and its genetic stability

Strain T129-J1 was selected as a potential probiotic mutant strain as it was able to grow in the jejunal residue and showed high survival in the static tube-based selection experiments. J-1 also showed high survival in the TIM-1 system. This indicates a high possibility that J-1 strain will also survive in the human gastric passage and show potential probiotic effects. The cumulative survival of *L. plantarum* J1 mutant and its survival after cultivation in stress-free conditions are given in **Figure 3.58**.

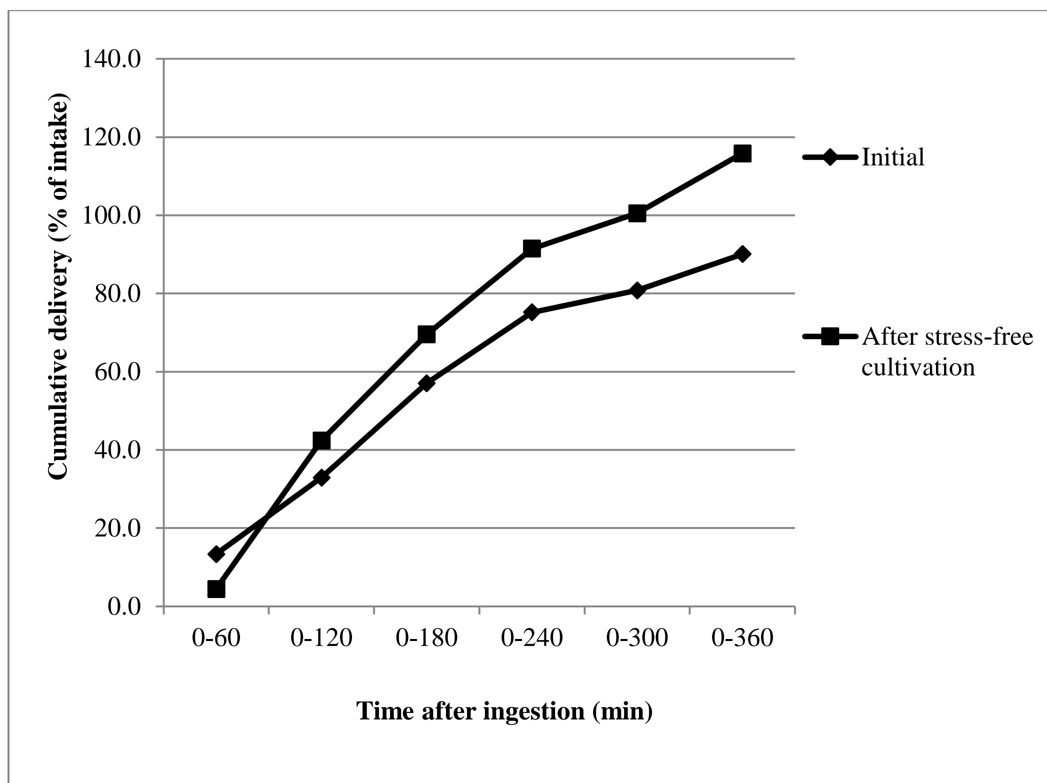

Figure 3.58 : Cumulative delivery of *L. plantarum*-J1 from stock and after stress-free cultivation.

The increase in the survival of *L. plantarum* mutant strain J1 through the gastric passage despite preculturing for 88h in stress-free conditions indicates its genetic stability.

3.3.8 Growth physiological analyses of the wild type strain T129 and its selected individual mutant J1

The wild type T129 strain and its mutant J1 were cultivated in shake flasks containing 6.5% (w/v) NaCl-containing 50% diluted MRS or 50% diluted MRS. Their growth was followed by OD_{580} and CDW measurements. Their substrate utilization and lactic acid production rates were determined by HPLC measurements.

3.3.8.1 OD_{580} measurements of cultivated cells

The OD_{580} nm data of T129 and its mutant strain J1 grown in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth are given in **Table 3.28.**

Table 3.28 : OD_{580} of T129 and its mutant J1 in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth.

Sampling time (h)	50% diluted MRS	50% diluted MRS + 6.5% (w/v) NaCl

	T129	J1	T129	J1
2	0.002±0.002	0.002±0.001	0.0864±0.01	0.084±0.013
4	0.012±0.002	0.022±0.004	0.0528±0.013	0.147±0.052
6	0.106±0.01	0.11±0.012	0.074±0.016	0.185±0.019
8	0.346±0.007	0.429±0.065	0.076±0.01	0.265±0.019
10	1.074±0.038	1.252±0.063	0.141±0.013	0.359±0.051
12	1.838±0.106	2.123±0.129	0.191±0.033	0.819±0.016
14	3.124±0.005	3.226±0.089	0.351±0.07	0.866±0.067
16	3.616±0.055	3.719±0.112	0.452±0.027	1.273±0.049
18	4.435±0.059	4.458±0.041	0.557±0.044	1.496±0.032
20	4.590±0.061	4.682±0.064	0.748±0.064	1.657±0.064
22	4.956±0.132	4.864±0.303	0.968±0.043	1.774±0.1
24	4.900±0.073	4.781±0.212	1.174±0.05	1.791±0.02
26	-	-	1.37±0.046	1.902±0.063
28	-	-	1.397±0.011	1.918±0.092
30	-	-	1.378±0.027	1.905±0.076

The growth curves of T129 and its mutant J1 grown in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth are given in **Figure 3.59** and **3.60**, respectively.

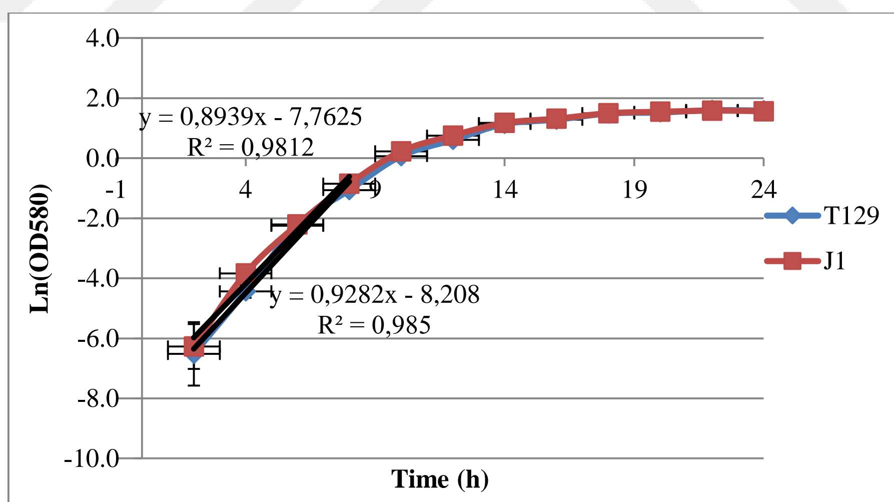

Figure 3.59 : Growth curves of T129 and its mutant J1 in 50% diluted MRS broth.

The maximum specific growth rate (μ_{max}) of T129 and its mutant strain J1 were calculated using the ln(OD_{580}) versus time graph. Under stress-free conditions, the slope of the trend lines in the exponential phase of growth yielded the maximum specific growth rate (μ_{max}) of T129 and its mutant J1 as were 0.928 h^{-1} and 0.894 h^{-1}, respectively. The generation times of these strains were calculated as 1.080 h and

1.116 h, respectively. The maximum specific growth rates of the wild-type and the mutant strain J1 were similar. Apparently, the mutations in J1 did not cause a decreased growth rate under stress-free conditions (**Figure 3.59**).

The maximum specific growth rates of T129 and its mutant J1 under NaCl stress conditions were calculated using the corresponding ln(OD_{580}) versus time graph (**Figure 3.60**). The maximum specific growth rate (μ_{max}) of T129 and its mutant strain J1 were 0.205 h^{-1} and 0.239 h^{-1} in 6.5% (w/v) NaCl-containing MRS broth, and their generation times were 4.878 h and 4.184 h, respectively. The mutant individual had a shorter lag phase than its wild-type and reached higher final OD_{580} values during cultivation. Additionally, its μ_{max} value was higher than that of the wild-type strain.

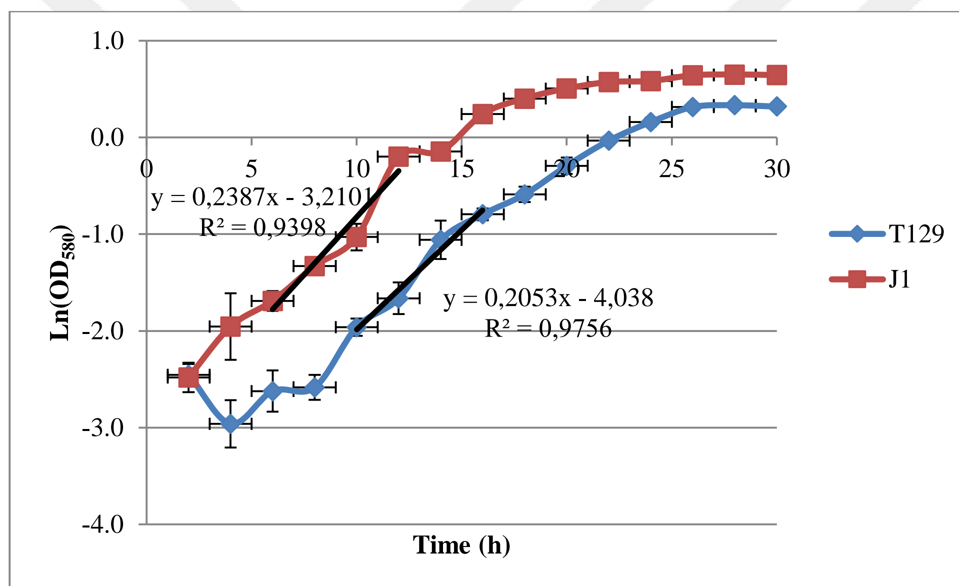

Figure 3.60 : Growth curve of T129 and its mutant J1 on 6.5% (w/v) NaCl-containing 10% diluted MRS broth.

Mutant J1 might have developed multiple mutations that modified the cell wall structure or improved cellular repair processes, as this strain was selected in an *in vitro* model of the upper gastrointestinal tract, where it faced various stress conditions such as acid bile and nutrient deprivation stresses.

3.3.8.2 CDW Measurements of T129 and its mutant strain J1

The CDW measurement results of T129 and its mutant strain J1 in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth are given in **Table 3.29.** The CDW profiles of T129 and its mutant J1 during growth in 50% diluted MRS broth and 6.5% NaCl-containing 50% diluted MRS broth are shown in **Figures 3.61** and

3.62, respectively. As expected, CDW data were consistent with OD_{580} data for the growth of T129 and its mutant strain J-1 in 50% diluted MRS and 6.5% (w/v) NaCl-containing 50% diluted MRS.

Table 3.29 : CDW (g/l) of T129 and its mutant T8 in 50% diluted MRS broth and 6.5% (w/v) NaCl-containing MRS broth.

Sampling time (h)	50% diluted MRS		50% diluted MRS + 6.5% (w/v) NaCl	
	T129	J1	T129	J1
2	0.033±0.058	0.067±0.058	0.067±0.058	0.067±0.058
4	0.067±0.058	0.1±0	0.067±0.058	0.133±0.058
6	0.067±0.058	0.067±0.058	0.067±0.058	0.167±0.058
8	0.3±0.1	0.267±0.153	0.033±0.058	0.2±0.1
10	1.067±0.231	1.233±0.252	0.1±0.1	0.3±0.1
12	2±0.1	2.567±0.153	0.267±0.058	0.6±0.1
14	2.933±0.058	3.167±0.153	0.433±0.153	0.733±0.058
16	3.7±0.3	4.667±0.306	0.5±0.1	1.233±0.058
18	4.767±0.252	4.767±0.208	0.6±0.1	1.433±0.231
20	4.7±0.173	4.767±0.115	0.767±0.058	1.6±0.1
22	4.8±0.265	4.7±0.173	0.967±0.115	1.667±0.153
24	5±0.2	4.7±0.265	1.333±0.152	1.767±0.153
26	-	-	1.333±0.115	1.933±0.153
28	-	-	1.5±0.1	1.9±0
30	-	-	1.4±0.173	1.933±0.115

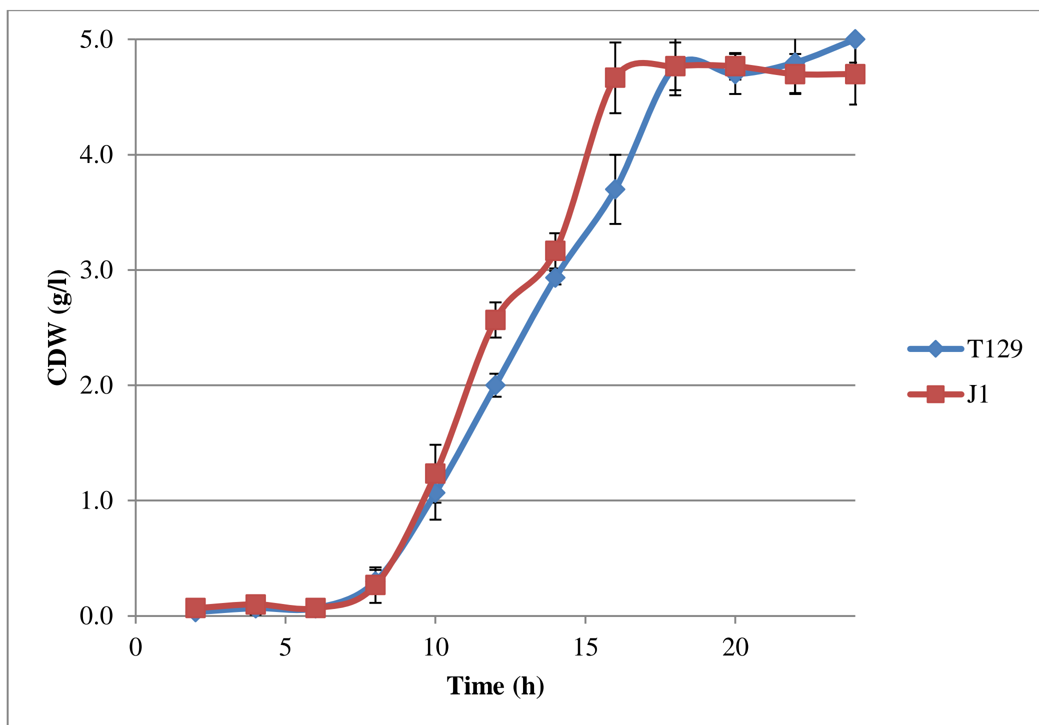

Figure 3.61 : CDW profiles of T129 and its mutant strain J1 during growth in 50% diluted MRS broth.

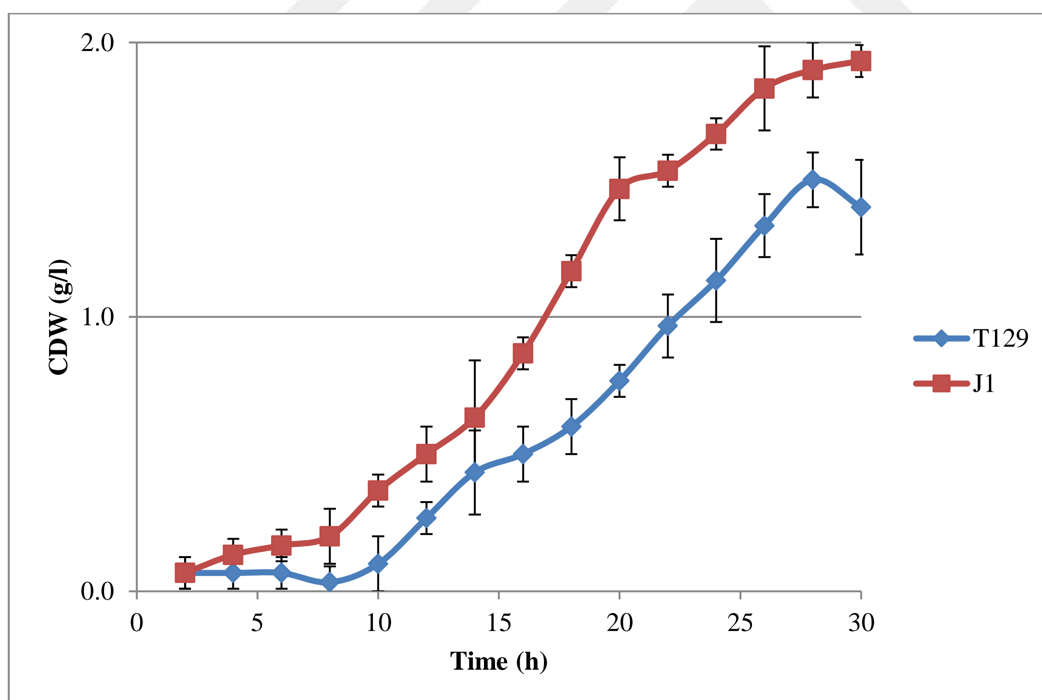

Figure 3.62 : CDW profiles of T129 and its mutant strain J1 during growth in 6.5% (w/v) NaCl-containing 50% diluted MRS broth.

3.3.8.3 Metabolite analyses of T129 and its mutant strain J1 by high performance liquid chromatography (HPLC)

Culture supernatants collected during growth physiology experiments with T129 and J1 were analysed for their glucose and lactate contents by HPLC. Glucose and lactate concentrations of T129 and J1 under control conditions are shown in **Figures 3.63** and **3.64**, respectively.

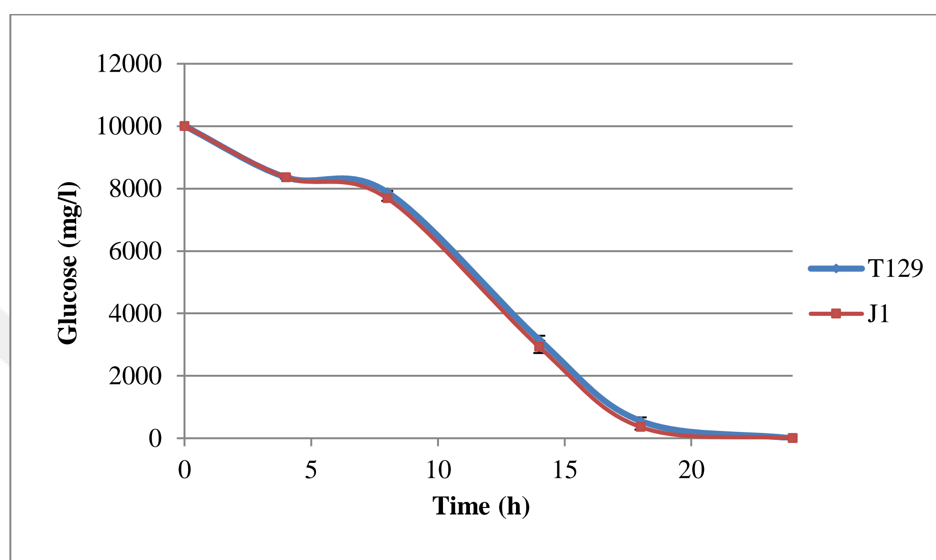

Figure 3.63 : Residual glucose concentrations of T129 and J1 under control conditions.

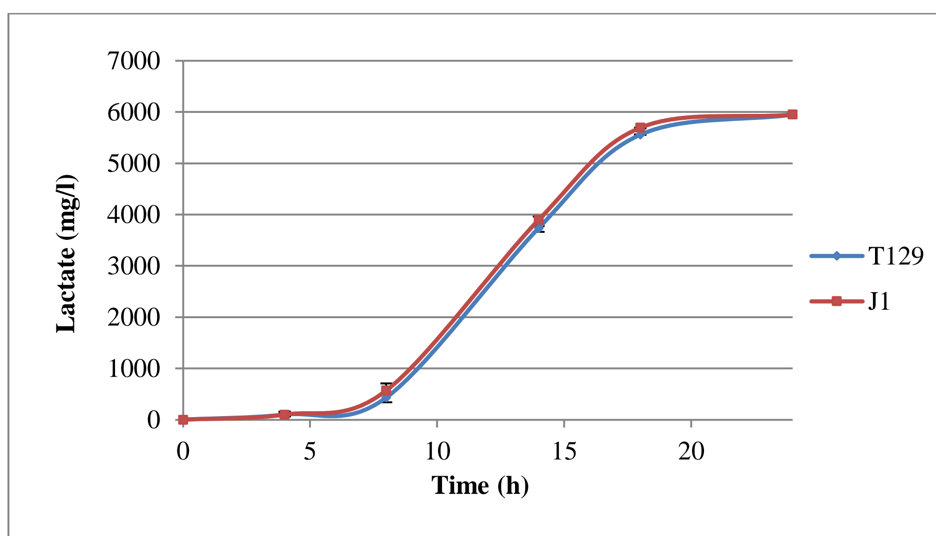

Figure 3.64 : Lactate concentrations of T129 and J1 under control conditions.

The glucose consumption and lactate production profiles were also consistent with OD_{580} data for both strains. The mutations in J1 did not cause a decreased growth rate under stress-free conditions. Both wild-type and the mutant J1 consumed all available glucose by the 20th hour of the experiment. Glucose depletion resulted in a

decrease in the growth rates of both strains followed by their entry into the stationary phase. The lactate concentrations up to 6000 mg/l did not have a significant effect on the growth of both T129 and its mutant J1.

Glucose and lactate concentrations of T129 and J1 during growth physiology experiments under 6.5% (w/v) NaCl stress are given in **Figures 3.65** and **3.66**, respectively.

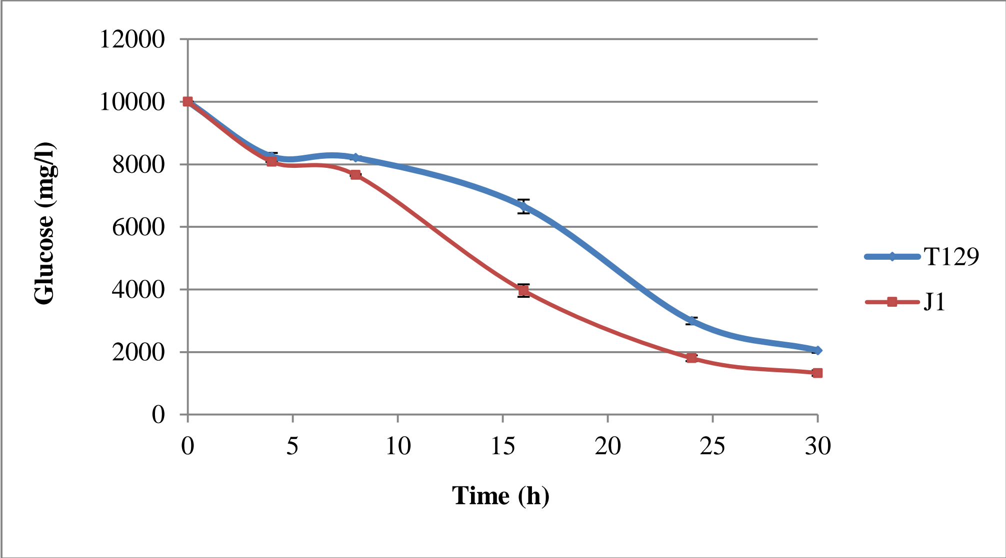

Figure 3.65 : Residual glucose concentrations of T129 and J1 under 6.5% (w/v) NaCl stress condition.

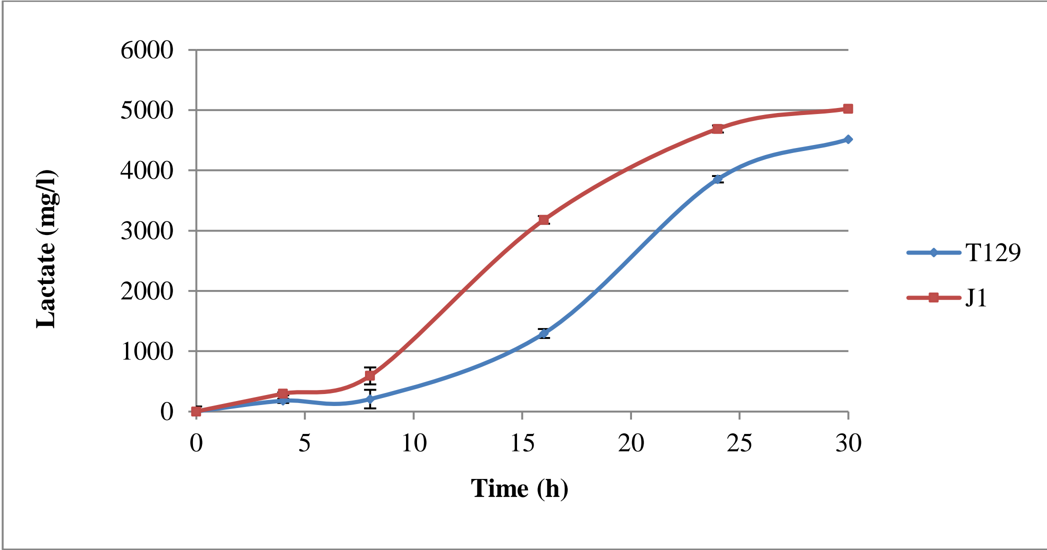

Figure 3.66 : Lactate concentrations of T129 and J1 during growth experiments on 6.5% (w/v) NaCl.

Lactate production was consistent with glucose consumption. This was again expected, as lactic acid is the primary metabolite of *L. plantarum*. The growth of the wild-type strain T129 was most likely suppressed by the combined effect of NaCl and the increasing acidity due to lactic acid production. The wild-type strain could grow up to 400 mg/l lactate in the presence of 6.5% (w/v) NaCl stress conditions, while it could grow up to 600 mg/l lactate under stress-free conditions. The growth of the strain J1 was not inhibited at 500 mg/l lactate present in 6.5 % (w/v) NaCl stress conditions. This could be due to the multiple stress resistance of J1, including both NaCl and acidity (lactate) stress.

3.3.9 Investigation of the genetic mechanism of increased survival in J1 strain

The complete genomes of T129 Rif^r wild-type and its mutant strain J1 were sequenced in order to investigate the genetic mechanism of increased survival of J1 during gastric passage.

Although the sequencing quality of the NGS run seemed reasonable for downstream analyses, genomic assembly and annotation, second FastQ pair of the J1 strain had relatively low quality sequences. All sequences were thus quality trimmed by the same protocol . Subsequent to the quality trimming process, 16975812 and 13373049 read pairs were remained in total for the wild type and J1 strains, respectively.

According to the first method, based on the equation $C = LN / G$, coverage was calculated to be ~ 995. ($G = 3603563$, $L = 110$, $N = 16297801 \times 2$)

In the second method, different coverage estimates were computed by adjusting the k-mer length. k = 21 was selected among others, since the estimated genome size was calculated as closest to the genome size of the reference strain (3603563 bp), by using this k-mer length. The coverage values were estimated to be 435x and 333x, by this method, for the two strains respectively.

Further bioinformatic analyses were performed to determine the variations between both genomes, and 32 point mutations were determined. The list of the 32 transversions found in the mutant strain J1 is given in **Table 3.30.**

Table 3.30 : The list of the 32 transversions found in the mutant strain J1.

Transversions	Contig	Nucleotide position from 5' position	Wild-Type	Mutant	Quality Score
			Leading strand(+) (5'- 3')		
1	chrNODE_95	23328	C	T	999.0
2	chrNODE_95	63361	C	T	999.0
3	chrNODE_418	8539	C	T	999.0
4	chrNODE_418	133579	G	A	999.0
5	chrNODE_470	94445	C	T	999.0
6	chrNODE_499	32042	G	A	999.0
7	chrNODE_499	61027	G	A	999.0
8	chrNODE_525	28130	C	T	999.0
9	chrNODE_525	103351	C	T	999.0
10	chrNODE_546	58486	C	T	999.0
11	chrNODE_795	20261	G	A	999.0
12	chrNODE_795	66956	G	A	999.0
13	chrNODE_795	72096	G	A	999.0
14	chrNODE_795	202729	C	T	999.0
15	chrNODE_86182	176372	G	A	999.0
16	chrNODE_861	198041	G	A	999.0
17	chrNODE_861	203591	G	A	999.0
18	chrNODE_861	239036	C	T	999.0
19	chrNODE_861	239565	C	T	999.0
20	chrNODE_940	83961	C	T	999.0
21	chrNODE_940	91359	C	T	999.0
22	chrNODE_940	105271	C	T	999.0
23	chrNODE_940	190697	G	A	999.0
24	chrNODE_941	124173	G	A	999.0
25	chrNODE_941	141495	C	T	999.0
26	chrNODE_942	5605	C	T	999.0
27	chrNODE_1032	70726	G	A	999.0
28	chrNODE_1032	77660	G	A	999.0
29	chrNODE_1192	7162	G	A	999.0
30	chrNODE_1192	123832	G	A	999.0
31	chrNODE_1192	132017	G	A	999.0
32	chrNODE_1192	150730	G	A	999.0

3.3.9.1 Genome annotation

A specialized genome annotation software was used to determine the expressed regions of the genome. The web-based Maker Genome Annotation software was used (http://weatherby.genetics.utah.edu/cgi-bin/mwas/maker.cgi). The genome annotations were consistent with the results of the manual nucleotide BLAST search. Ten point mutations were determined to be within the expressed regions of the annotated genome. These mutations are given in **Table 3.31**.

Table 3.31 : List of transversions at annotated genes

Transversion	Position of the Gene	Description	Reference
2	* - strand (G →A)	Phosphoribosylformylglycinamidine synthase subunit PurL	WP_003642590.1
5	- strand (G →A)	Glutamine ABC transporter, permease protein	WP_003644017.1
8	+strand (C →T)	DNA repair protein RecN	WP_003640355.1
9	+strand (C →T)	Ribosome small subunit-dependent GTPase A	WP_003640424.1
15	- strand (C →T)	Molecular chaperone DnaK	WP_003640711.1
16	- strand (C →T)	DNA-directed DNA polymerase III, alpha chain PolC-type	WP_003640729.1
17	- strand (C →T)	Phosphatidate cytidylyltransferase	WP_003640734.1
19	* +strand (C →T)	Cell surface protein	WP_003643429.1
21	- strand (G →A)	Cardiolipin synthetase	WP_015381020.1
23	- strand (G →A)	ATP-dependent zinc metalloprotease FtsH	WP_003643854.1

(*) +strand and – strand refer to leading strand and lagging strand, respectively.

3.3.9.2 Potential effects of the mutations on the protein structure in J1

Transversion-2

Transversion 2 resulted in a transition from G to A at the 681st nucleotide of the phosphoribosylformylglycinamidine synthase subunit *purL* gene. This changed the codon from GCG to GCA. Both GCG and GCA code for alanine and their codon usage was found to be quite close with 22.2‰ and 21.2‰, respectively (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=220668). Thus, this

transversion is not expected to result in a change in the translation of this gene (Angov, E. 2011).

221 R D G I N G **A** S F A S

Mutant 661 CGT GAC GGC ATT AAC GGT GC**A** TCC TTT GCC TCT

221 R D G I N G **A** S F A S

Wild-type 661 CGT GAC GGC ATT AAC GGT GC**G** TCC TTT GCC TCT

Transversion-5

Transversion 5 resulted in a transition from G to A at the 385th nucleotide of the glutamine ABC transporter, permease protein gene. This changed the codon from GCA to ACA. While GCA is coding for alanine in the wild-type, ACA is coding for threonine in the mutant.

121 A N G M T Y W Q **T** M S K

Mutant 361 GCT AAT GGG ATG ACG TAC TGG CAG **A**CA ATG AGT AAA

121 A N G M T Y W Q **A** M S K

Wild-type 361 GCT AAT GGG ATG ACG TAC TGG CAG **G**CA ATG AGT AAA

Domain search of this protein revealed that this mutation is not directly at the active site, but only one amino acid away from the dimer interface and three amino acids away from the ABC ATPase subunit interface. Smart BLAST Search revealed that this residue is not highly conserved: this residue is replaced by serine in *L. pentosus* a bacterium closely related to *L. plantarum*. Further studies would be necessary to determine the effect of this point mutation on glutamine transport activity.

Transversion-8

Transversion 8 resulted in a transition from C to T at the 349th nucleotide of the DNA repair protein *recN* gene. This changed the codon from CCT to TCT. While CCT is coding for proline in the wild-type, TCT is coding for serine in the mutant strain.

107 H Q E L M Q **S** E K H

Mutant 331 CAT CAA GAG TTG ATG CAA **T**CT GAA AAA CAC 360

107 H Q E L M Q **P** E K H

Wild-type 331 CAT CAA GAG TTG ATG CAA **C**CT GAA AAA CAC 360

BLAST domain search of this protein revealed that this mutation is at the active site of the Q- loop\lid region. Proline is an iminoacid and has no available H for hidrogen bond formation. Proline or glycine also play a crucial role in α helix formation, since the N-Cα backbone cannot rotate. It is common that proline residues result in a bend in the protein secondary structure (Nelson and Cox, 2000). Thus, it is highly possible

that this mutation has altered the function of Q-loop\lid domain of the Rec N protein. The proline residue is highly conserved among *Lactobacillales*. But it is not conserved among *Bacillales*. The Q-loop/lid region is responsible for ATP hydrolysis activity in the RecN protein, which forms dimers that stabilize the DNA and probably increase the affinity of DNA ligase by binding double-stranded broken DNA. It has been observed that *Bacillus subtilis* RecN has a modest APTase activity which could be in parallel with the altered Q-loop/lid region. Altered ATPase activity would have resulted in a RecN protein with decreased activity in the mutant (Reyes, E. D. 2011), (http://biocyc.org/gene?orgid=ECOLI&id=EG10831).

Transversion-9

Transversion 9 resulted in a transition from C to T at the 193rd nucleotide of the ribosome small subunit-dependent GTPase A gene. This changed the codon from CCG to TCG. While CCG is coding for proline in the wild-type, TCG is coding for serine in the mutant strain.

	60	P	A	A	F	**S**	A	V	G	D	W
Mutant	181	CCC	GCG	GCA	TTT	**T**CG	GCG	GTT	GGG	GAT	TGG
	60	P	A	A	F	**P**	A	V	G	D	W
Wild-type	181	CCC	GCG	GCA	TTT	**C**CG	GCG	GTT	GGG	GAT	TGG

BLAST domain search of this protein revealed that this mutation is not directly at the active site. It is at the RNA binding domain of the ribosome small subunit-dependent GTPase A gene. It is 16 amino acids away from the RNA binding site. Smart BLAST search revealed that this proline residue is highly conserved among different bacteria groups of firmicutes. The transition probably radically altered the secondary structure of this protein (http://biocyc.org/gene?orgid=ECOLI&id=G7841-MONOMER).

Transversion-15

Transversion 15 resulted in a transition from C to T at the 1215th nucleotide of the molecular chaperone DnaK gene. This changed the codon from AAC in the wild-type to AAT in the mutant. Although both AAC and AAT are coding for asparagine, their codon usage differs. The codon usage of AAC is 17.5 ‰, whereas the codon usage of AAT is 22.6 ‰ (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=220668). The gene expression of the molecular chaperone could have increased in the mutant *L. plantarum* (Angov, E 2011).

401 T A A D **N** Q P A V D

Mutant 1201 ACG GCG GCT GAT AA**T** CAA CCA GCT GTT GAC

401 T A A D **N** Q P A V D

Wild-type 1201 ACG GCG GCT GAT AA**C** CAA CCA GCT GTT GAC

Transversion--16

Transversion 16 resulted in a transition from C to T at the 1409th nucleotide of DNA-directed DNA polymerase III, alpha chain *polC*-type gene. This changed the codon from ACC to ATC. While ACC is coding for threonine in the wild-type, ATC is coding for isoleucine in the mutant strain.

461 E T T T N L T S I **I**

Mutant 1381 GAA ACA ACC ACT AAT TTG ACG AGT ATC A**T**C

461 E T T T N L T S I **T**

Wild-type 1381 GAA ACA ACC ACT AAT TTG ACG AGT ATC A**C**C

Domain search of this protein revealed that this mutation is not directly at the active site, but it is in the exonuclease domain (DNA-polymerase alpha and epsilon chain, ribonuclease T and other exonucleases). It is 28 amino acids away from the active site site of this domain. The partial model structure of *L. plantarum* WCFS1 DNA polymerase III PolC-type from residues 421- 586 is given in **Figure 3.67** (http://www.proteinmodelportal.org/?pid=modelDetail&provider=NYSGXRC&template=2p1jA&pmpuid=1000818856178&range_from=1&range_to=1437&ref_ac=Q88VK2&mapped_ac=Q88VK2&zid=async).

Threonine residues are highly conserved at the 469th and the 471st residues, probably forming hydrogen bonds. The mutation at the 469th residue would thus destabilize the turn at the secondary structure. This structural change could destabilize the exonuclease activity of DNA polymerase III.

Transversion--17

Transversion 17 resulted in a transition from C to T at the 223rd nucleotide of the phosphatidate cytidylyltransferase gene. This changed the codon from CCA to TCA. While CCA is coding for proline in the wild-type strain, TCA is coding for serine in the mutant strain.

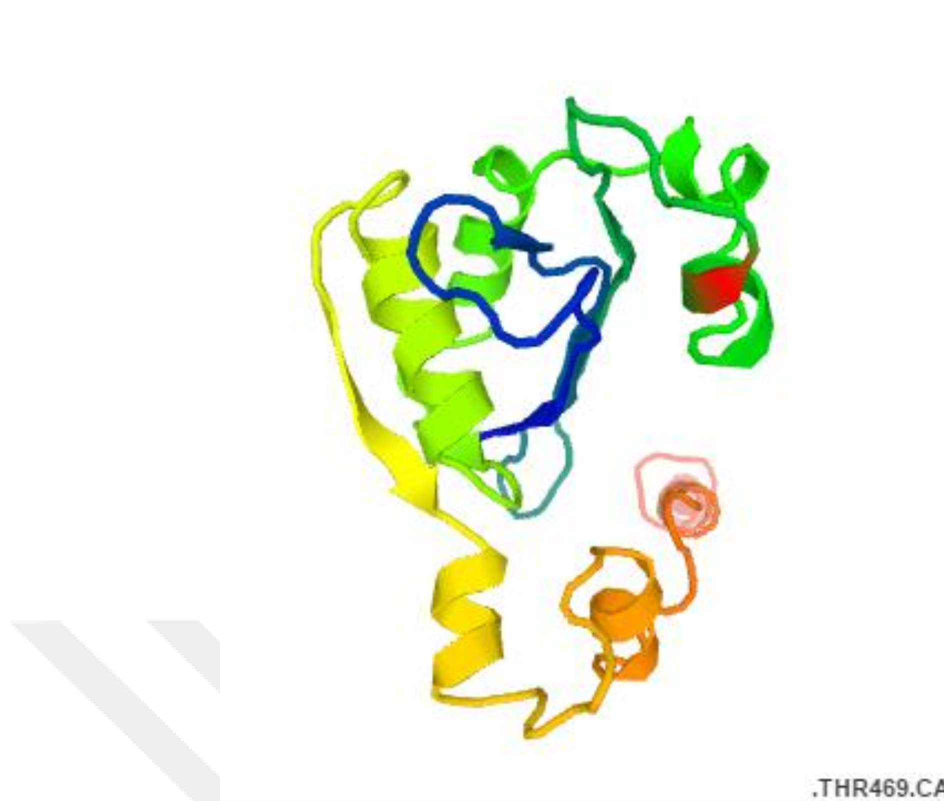

Figure 3.67 : DNA polymerase III PolC-type from residues 421- 586.

	71	F	N	G	L	**S**	D	Q	L	N	K
Mutant	211	TTC	AAT	GGC	TTA	**T**CA	GAC	CAA	TTA	AAC	AAG
	71	F	N	G	L	**P**	D	Q	L	N	K
Wild-type	211	TTC	AAT	GGC	TTA	**C**CA	GAC	CAA	TTA	AAC	AAG

BLAST Domain search did not indicate any active site for this protein, thus it was not possible to interpret the possible effect of this mutation on protein structure at the domain level.

Smart BLAST search revealed that this proline residue is highly conserved among *Lactobacillales*. Although it is not conserved in *Bacillales,* glycine is present in them, suggesting a start of the α helix structure at this point. Thus, this transition has probably radically altered the secondary structure of this protein by destabilizing the consequent α helix structure (Nelson and Cox, 2000).

Transversion--19

Transversion 19 resulted in a transition from C to T at the 308th nucleotide of the ErfK family cell surface protein gene. This changed the codon from ACC to ATC. While ACC is coding for threonine in the wild-type strain, TCA is coding for isoleucine in the mutant.

76 N A **I** P K G D F V I

Mutant 301 AAT GCA A**T**C CCC AAA GGC GAT TTC GTT ATC

76 N A **T** P K G D F V I

Wild-type 301 AAT GCA A**C**C CCC AAA GGC GAT TTC GTT ATC

Although the BLAST domain search did not indicate any active site for this protein and it was not possible to interpret the distance of the mutation to the active site, it revealed that this mutation is at the L,D-transpeptidase catalytic domain of the YkuD super family. The threonine residue is remarkably well preserved among *Bacillales* and even in *Clostridium difficile*. The 3D structure of YkuD transpeptidase from *Bacillus subtilis* is given in **Figure 3.68** (http://www.proteinmodelportal.org/?pid=modelDetail&provider=SWISSMODEL&template=1y7mB&pmpuid=1000801262239&range_from=1&range_to=164&ref_ac=O34816&mapped_ac=O348zid=async). It is difficult to predict the effect of the threonine residue as no specific secondary structure is observable. However, it can be generally predicted that the change of a well preserved amino acid residue would have some effect on protein function.

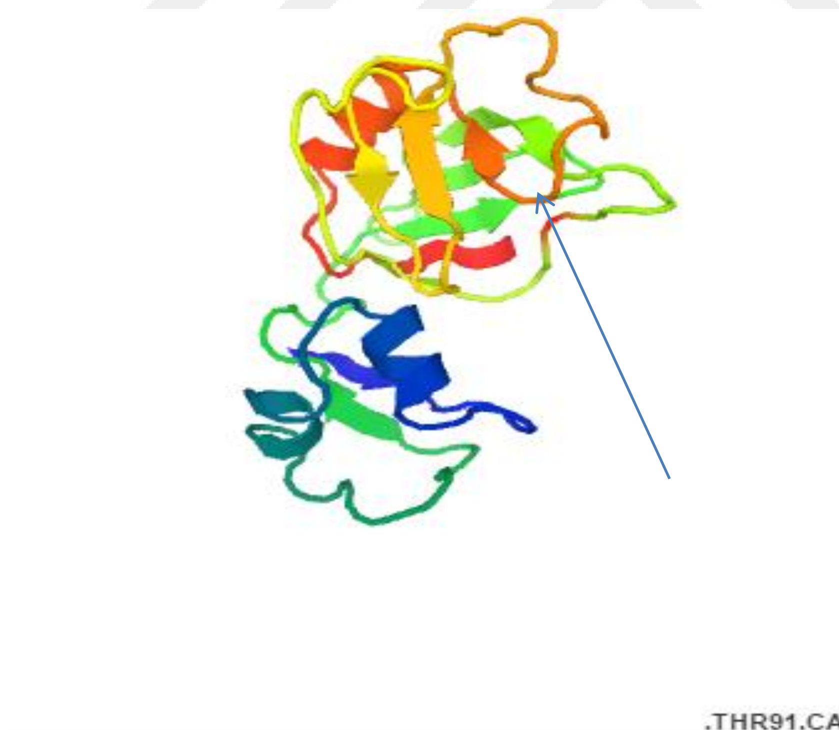

Figure 3.68 : Putative L,D-transpeptidase YkuD from *Bacillus licheniformis* ATCC 14580 strain.

Transversion--21

Transversion 21 resulted in a transition from G to A at the 713th nucleotide of the cardiolipin synthetase gene. This changed the codon from GGT to GAT. While GGT is coding for glycine in the wild-type strain, GAT is coding for aspartic acid in the mutant.

	231	Y	V	G	G	F	N	V	**D**	D	Q	
Mutant	691	TAC	GTC	GGT	GGT	TTT	AAC	GTT	G**A**T	GAC	CAG	720
	231	Y	V	G	G	F	N	V	**G**	D	Q	
Wild-type	691	TAC	GTC	GGT	GGT	TTT	AAC	GTT	G**G**T	GAC	CAG	720

BLAST domain search of this protein revealed that this mutation is not directly at the active site, but only two amino acids away from the catalytic domain, repeat 1, of bacterial cardiolipin synthase domain. **Figure 3.69** demonstrates the mutated residue and active site on model cardiolipin synthase 2 of the *Enterococcus faecalis* T2 strain (http://www.proteinmodelportal.org/?pid=modelDetail&provider=MODBASE&template=1f0iA&pmpuid=1000806633357&range_from=1&range_to=292&ref_ac=C7CXF4&mapped_ac=C7CXF4&zid=async). It is highly possible that the mutation might have resulted in some changes in cardiolipin synthase. Further work has to be done to determine the effect of this mutation on enzyme activity and specificity.

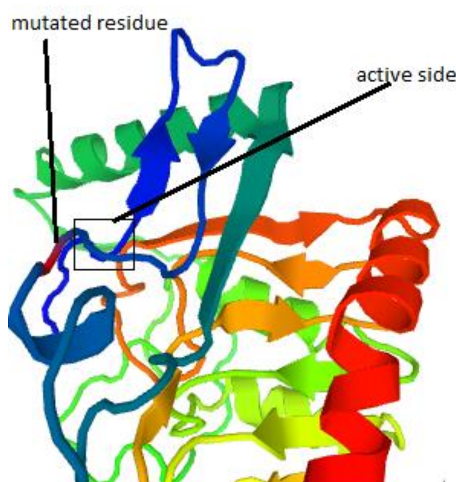

Figure 3.69 : Cardiolipin synthetase 2 of *Enterococcus faecalis* T2.

Transversion--23

Transversion 23 resulted in a transition from C to T at the 1033rd nucleotide of the ATP-dependent zinc metalloprotease *ftsH* gene. This changed the codon from CCT

to TCT. While CCT is coding for proline in the wild-type strain, TCT is coding for serine in the mutant.

	341	D	V	L	D	**S**	A	L	L	R	P
Mutant	1021	GAT	GTA	CTT	GAT	**T**CT	GCC	TTA	CTT	CGT	CCA
	341	D	V	L	D	**P**	A	L	L	R	P
Wild-type	1021	GAT	GTA	CTT	GAT	**C**CT	GCC	TTA	CTT	CGT	CCA

BLAST domain search of this protein revealed that this mutation is at the AAA+ (ATPases Associated with a wide variety of cellular Activities) domain. This mutation is not at the active site of this domain, but it is 7 amino acids away from both the arginine finger and the ATP-binding active site. Thus, it could have possibly affected the structure and the activity of this protein. **Figure 3.70** indicates the position of the conserved proline residue at AAA+ domain in *E. coli* (http://www.ebi.ac.uk/pdbe/entry/pdb/1lv7/protein/1) protein databank.

Proline is an imino acid and is mainly present at the β turn or bending structures between α helix structures (Nelson and Cox 2000). Thus, the mutation would change the conformation of the α helix structure and alter the activity of the FtsH protein.

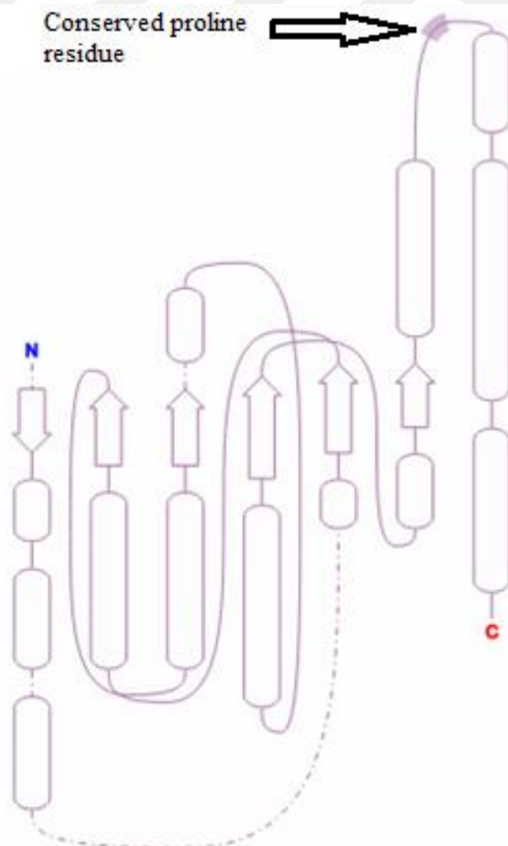

Figure 3.70 : The position of the conserved proline residue in the wild-type AAA+ domain.

3.3.9.3 Proposal of a genetic mechanism for resistance, based on the mutation data

Glutamine ABC transporter, permease protein (GlnP):

Antigen binding cassette (ABC) type transporters are among the largest protein families. ABC type transporters facilitate active import or export of molecules. These active transporters are present in all three domains of life (bacteria, archaea, and eukaryotes). ABC transporters enable transport of ions, sugars, lipids, sterols, peptides, proteins, drug and amino acids at the expense of ATP by bacteria. Three components make up the functional bacterial ABC transporter. These are; two integral membrane proteins, two ATP binding and hydrolysing peripheral proteins, and one periplasmic protein that binds the targeted substrate. The genes that express the proteins required for a functional ABC are present in operons. Glutamine ABC transporter permease protein functions as the membrane proteins of the bacterial glutamine importer (Biemans-Oldehinkel et al., 2006, Xie et al., 2004).

It has previously been reported that cytoplasmic glutamine concentration has a terminal effect on nitrogen metabolism in lactic acid bacteria. It has also been reported that lactic acid synthesis was downregulated during heat stress. It was further observed that lactococci with inactivated glutamine transport genes *glnP* and *glnQ* had strong lactic acid resistance (Xie et al., 2004). Studies done on *L. plantarum* WCFS1 revealed that *L. plantarum* downregulates nitrogen metabolism in the presence of gallic acid stress. Nitrogen metabolism is regulated by glutamine and ammonia levels in the cell. Glutamine synthase *glnA* and *glnR* are in the same operon. Thus, *glnR* is transcribed simultaneously with *glnA*. This operon is self-regulating by feedback inhibition, since GlnR is activated by dimerization and decreases expression of *glnA* and other genes related to nitrogen metabolism (Reverón et al., 2015). Although literature describes a link between decreased GlnP activity and acid stress, and it would make sense that a strain with increased acid stress resistance would have a higher survival during gastric passage; it is not possible to relate increased survival to this mutation since the mutated residue is not preserved well even among *Lactobacillus* species.

DNA repair protein RecN

RecN is responsible for the repair of double stranded DNA by binding DNA. Although several mechanisms of action were proposed, it has been demonstrated that RecN filaments form on DNA that result in bringing DNA in close proximity and facilitate alignment of DNA. It has further been shown that RecN increases the efficiency in DNA ligation by decreasing the occurrence of self-circularization. It is suggested that RecN is able to interact with DNA without the need of ATP but requires ATP for dissociation from the formed RecN DNA complex (Reyes, E. D. 2011). Insertional mutations in *recN* gene were related to increased acid and heat shock survival: *Lactococcus lactis* strains with insertional mutations in the *recN* gene were related to increased acid and heat shock resistance. The increased heat shock resistance of the *recN* mutant was related to increased heat shock protein levels (Rallu et al., 2000).

The mutation is in the *recN* gene, in the region coding for the Qloop/lid region of *recN*. Qloop /loop lid region is responsible for binding ATP which would be needed to dissociate the formed RecN complex from DNA. Thus, it can be concluded that a mutation at the ATP-binding active site of the J1 strain would decrease the RecN complex formation and RecN decomplex forming cycle. Thus, the increased RecN deposition around DNA could have a protective effect and confer stress resistance to cells. But increased stress response by delayed DNA repair could also have an effect, since SOS stress response is activated when the RecA protease forms a filament with single stranded DNA and dissociates the LexA dimer that suppresses SOS response genes (Butala et al., 2011).

Ribosome small subunit-dependent GTPase A

Ribosome small subunit-dependent GTPase A (RsgA) is a protein with GTP hydrolysis activity. The RsgA protein has three structural domains: the oligonucleotide binding (OB) fold binds oligonucleotides or oligosaccharides, the circular domain has GTPase acticity, and the last domain comprises of a zinc-binding motif. RsgA is active in ribosome maturation. Studies made with *E. coli* showed that *rsgA* deletion mutants were more salt-resistant than the wild-type but had poorer growth in stress-free conditions. This phenomenon was explained by the presence of different GTPases in the cell and that the use of alternative GTPases could be

advantageous for the cell depending on the culture conditions. It could be assumed that under stress conditions, the J1 mutant might have benefited from the *rsgA* gene (Hase et al., 2009).

DNA-directed DNA polymerase III, alpha chain PolC-type

DNA polymerase III is responsible for bacterial genomic DNA replication. The mutation in the proofreading domain of DNA polymerase would have decreased the proofreading activity of the J1 strain. This mutation could increase the adaptability of the strain to certain stressful environmental conditions by increased failure rate in DNA synthesis. However, it is known that this has not been the case, as all mutations observed were ftom G to A or C to T, as expected for EMS mutagenesis. Another possible mechanism for a disfunctioning exonuclease activity could be the delay in DNA repair and prolonged activation of bacterial SOS response, as proposed for RecN (Timinskas et al., 2013; Echols et al., 1983).

Phosphatidate cytidylyltransferase

Phosphoglycerides make up the majority of bacterial membrane lipids. Sn1 and 2 positions of glycerol are esterified with a fatty acid and Sn-3 position is esterified with a phosphate group in the simplest form of phosphoglycerides. The synthesis of phosphoglycerides starts with the activation of the Sn-3 position with CTP. Phosphatidate cytidylyltransferase catalyses the transfer of a CDP group to the Sn-3 position of a 1,2-diacyl-sn-glycerol 3-phosphate and releases a phosphate. The CMP group is released by further enzymes and yields a phosphorylated lipid. The GDP ester of 1,2-diacyl-sn-glycerol is also an important building block of polyglycerol phosphate polymers found in Gram-positive cell membrane. The phosphatidate cytidylyltransferase and the conserved initial steps in lipoteichoic acid synthesis are described in **Figure 3.71** (Chapot-Chartier and Kulakauskas, 2014; Percy and Gründling 2014).

The commonly shared initial steps of teichoic acid synthesis show great variation among species. Thus, a decreased activity in phosphatidate cytidylyltransferase would have an impact on bacterial cell wall structure. The resulting cell wall structure could have increased the resistance of the mutant strain during passage through the upper digestive tract.

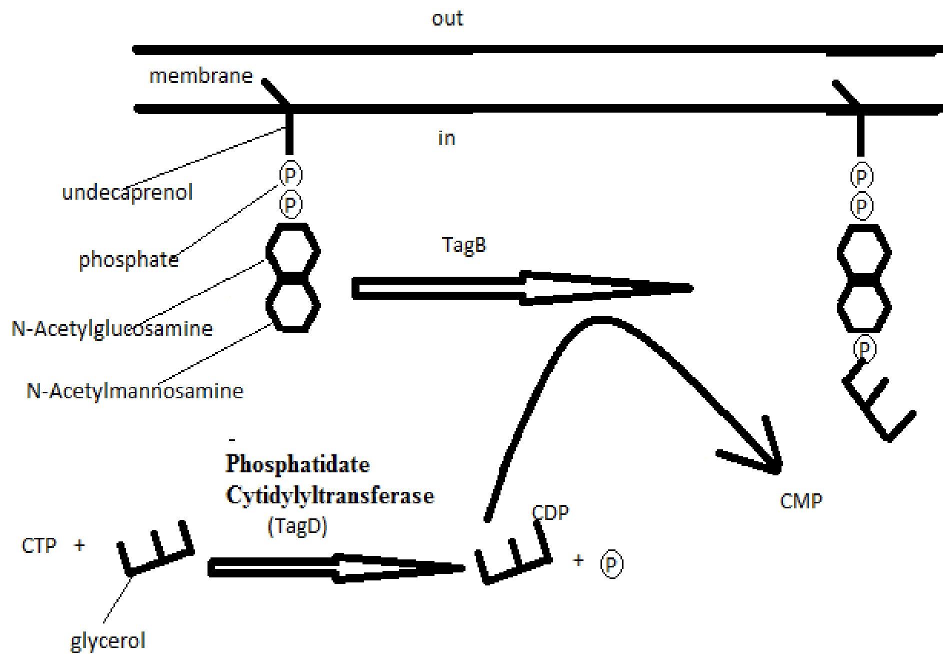

Figure 3.71 : The Phosphatidate cytidylyltransferase and the conserved initial steps in lipoteichoic acid synthesis.

ATP-dependent zinc metalloprotease FtsH

This cell surface protein degrades membrane proteins with a 10-20 amino acids long nonpolar destabilizing tail by starting hydrolysis at either C or N terminus of the protein. Thus it acts as a quality control mechanism for membrane proteins. It is also active by protecting the cell, as it also hydrolyses phage proteins. FtsH also has a function in regulating lipopolysaccharide (LPS) levels in *E. coli* (Ogura et al., 1999). But lactic acid bacteria are Gram-positive and do not produce LPS. Thus, it has been reported thet FtsH is not essential in Gram-positive bacteria but markedly decreases stress resistance in *B. subtilis*. Although it is known that FtsH has also a regulatory function in Gram-positive bacteria, the mechanism is still not fully elucidated (Deuerling et al., 1997). It has been shown that *ftsH* mutations are lethal in *E. coli* due to overproduction of lipopolysaccharides and that this effect could be reverted by a mutation in phosphatidate cytidylyltransferase (Ogura et al., 1999). Thus, FtsH could also have an effect on the phosphatidate cytidylyltransferase activity in lactic acid bacteria.

Bove et al. (2012) have demonstrated that FtsH overproduction or inactivation both resulted in increased bile salt stress tolerance and decreased biofilm formation ability due to changes in cell wall composition. The increased survival of the mutant J1 strain during the passage through the gastrointestinal tract could be partially due to

the increased bile resistance conferred by this mutation. The absence of clumping in J1 strain could also be attributed to this mutation. The J1 strain grows similar as wild type in stress-free conditions, which could be due to the protective effect of a second mutation, as previously reported for *E. coli* by Ogura et al. (1999). The hypothesis that both mutations have resulted in a more stress-resistant cell wall structure should be investigated further.

ErfK family cell surface protein

ErfK could be active in peptidoglycan cross-linking in *L. sakei* and *Enterococcus faecium* (Hüfner et al., 2007). A mutation in *erfK* could also have an effect on peptidoglycan cross-linking in the J1 strain. This change in cross-linking could have increased the stress resistance of J1 strain. Studies made with *L. sakei* showed that peptidoglycan cross-linking is regulated in stressful environments during meat fermentation (Hüfner et al., 2007).

It should further be investigated if mutations in phosphatidate cytidylyltransferase, ATP-dependent zinc metalloprotease *ftsH*, and cell surface protein *erfK* confer an increased survival of *L. plantarum* during passage through the upper gastrointestinal tract or if they form a more resistant cell membrane and cell wall in combination.

Cardiolipin synthetase

The phosphatidyl group of a phosphatidylglycerol is transferred to a second phosphatidylglycerol by cardiolipin synthetase. This enzymatic reaction results in the formation of diphosphatidylglycerol (cardiolipin). Phosphatidate cytidylyltransferase is active in the synthesis of phosphatidylglycerol. Thus, it is highly possible that these two mutations both affect cardiolipin synthesis in the cell. Intracellular cardiolipin synthesis is specially related to osmotic stress in prokaryotes. It has been demonstrated that cardiolipin synthase has an effect on bacterial phospholipid composition (Romantsov et al., 2009). This mutation could also have an effect on the bacterial cell wall as both cardiolipin synthase and teichoic acid synthesis pathways use the product of phosphatidate cytidylyltransferase. Further studies are required to examine the cell wall and cell membrane structures of *L. plantarum* J1 strain to better understand the mutation that has led to an increased survival.

Interestingly, four genes with functions in cell membrane and cell wall structure were altered in this mutant strain. This is in fact not unexpected as both bile salt and acid stress would firstly be counteracted by the cellular membrane and cell wall. This

finding also indicates the power of evolutionary engineering in the optimization of complex cellular processes.

3.3.9.4 Determination of the genetic background of the increased rifampicin resistance in the wild type strain

The *rpoB* sequence of rifampicin resistant T129 and its mutant strain J1 were compared. The *rpoB* sequences of both strains were determined to be the same as expected. The obtained *rpoB* gene sequence was BLAST searched and a point mutation was observed in the 1574th residue of the rpoB gene. This point mutation had changed C in all reference *Lactobacillus* strains to T in rifampicin resistant *L. plantarum* T129. This point mutation changed the 525th codon from CCT to CTT. This mutation changed the translation of this codon from P to L (Goldstein, 2014).

This mutation was expected as rifampicin is known to inhibit transcription by binding the β subunit of RNA polymerase. Most of rifampicine resistant mutants belonging to various species were reported to have a point mutation in cluster I covering aminoacids between 507th to 533rd of the *rpoB* gene (Goldstein, 2014).

4. CONCLUSIONS AND RECOMMENDATIONS

Olives are the fruits of the *Olea europaea* tree. These fruits are inedible and bitter mainly due to the presence of an olive polyphenol, oleuropein. Thus, they have to be debittered. This is mainly done by removing the bitterness from the whole fruit to obtain table olives, or the oil from this fruit is pressed to obtain an edible non-bitter oil, since the main bitter compounds are left in the aqueous phase (Rivas et al., 2000, Papoff et al., 1996).

Table olives are processed by two main strategies called the Spanish style and the Californian style. In the Spanish style, the unripe, green olives are treated with lye and oleuropein is broken down. The lye is then rinsed and the debittered olives are fermented mainly by lactic acid bacteria. In the Californian style, the ripe olives are fermented in brine solution anaerobically until the bitterness is removed and then fermented in an aerated or non-aerated milder brine solution by lactic acid bacteria. Although both strategies vary in their ways of removing the bitterness, they both depend on fermentation to gain the desirable properties (Rivas et al., 2000; Brenes et al., 1992; Ciafardini et al., 1994).

Evolutionary engineering enables changes of the microbial genome and metabolism at diverse points and optimizes the function or properties of the microorganism of interest by a nature-driven force. This is quite difficult for rational metabolic engineering which needs extensive genetic and metabolic information about the microorganism to be improved. The only requirement in evolutionary engineering is, however, to determine and use the right selection criteria that are easily screenable and give rise to mutants with the desired phenotypes (Koffas and Cardayre 2005). Thus, evolutionary engineering was used in this study to develop mutant microorganisms with improved resistance against major stress factors observed during olive fermentation processes.

4.1 Resistance of Strains Against Olive Polyphenols and Oleuropein

The first aim of this study was to obtain oleuropein-resistant lactic acid bacteria strains, (preferably *L. plantarum* strains) by using evolutionary engineering approach. During initial screening experiments, it was determined that oleuropein did not inhibit the growth of most of the tested *L. plantarum* strains. It only extended the lag phase of growth in some of the tested strains. Although oleuropein was not inhibitory on its own, it decreased the NaCl resistance of selected strains. Thus, the hypothesis was modified such that NaCl and not oleuropein is the major stress factor during olive fermentation and a strain with high NaCl resistance would also resist the combined stress of NaCl and oleuropein.

4.2 Evolutionary Engineering of NaCl and Oleuropein Resistance in Lactic Acid Bacteria to be Used as Starter Cultures.

Mutant populations obtained by evolutionary engineering of both wild-type strains T129 and DSMZ 10492 grew up to 6.5% (w/v) NaCl. Repeating the same stress conditions at high stress levels revealed to be a good strategy during selection of mutant populations of lactic acid bacteria. After 7.5% (w/v) NaCl, it was no longer possible to obtain high enough survival rates upon 24 h of cultivation. Thus, the cultivation time was increased to 48 h. Increasing the cultivation time along with the application of the same stress conditions for a few successive populations consequently revealed to be a good strategy during selection of mutant populations of lactic acid bacteria resistant to high stress levels. The selection of mutant populations was continued until final mutant populations with 10% (w/v) NaCl resistance were obtained.

Most of the isolated individuals from the final mutant populations were resistant to 10% (w/v) NaCl and oleuropein. However, some mutants derived from DSMZ 10492 were resistant to 10% (w/v) NaCl, but not to 10%(w/v) NaCl and oleuropein. The comparative genomic study of these individuals could reveal the interaction between oleuropein and NaCl resistance mechanism in lactic acid bacteria. It was further observed that some mutant individuals derived from DSMZ 10492 (D2, D3, D4, D5, D7, **Table 3.10**) and T129 (T1, T6, T7, T10, **Table 3.6**) grew faster in 4 g/l oleuropein and 10% (w/v) NaCl than in 10% (w/v) NaCl alone.

Cross-resistance studies were performed to determine the resistance of mutant individuals against other olive processing-related stress factors. The results revealed that resistance to increased NaCl stress did not increase the resistance to other olive-processing-related stress types to the same extent. The mutant individuals had similar resistance levels to their wild-type strain under selected stress conditions. Most strains were resistant against pH 9 and ferrous gluconate, whereas they were more sensitive against pH 4. Some mutants of DSMZ 10492 were sensitive against pH 9. Comparative genomic studies can be performed to better understand the molecular mechanisms of alkali stress resistance in lactic acid bacteria.

T8 and D2 mutant strains were chosen for further physiological analyses, according to their cross-resistance results. The physiological analyses of the mutants and their wild-type strains revealed differences in their growth characteristics. Since T8 grew only slowly under stress-free conditions, it showed some physiological growth advantages in the presence of NaCl stress. D2 strain grew similar as the wild type strain in stress-free conditions, but also preserved its NaCl resistance. Further detailed studies should be performed to compare the genetics of both strains.

4.3 Engineering Lactic Acid Bacteria Against Upper Gastro-Intestinal Stress Using the TIM-1 System.

The second aim of this study was to increase the resistance of lactic acid bacteria against upper gastro-intestinal stress for a more efficient use of these bacteria as probiotics. For this purpose, it is essential to make these experiments in a dynamic *in vitro* model of the stomach and small intestine such as the TIM-1 system, as it is not possible to cultivate strains during selection experiments and it is necessary to reintroduce the whole ileal efflux. The survival of *L. plantarum* J1 strain obtained by the TIM-1 selection was superior to its wild-type strain and it also showed antimicrobial activity against certain *Enterococcus* strains. Physiological studies revealed that J1 strain grew similar as its wild-type strain at stress-free conditions, but it had increased resistance against 6.5% (w/v) NaCl. This increased survival at moderate NaCl stress conditions can be useful in its application with NaCl-containing foods such as olives, pickles or ayran. Further studies have to be performed to determine the applicability and shelf-life of this strain for different foods.

Comparison of whole genome sequences of the wild-type T129 Rif^r and J-1 revealed that the mutations were point mutations, typical to EMS mutagenesis. Genome annotation studies revealed point mutations in the genes coding for phosphoribosylformylglycinamidine synthase subunit PurL, Glutamine ABC transporter, permease protein, DNA repair protein RecN, ribosome small subunit-dependent GTPase A, molecular chaperone DnaK, DNA-directed DNA polymerase III, alpha chain PolC-type phosphatidate cytidylyltransferase, cell surface protein, cardiolipin synthetase, and ATP-dependent zinc metalloprotease FtsH.

BLAST analysis of these mutations may suggest that the stress resistance of J1 might potentially result from two main molecular mechanisms: the modification of the cellular metabolism and the modification of the cell wall structure.

Cellular metabolism of the J1 mutant strain is modified through the accumulation of several mutations. It has previously been shown that the inactivation of the glutamine ABC transporter resulted in an increased acid resistance in *Lactococci* (Xie et.al 2004). Mutations in *recN* ATPase subunit could have delayed the dissociation RecN from DNA and protected DNA from damage. Ribosome small subunit-dependent GTPase was found to be effective in ribosome maturation. Studies in *E.coli* have revealed that NaCl resistance was gained by the activation of alternative ribosome maturation pathways in the absence of ribosome small subunit-dependent GTPase (Hase et al., 2009). Although *E.coli* and lactic acid bacteria have quite different physiologies, the activation of alternative GTPases could also have a protective effect in lactic acid bacteria. The mutation in DNA-directed DNA polymerase III could have prolonged activation of bacterial SOS response (Timinskas et al., 2013; Echols et al., 1983).

The cell wall structure of the mutant strain J1 might have been altered through, mutations in phosphatidate cytidylyltransferase, cell surface protein, cardiolipin synthetase and ATP-dependent zinc metalloprotease *ftsH* genes. All these genes are effective on cell wall composition and structure. Thus, it can be assumed that the cell wall structure has been modified in a way that made the J1 strain more resistant to acid and bile salts. Further physiological studies on the cell wall structure and composition would be needed to test this hypothesis. The effect of the individual mutations can further be studied by preparing and analysing knock-out mutants of T129. Further *in vivo* studies are also required to investigate the probiotic potential of this strain.

To conclude, this study is so far the first application of evolutionary engineering, a powerful strategy to improve microbial properties, to obtain NaCl and oleuropein-resistant robust potential starter cultures for olive fermentation processes. Highly resistant and genetically stable mutant strains were successfully obtained using this non-GMO approach, which is highly acceptable for food processes. Additionally, the use of a dynamic, *in vitro* digestion system model (TIM-1) to evolve lactic acid bacteria for potential probiotic applications was also applied for the first time in this study. The comparative genomic analysis of the mutant strain J1 obtained from the TIM-1 selection and its wild-type strain revealed mutations in various critical genes. To understand the complex molecular mechanisms of stress resistance and robustness in lactic acid bacteria, detailed transcriptomic and proteomic analyses would also be necessary.

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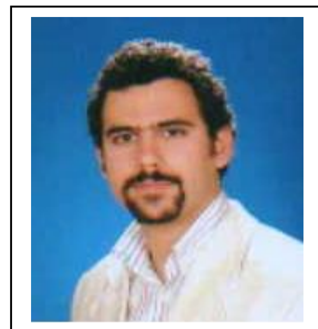
CURRICULUM VITAE



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PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- **Öztürk T**., Venema K., Çakar, Z. P. and Borcakli, M.,2016, (2016). Viability investigation of a potential probiotic olive strain using an *in vitro* digestive system model. *Zeytin Bilimi 6,1*, 19-24
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