EFFICACY OF NATURAL ANTIMICROBIALS ON FOOD-BORNE PATHOGENS AND THEIR APPLICATIONS

A Thesis Submitted to
the Graduate School of Engineering and Science of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Biotechnology

by
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October 2008
İZMİR

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ACKNOWLEDGMENTS

I would like to express my sincere thanks and appreciation to my advisor Assist. Prof. Dr. Ferda SOYER and also to my co-advisor Assist. Prof. Dr. Banu Özen for their patience, guidance and support during my graduate studies. I will always remember them as wonderful advisors, great role models and good friends.

I would also like to thank my graduate committee members; Assist. Prof. Dr. Gülşah Şanlı, Assoc. Prof. Dr. Anne FRARY, Assoc. Prof. Dr. Halil İbrahim ATABAY for their contributions to my thesis.

I would like special thanks to Hasan TÜRE who I have worked with in the Molecular Pathogen Laboratory sometimes without sleeping, sometimes at the weekends and vacations for three years. None of this would have been possible without his help and our team work. I would also like to thank another lab. colleague Hande KARAOSMANOĞLU for her understanding and valuable help and all my graduate friends for their friendship and motivation. You are all truly my friends and colleagues. I will never forget you all.

Finally, I could not find any words to express my gratitude, when it comes to my family. I would like to thank with my all heart to my father Ahmet EROĞLU whom I dedicated my all success to the memory. I would also like to thank my mother Nefise EROĞLU who let me never alone through my life. A special thank to my brother Ercan EROGLU who supported me financially and emotionally during my education, to my sister Emine DERELİ and to her family for their trust in me, patience, love and understanding through the years.

ABSTRACT

EFFICACY OF NATURAL ANTIMICROBIALS ON FOOD-BORNE PATHOGENS AND THEIR APPLICATIONS

Microbial contamination of food products increases the risk of food-borne infections and intoxications. In recent years, more studies have been performed for development of natural alternatives to control the growth of pathogenic microorganisms in food rather than chemical origin. In the present study, natural antimicrobial agents from different sources, activated lactoferrin-(ALF), rosemary extract-(RE), jenseniin-G (JG) and natamycin-(NA) were used.

The antimicrobial activity of agents (ALF-RE-JG) and their combinations against *L. monocytogenes*, *E. coli* O157:H7 and *S.* Enteritidis, and NA against *A. niger* and *P. roquefortii* was tested. These antimicrobials, found effective *in vitro*, applied also on meat and cheese samples. The minimum inhibitory concentrations (MIC) of ALF, RE and JG against pathogens were determined via disc diffusion and microtiter plate growth assay. Dipping method was applied on meat samples to test the efficacy of antimicrobials. MIC of NA was determined via disc diffusion assay. NA was applied on cheese samples to test the efficacy against both molds by dipping method.

The data from microtiter well plate assay showed that ALF is effective on three pathogens. Rosemary extract inhibited only growth of *L. monocytogenes* and JG did not show any significant activity on the growth of same pathogens. RE enhanced the activity of ALF on *E. coli* O157:H7. Neither individual activity nor synergistic activity was determined by disc diffusion assay. The data from disc diffusion assay revealed that the effective NA concentration on both molds was 750 ppm. Some of dipping applications on both meat and cheese displayed promising results.

ÖZET

DOĞAL ANTİMİKROBİYALLERİN GIDA KAYNAKLI PATOJENLER ÜZERİNE ETKİSİ VE UYGULAMALARI

Gıda kaynaklı patojen mikroorganizmalardan kaynaklanan enfeksiyon ve gıda zehirlenmeleri her yıl ölümlere varan ciddi sağlık problemlerinin yanısıra iş kaybına ve buna bağlı olarak ekonomik kayıplara sebep olmaktadır. Günümüzde, gıdalardaki bu patojen mikroorganizmaları yok etmek için çeşitli sentetik antimikrobiyal maddeler kullanılmaktadır. Fakat, bu gibi kimyasal maddelerin kanserojen, allerjenik reaksiyonlar gibi yan etkilere sahip olması doğal kaynaklı koruyucu maddelere olan ilgiyi arttırmıştır. Bu çalışmada, farklı kaynaklardan elde edilen; aktive laktoferrin (ALF), biberiye özütü (BÖ), bir bakteriosin olan Jenseniin-G (JG) ve Natamycin (NA) kullanılmıştır.

Bu çalışmanın amacı, bu doğal maddelerin gıda kaynaklı patojen bakterilere ve gıdalarda bozulmalara neden olan küflere karşı olan etkilerinin belirlenmesidir. Öncelikle, Microtiter plate ve disk difusyon yöntemi ile bireysel olarak etki eden en düşük konsantrasyonları araştırılmış daha sonra etkili olan maddelerin kombinasyonlar halinde etkileşimlerine bakılmıştır. Son olarak, aktivite gösteren maddelerin batırma yöntemi kullanılarak et ve peynir gibi gıda örnekleri üzerindeki antimikrobiyal aktiviteleri test edilmiştir.

Microtiter plate yöntemi ile elde edilen veriler ışığında, ALF' nin *L. monocytogenes*, *E. coli* O157:H7 ve *S.* Enteritidis üzerindeki etki eden en düşük konsantrasyonu sırasıyla; 0.1%, 1% and 0.5%' dir. BÖ sadece 15%'lik konsantrasyon ile *L. monocytogenes* üzerinde etki gösterirken JG her üç patojen üzerinde antimikrobiyal etki sergilememiştir. Fakat, Biberiye özütü ALF ile birlikte test edildiğinde ALF'nin antimikrobiyal özelliğini arttırmıştır. ALF, BÖ, JG tek tek ve bazılarının kombinasyonlar halinde disk difusyon yöntemiyle denenmesiyle bakteriler ve küfler üzerinde antimikrobiyal etki bulunamamıştır. Fakat aynı yöntemle NA her iki küf üzerinde de etkili antifungal aktivite sergilemiştir. Etkili maddeler batırma yoluyla et ve peynirler üzerinde bazı olumlu sonuçlar vermiştir.

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ABBREVIATIONS

E. coli O157:H7	*Escherichia coli* O157:H7
L. monocytogenes	*Listeria monocytogenes*
S. Enteritidis	*Salmonella* Enteritidis
A. niger	*Aspergillus niger*
P. roquefortii	*Penicillium roquefortii*
R. officinalis	*Rosmarinus officinalis*
P. jensenii	*Propionibacterium jensenii*
ALF	Activated lactoferrin
LF	Lactoferrin
JG	Jenseniin-G
RE	Rosemary extract
NA	Natamycin
ETEC	Enterotoxigenic *E. coli*
EIEC	Enteroinvasive *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EPEC	Enteropathogenic *E. coli*
LB	Lauria-bertani
BHI	Brain heart infusion
TSB	Tryptic soy broth
TSA	Tryptic soy agar
XLD	Xylose lysine deoxycholate
SMAC	Sorbitol Mac conkey
CT	Cefixime tellurite
DRBC	Dichloran rose bengal chloramphenicol
OD	Optical density
NCTC	National culture type of collection
ATCC	American type culture collection
RSKK	Refik Saydam Kültür Kolleksiyonu
RSHM	Refik Saydam Hıfzısıhha Merkezi
ABPA	Allergic bronchopulmonary aspergillosis
TTP	Thrombic thrombocytopenic purpura

AFM	Atomic force microscopy
LPS	Lipopolysaccharide
GRAS	Generally Recognized as Safe
WHO	World Health Organisation
FAO	Food and Agriculture Organisation
CDC	Centers for Disease Control and Prevention
USDA	United States Department of Agriculture
FDA	Food Drug Administration
IZTECH	Izmir Institute of Technology
a_w	Water activity
d-water	De-ionized water
l	Liter
ml	Milliliter
µl	Microliter
g	Gram
min	Minute
sec.	Second
rpm	Revolutions per minute
ppm	Parts per million
AU/ml	Activity unit per milliliter
cfu	Colony forming unit
wt/vol	Weight per volume
vol/vol	Volume per volume

CHAPTER 1

LITERATURE REVIEW

1.1. Food-Borne Pathogenic and Spoilage Microorganisms

Consumption of foods contaminated with pathogenic microorganisms or their toxin causes food-borne diseases. Many microorganisms such as bacteria, molds and viruses can grow on foods in mild temperature and spoil the food. Particularly, bacteria can multiply quickly and produce harmful substances or toxic chemicals on foods stored in abused conditions.

There are more than 250 different types of food-borne diseases caused by a variety of bacteria, viruses or parasites (CDC 2008-a). Overall, bacteria are responsible for 30% of all food-borne diseases and 72% of food-borne diseases related deaths. Among bacterial pathogens, in particular, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella* spp. are responsible for the largest number of food borne diseases, cases, outbreaks and deaths (Oussalah, et al. 2007). The highest mortality rate belongs to *Salmonella* spp. with 31% which is followed by *L. monocytogenes* with 28% and *E. coli* O157:H7 with 3%. (Mead, et al. 1999). In the USA, 73000 cases and 61 deaths were reported by USDA-FSIS in 2002 due to *E. coli* O157:H7 infections (Gupta and Ravishankar 2005). According to statistics, only *Salmonella*, *Listeria*, *Campylobacter* and *E. coli* both O157:H7 and non-O157 among the bacterial food-borne pathogens were responsible for 6 million illnesses and nearly 9000 deaths in 1999 (Mead, et al. 1999). Although it is poorly reported, the statistics show that 7875 hospitalization and 324 deaths were caused by food borne illnesses in Turkey in 2002 (Ozelhastaneler 2008).

Many species of fungi including *Aspergillus* spp. and *Penicillium* spp. are utilized in food industry for enzyme and additive production. However these molds are also responsible for food spoilage and mycotoxins production which are poisonous for human on foods (Ray 2001). According to Turkish statistic, *Aspergillus* spp. and *Penicillium* spp. play main role for food spoilage in Turkey (Topal 2004).

1.1.1. Food-Borne Pathogenic Bacteria

1.1.1.1. *Listeria monocytogenes*

Listeria monocytogenes is a small (1-2 μm in length and 0.5 μm in width) Gram-positive, rod shaped (Figure 1.1), non-sporeforming and facultative anaerobic food-bone pathogenic bacteria (Salyers and Whitt 2002). Although the optimum growth temperature is 30-37 ^{o}C, it has ability to grow at low and high temperatures of between 1 to 45 ^{o}C (Singh, et al. 2003). This organism can also multiply readily in aerobic or microaerophilic, salty (100 g/l in medium), relatively low a_w (below 0.93) conditions and in a wide range of pH (4.4-9.6) (Gandhi and Chikindas 2006, Singh, et al. 2003, Low and Donachie 1997, Farber and Peterkin 1991). Most bacteria can not grow under/at the 4 ^{o}C (refrigeration temperature), While *Listeria* can maintain its survival down to the temperature of -7 ^{o}C (Ramaswamy, et al. 2007). Having ability to survive at refrigeration temperature and in minimal nutrients make it dangerous as a food-borne pathogen. *Listeria* can be commonly found and can multiply on plants, in the soil and water, therefore fresh foods can become contaminated with *Listeria* easily at any stage of food growing or processing (Atlas 1995). It is generally isolated from raw meat, soft cheese, ready to eat poultry, unpasteurized milk and milk products, unprocessed vegetables, sandwiches and seafood products (Table 1.3). The bacterium *L. monocytogenes* and the disease listeriosis were first recognized by Murray et al. in animal laboratory in 1924. However, the first listeriosis cases resulted from food-borne

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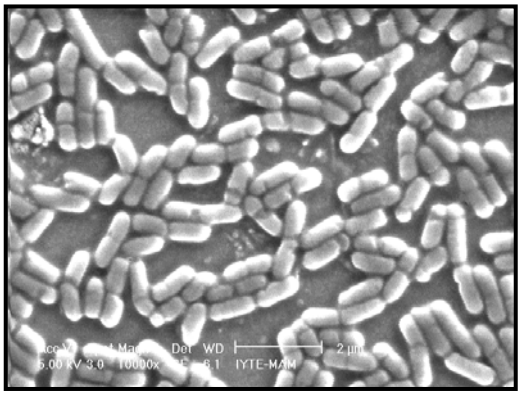


Kingdom:	Bacteria
Phylum:	Firmicutes
Class:	Bacilli
Order:	Bacillales
Family:	Listeriaceae
Genus:	*Listeria*
Species:	*L. monocytogenes*

Figure 1.1. a-) SEM photography of *L. monocytogenes* at the magnification of 10000 b-) Taxonomic classification (Source: NCBI 2008-c).

transmission was not demonstrated until 1981 (McLauchlin, et al. 2004, Meng and Doyle 1998). *Listeria monocytogenes* is capable of growing on foods, in particular on meats, under severe conditions such as acidic, salty, heat and cold environments for long period of time (Madigan, et al. 2003, Singh, et al. 2003, Ye, et al. 2008). Epidemiological studies showed that contaminated foods are the main sources of transmission of listeriosis that is a serious sporadic gastrointestinal food infection. Besides listeriosis, *L. monocytogenes* can cause fatal non-enteric diseases, meningitis, septicemia, miscarriage and encephalitis, especially in the children, pregnant, elderly and immunosuppressive (person with AIDS, cancer, diabetes or leukemia) individuals (Liu 2006, Low and Donachie 1997). Mortality rate and sources of some *L. monocytogenes* outbreaks from different parts of the world between 1980 and 1999 are given in Table 1.1. Once *Listeria* enters the body through intestinal track by digesting contaminated foods, it invades host's monocytes, macrophages or leucocytes and it grows and then spreads to other vital organs such as brain. The incubation period of *Listeria* is so long, from 11 to 70 days (average 31 days). Incidence of listeriosis infection in humans is approximately 0.7 cases per 100000 populations. However, this number is more drastic in infants (10 cases per 100000 population) and the elderly (1.4 cases per 100.000 population) (Salyers and Whitt 2002, Schlech 2000). Despite the low incidence of listeriosis, it has high mortality rate approaching 40%. Recent studies revealed that number of the food stuff contaminated with *L. monocytogenes* is not negligible. Although *Listeria* is susceptible to radiation and heat treatment, it can readily contaminate the raw foods or processed foods during handling due to widespread distribution of this pathogen (Singh, et al. 2003, Madigan, et al. 2003). The best way to fight with listeriosis is periodic surveillance of food producers and education of consumers and employees in food manufactory (Gilot, et al. 1997).

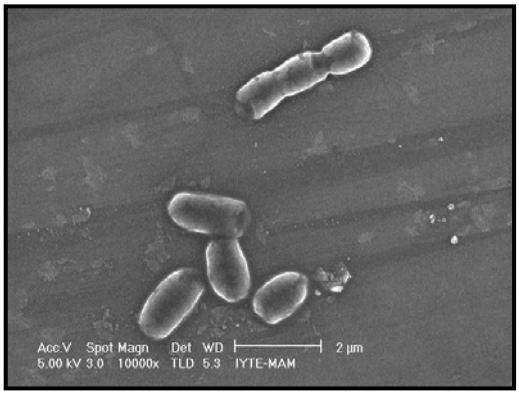
Table 1.1. Major outbreaks of *L. monocytogenes* infections between 1980-1999 (Source: Schlech 2000)

Years	Location	Number of Cases	Perinatal Cases %	Mortality Rates %	Sources
1980-1981	Maritime Province	41	83	34	Coleslaw
1983	New England	49	14	29	Pasteurized Milk
1983-1984	Switzerland	57	9	32	Soft Cheese
1985	Western United States	142	65	34	Mexican-Style Cheese
1986-1987	Pennsylvania	36	11	44	Unknown
1989	Connecticut	10	20	10	Shrimp
1993	Italy	39	-	-	Rice Salad
1992	France	38	82	32	Pork
1994	Illionis	45	-	-	Chocolate Milk
1997	Italy	1566	-	-	Corn Salad
1998-1999	United States	101	12	21	Hot Dogs, Deli Meats
1999	France	32	28	31	Pork Tongue

1.1.1.2. *Escherichia coli* O157:H7

Escherichia coli is a Gram-negative, non-sporeforming, rod-shaped (Figure 1.2), mesophilic and facultative anaerobic, enteric bacterium (Tortora, et al. 2004, Cliver 1990). Not all strains of *E. coli* are pathogenic for humans, even harmless strains are normally found in a healthy people's intestine. Pathogenic strains are split into four main groups: entero pathogenic *E. coli* (EPEC), entero toxigenic *E. coli* (ETEC), entero invasive *E. coli* (EIEC) and entero hemorrhagic *E. coli* (EHEC) based on their virulence

a-)



b-)

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gamma Proteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	*Escherichia*
Species:	*E. coli*

Figure 1.2. a-) SEM photography of *E. coli* O157:H7 at the magnification of 10000 b-) Taxonomic classification (Source: NCBI 2008-b).

mechanism. *E. coli* O157:H7, the well-known member of EHEC class, has been regarded as one of the most dangerous food-borne pathogens since it was first recognized as a food-borne pathogen in 1982 (Cliver 1990, Meng and Doyle 1998, Duffy, et al. 2006, Koohmaraie, et al. 2005). It is named due to its 157^{th} somatic (O) antigen and its 7^{th} flagellar (H) antigen. Serotype O157:H7 among *E.coli* strains do not have ability to ferment sorbitol, so colorless colonies on sorbitol MacConkey agar (SMAC) belong to *E. coli* O157:H7. For more reliable selection, cefixime tellurite (CT) can be added to SMAC agar (Mead and Griffin 1998). *E. coli* strains do not require complex nutrients and can multiply easily under the mild conditions. Besides being acid and low a_w tolerant, it is even able to survive in acidic and semi-dry foods that are considered as safe from food-borne pathogens (Buchanan and Doyle 1997). However, it does not have a tolerance to salt concentration higher than 6.5% (Duffy, et al. 2006). The major reservoir of *E. coli* O157:H7 is the intestines of cattle but it can also be nestled in sheep, goats, pigs, horses, dogs, poultry and deer. Thus, feces of these animals could pollute the environment easily (Mead and Griffin 1998). *E. coli* O157:H7 could survive in the environment for more than 10 months. Therefore, it most likely contaminates foods which are in contact with the contaminated environment or meat during slaughtering of cattle and other farm animals (Karch 2005). Grinding of meat would cause penetration of bacteria into minced meat that would be protected from heat treatment or cooking (Salyers and Whitt 2002). In many countries in the world, animal manures, especially bovine are used as pond fertilizer. Thus, pond water contains risk factor of pathogenic *E. coli* strains (Ozer and Demirci 2006). The major sources of this pathogen are uncooked and undercooked ground beef, lamb, dry cured salami, pork, poultry, and raw milk, raw vegetables, unpasteurized fruit juices and water (Table 1.3). Transmission of this bacterium is primarily through foods and water, and then directly from person to person or animal to person (Duffy, et al. 2006, Mead and Griffin 1998, Buchanan and Doyle 1997). In a traced outbreak in Washington State and California, the infection dose for this pathogen was determined as fewer than 50 organisms (Tilden, et al. 1996). It firstly invades and colonizes in small intestine. After 3-4 days incubation period, hemorrhagic colitis complaints appear as severe abdominal cramps and watery diarrhea. It can cause mortality in children (Figure 1.3).

In the case of entering blood stream, it spreads and infects other organs. When the disease involves the kidney, it causes acute renal failure called hemolytic uremic syndrome (HUS) (Figure 1.3). Especially, children under 5 years old, elderly and

immunosuppressive people are under the mortal risk of HUS and mortality rate is 3-5% (Table 1.2) (Duffy, et al. 2006, Krasner 2002, Alcamo 2003, Meng and Doyle 1998). In 2003, statistical data collected by FoodNet showed that 52 HUS cases observed on people aged less than 15 years old (occurrence rate is 0.6 per 100000 persons aged less than 15). Moreover, 36 of these 52 HUS cases are children under 5 years of age (occurrence rate is 1.3 per 100000) (CDC 2007-a). The third syndrome leaded by *E. coli* O157:H7 is thrombic thrombocytopenic purpura (TTP) that has also very high mortality rate. Although this syndrome resembles HUS, it causes serious damages to brain where red blood cells are destroyed instead of renal damage (Buchanan and Doyle 1997). 4744 EHEC cases were observed in 1999. The case number declined below 1 case per 100000 persons in 2004 (CDC 2007-a). The most effective ways of protection from *E. coli* O157:H7 diseases are consumption of well cooked foods, especially, meat products and preventing cross-contamination.

Table 1.2. Probability of illness from consumption of a single serving of beef contaminated with *E.coli* O157:H7 for elderly people (Source: Duffy, et al. 2006)

Beef	Location	Population group	Illness	HUS	Mortality
Ground beef	North America	Average adult	5.1×10^{-5}	-	-
Ground beef	North America	Children	3.7×10^{-5}	3.7×10^{-6}	1.9×10^{-5}
Ground beef	Australia	Average adult	6.4×10^{-4}	6.44×10^{-5}	7.72×10^{-5}
Ground beef	Australia	Children	4.6×10^{-4}	4.6×10^{-5}	2.3×10^{-5}
Ground beef	USA	Average population	9.6×10^{-7}	4.2×10^{-9}	5.9×10^{-5}
Ground beef	USA	Average population			
		June-September	1.7×10^{-6}	-	-
		October-May	6.0×10^{-7}	-	-
Ground beef	USA	Children	2.4×10^{-6}	-	-
Beef Burgers	Ireland	Average adult	1.1×10^{-6}	-	-

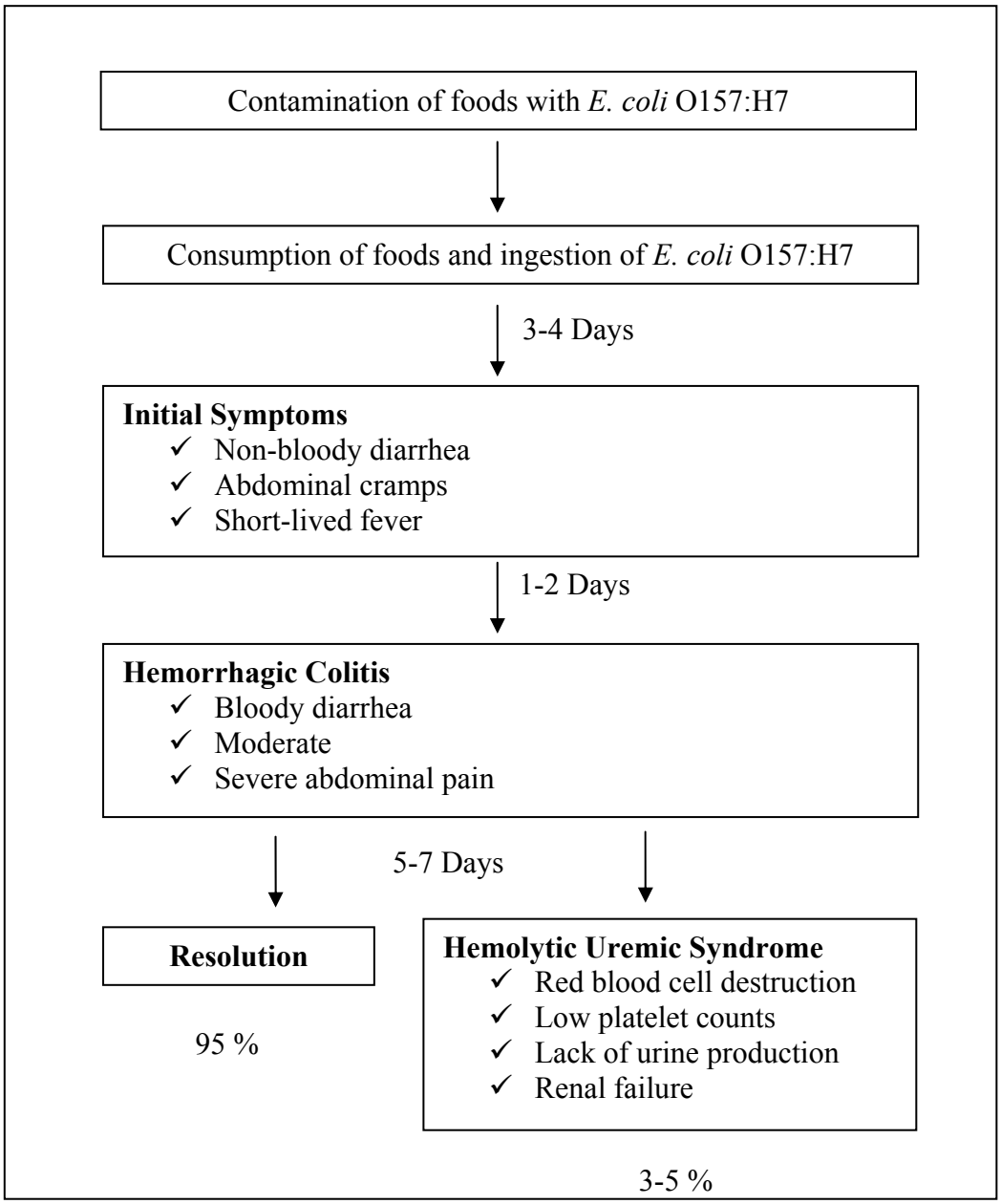


Figure 1.3. Pathogenicity and clinical aspects of *E. coli* O157:H7 infection (Source: Mead and Griffin 1998, Buchanan and Doyle, 1997).

1.1.1.3. *Salmonella* Enteritidis

Salmonella Enteritidis is a mesophilic, Gram-negative, motile, rod shaped (Figure 1.4), non-sporeforming and facultative anaerobic bacterium like other closely related enteric bacteria (Tortora, et al. 2004, Salyers and Whitt 2002). Optimal growth temperature and pH of *Salmonella* is 35-37 ^{o}C and 6.5-7.5, respectively. However, it can survive at the temperature range of 5-45 ^{o}C and at pH 4-9. It is one of the most well known bacterium among *Salmonella* serovars causing gastrointestinal disease called

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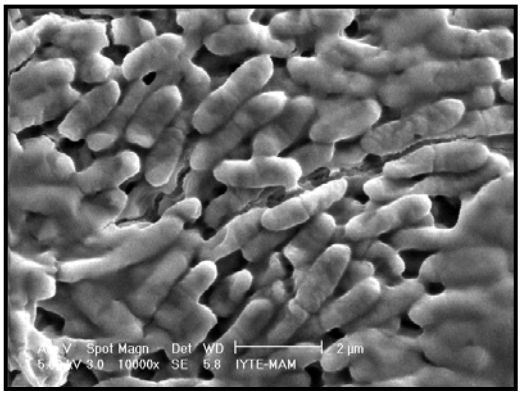


Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gamma Proteobacteria
Order:	Enterobacteriales
Family:	Enterobacteriaceae
Genus:	*Salmonella*
Species:	*S.* Enteritidis

Figure 1.4. a-) SEM photography of *Salmonella* Enteritidis at the magnification of 10000 b-) Taxonomic classification (Source: NCBI 2008-c).

"salmonellosis". In the United States, salmonellosis is the most common food-borne disease and nearly 40000 culture-confirmed cases are reported annually. However, it is believed that real occurrence exceeds this statistical number and reaches approximately to 1.4 million yearly because many patients do not visit phycians or neglect laboratory testing (CDC 2007-b, Rabsch 2001, Koohmaraie, et al. 2005). *S.* Enteritidis is responsible for 14% of all salmonellosis cases caused by other *Salmonella* serovars. According to 2003 surveillance data in the US, 4890 cases due to *S.* Enteritidis infection were reported, but the real number was estimated as 185829 (Figure 1.5) (Braden 2006). Rodents are the main animal reservoir for *S.* Enteritidis; therefore, this bacterium is introduced into poultries from rodents (Rabsch 2001). The organism contaminates eggs internally by transovarion transmission and these pathogens pass into the eggs before the egg shell forms (Braden 2006, Krasner 2002). Poultry products such as chickens, raw eggs and egg products (ice cream, liquid egg, mayonnaise) are common

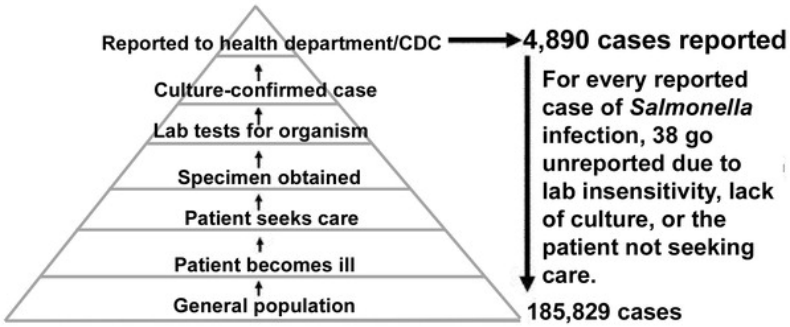


Figure 1.5. Reported cases and estimated real number caused by *S.* Enteritidis in the US, 2003 (Source: Braden 2006).

transmission vehicles for salmonellosis. *Salmonella* species naturally found in the gastrointestinal tract of human and animals. The symptoms of salmonellosis are fever, nausea, vomiting, diarrhea and severe abdominal cramps (Table 1.3) (CDC 2008-b, Alcamo 2003). Salmonellosis can affect any age group and mortality rate is not high in population (about 1%) but this rate increases among elder and infants. *S.* Enteritidis displays sporadic distribution and outbreaks of it reached the highest rate (3.9 per 100000 person) in 1995 and then declined to 1.98 per 100000 person in 1999 (Patrick 2004). The prevention methods of this pathogen are proper refrigeration, cooking (at least 82 ^{o}C for poultry), and pasteurization of eggs and juices (Tortora, et al. 2004, McCaron 1998).

Table 1.3. Diseaes, Symptoms and transmissions of food-borne bacteria: *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* (Source: Krasner 2002)

Causative Bacteria	Diseases	Symptoms	Transmission
***E. coli* O157:H7**	HUS, Hemorhagic Colitis, Thrombocytopenia	Diarrhea, Nausea, Abdominal cramps	Uncooked, undercooked ground beef lamb pork poultry , dry cured salami, raw milk, raw vegetables, unpasteurized juices water
***S.* Enteritidis**	Salmonellosis	Nausea, Vomiting, Abdominal cramps	Poultry products such as chicken, undercooked or raw eggs, egg products: ice cream, liquid egg, mayonnaise
L. monocytogenes	Listeriosis, fatal nonenteric diseases, meningitis, septicemia, miscarriage, encephalitis	Sore throat, fever, diarrhea miscarriage, death	Raw meats, soft cheese, ready to eat poultry, unpasteurized milk and milk products, unprocessed vegetables, seafood products

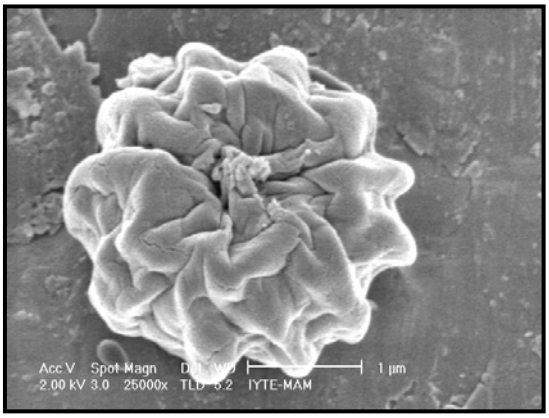
1.1.2. Spoilage Molds

Molds are microscopic fungi that live in/on plants, animals and human. Most of them have filamentous structure and produce spores that are distributed via air, water and insects. Although many genera and many species from different genera such as *Aspergillus*, *Penicillium*, *Mucor* and *Rhizopus* are utilized in food industry for enzyme and additive production, most molds are also known as responsible for food spoilage and mycotoxin production on foods (Ray 2001). Systematic distribution of molds in foods is 65% Penicillium, 19% Aspergillus, 2% Fusarium and 2% Zygomycetes in Turkey (Topal 2004).

1.1.2.1. *Aspergillus niger*

A. niger, the most prominent member of *Aspergillus* species, is a saprophytic filamentous fungi that grow aerobically on organic material. *A. niger* has no sexual reproduction cycle, filaments bears vegetative production parts on which black spores generated (Figure 1.6). The optimum growth temperature of *A. niger* is 35-37 ^{o}C. However, it can grow at the range of 6-47 ^{o}C. The minimum a_w for *A. niger* is 0.88 and capable of growing at pH between 1.4-9.8 (Schuster, et al. 2002). It is commonly found on decaying plant material, in soil and can also isolated from air, human, kashar cheese, dust, and some olives and seeds (Asan 2004).

a-)



b-)

Domain:	Eukaryota
Kingdom:	Fungi
Phylum:	Ascomycota
Subphylum:	Pezizomycotina
Class:	Eurotiomycetes
Order:	Eurotiales
Family:	Trichocomaceae
Genus:	*Aspergillus*
Species:	*A. niger*

Figure 1.6. a-) SEM photography of *A. niger* spore at the magnification of 25000 b-) Taxanomic classification (Source: NCBI 2008-d).

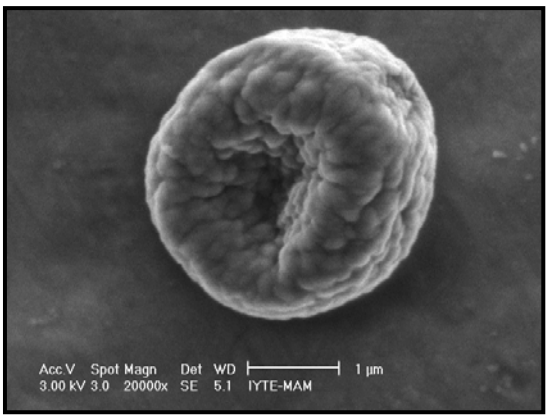
A. niger has industrial importance for production of useful metabolites such as citric, gluconic and fumaric acid and enzymes such as acidamylases, asparaginases, beta-galactosidases, glucoamylases, glucose oxidase, glycosidases, lipases, phospholipases, proteases, phytases and several hemicellulases including arabinases, arabinofuranosidases, pectin methylesterases, polygalacturonases, and xylanases. *A. niger* has been accepted as an important industrial fungi and its products received GRAS status by FDA (Punt, et al. 2002, Schuster, et al. 2002). However, in rare cases serious pulmonary disease called "aspergillosis" can be observed when the immunosuppressive patients exposed to intense spore dust. Aspergillosis is responsible for 9% of death cases following lung transplantation (Xavier, et al. 2008). Many *Aspergillus* species including *A. niger* can cause allergic bronchopulmonary aspergillosis that is the most frequent type of aspergillosis and caused by hypersentivity to Aspergillus antigen (Hoshino, et al. 1999, Kantarcioglu, et al. 2003). Another infection observed in the tropical area is trivial ear problem known as otomycosis. The fungal mycelia grow in the skin of external ear canal and cause local inflammation. *A. niger* is not considered as pathogen but produces secondary metabolites called 'mycotoxin' such as ochratoxin A, naphtho-Y-pyrones, malformins, nigerazine B, nigragillin and oxalic acid (Asan 2004, Blumenthal 2004). Some of these secondary metabolites are not only toxic to human and animals but also changes the organoleptic properties of food such as cheese and nuts (Sebti 2005).

1.1.2.2. *Penicillium roquefortii*

Penicillium roquefortii is a saprophytic fungus growing on decaying organic plant matters and asexually multiplying with spores namely conidia (Figure 1.7). It is tolerant to temperature as low as 2 ^{o}C, weak acids and low oxygen concentration (Doyle 2001). Spores play an important role in the life cycle of *P. roquefortii* (EPA 1997). They can readily disperse via air and can be dormant in unfavorable environment for years. These spores that are produced in high numbers are capable of germinating in mild environment. It can be isolated from the soil, air and, kashar and tulum cheese (Asan 2004).

P. roquefortii has been used in ripening period of roquefort, gorgonzola, stilton and blue cheese sinces ancient times and gives special flavor and fragrance to them

a-) **b-)**



Domain:	Eukaryota
Kingdom:	Fungi
Phylum:	Ascomycota
Subphylum:	Pezizomycotina
Class:	Eurotiomycetes
Order:	Eurotiales
Family:	Trichocomaceae
Genus:	*Penicillium*
Species:	*P. roqueforti*

Figure 1.7. a-) SEM photography of *Penicillium roquefortii* spore at the magnification of 20000 b-) Taxanomic classification (Source: NCBI 2008-e)

(Ray 2001). It is considered as an important contaminant. *P. roquefortii* is an opportunistic pathogen but number of cases is very low. Mycotoxins that are poisonous for people may be produced by this fungus in moderate conditions. Important secondary metabolites (toxins) produced by *P. roquefortii* are roquefortune C, isofumigaclavine A and B, PR-toxin, dihydroroquefortine, festuclavine, marcfortine A, patulin, penicillic acid, citrinin, botryodiplodin, siderophores, betaines and mycophenolic acid (Asan 2004 and EPA 1997). In a work by Chen et al. (1982) PR-toxins displayed acute toxicity on mice and rats. In another case reported by Campbell et al. (1983), a patient who worked in blue cheese production showed hypersensitivity pneumonitis to *Pencillium roquefortii*.

1.2. Natural Antimicrobial Agents

1.2.1. Lactoferrin and Activated Lactoferrin

Lactoferrin (LF) is an 80-kDa, iron-binding glycoprotein present in milk and exocrine secretions including saliva, tears, mucus and seminal fluids of mammals (Diarra, et al. 2002, Appelmelk, et al. 1994). It was first isolated from bovine milk in 1939 and then from human milk in 1960 by Johannson (Levay and Viljoen 1995). The concentration of lactoferrin in milk changes among different species. Amount of

lactoferrin in human milk (1-2 mg/ml) and milks from pigs and mice are very high (Recio and Visser 2000, Lonnerdal and Iyer 1995). However, in bovine milk, the concentration of LF is changing from 100 to 300 µg/ml (Sanches and Watts 1998). Lactoferrin polypeptide consist of about 600-800 contiguous amino acid residues depending on its source. For example, human lactoferrin contains 703 amino acids. Three dimensional structure of lactoferrin shows two lobes: N-terminus (residues 1-333) and C-terminus (residues 345-703) which have an iron-binding domain and glycan chain (Figure 1.8-a). N and C lobes exhibit different tertiary structure and iron binding capacity. Besides Fe^{+2} and Fe^{+3} ions, it can also bind to Cu^{+2}, Mn^{+2} and Zn^{+2} ions.

a-) **b-)**

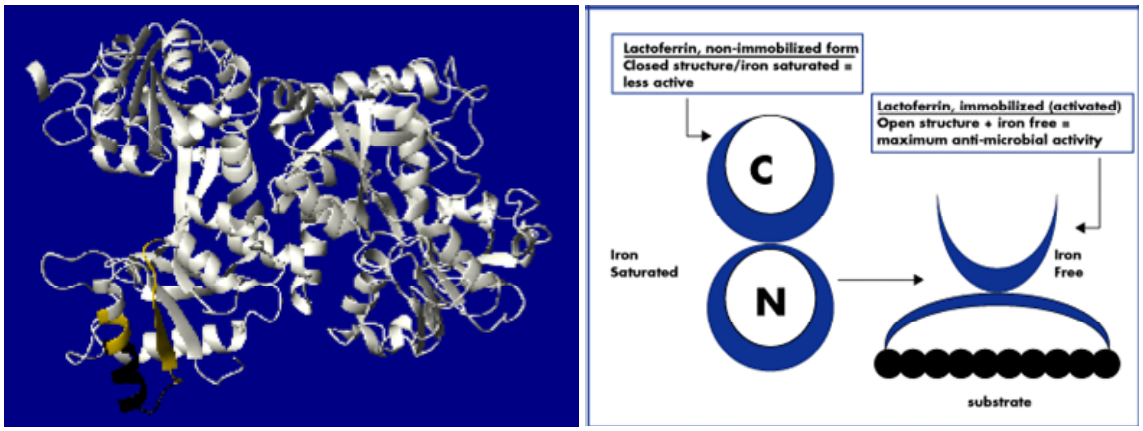


Figure 1.8. a-) Structure of Lactoferrin b-) Activated form of Lactoferrin (Source: Activatedlactoferrin 2006)

Binding to iron change three-dimensional conformation of this molecule (Oztas and Ozgunes 2005, Gonzalez-Chavez, et al. 2008). Its isoelectric point is between 8.4-9; therefore it is very cationic. Apo-lactoferrin at pH 4.0 is highly stable and resistant to heat up to 90 ^{o}C (Farnaud and Evans 2003, Abe, et al. 1991). LF exhibits defense and various biological activities such as antibacterial activities (Dionysius, et al. 1993, Bortner, et al. 1986, Kalmar and Arnold 1988, Nibbering, et al. 2000), antiviral activities (van der Strate, et al. 2001, Ikeda, et al. 2000), antifungal activities (Liceaga-Gesualdo, et al. 2001), antiparasitic activities (Omata, et al. 2001, Cirioni, et al. 2000), antioxidant activities (Chiu and Kuo 2007) imminomodulatory activities, anti-inflammatory activities (Legrand, et al. 2005), anticancer activities (Oztas and Ozgunes

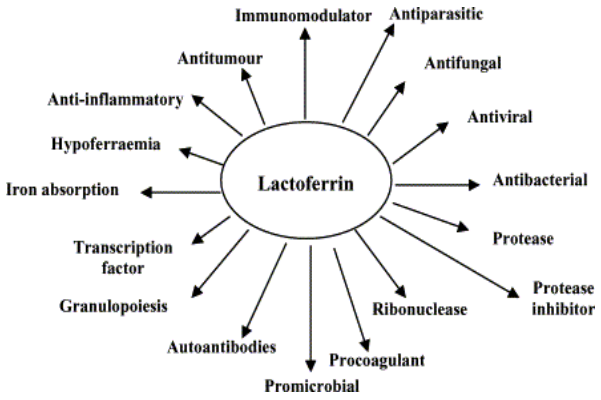


Figure 1.9. Proposed roles of lactoferrin.
(Source: Farnaud and Evans 2003)

2005), modulation of cell growth and inhibition of some bioactive compounds by binding (Wakabayashi, et al. 2006) (Figure 1.9). Two mechanisms are proposed for antimicrobial activity; the first, LF sequester iron with high affinity and bacterial growth is inhibited by the iron deficiency (Arnold, et al. 1977). After elucidation of globular structure of LF, it is predicted that LF has additional antibacterial mechanism for bacteria. Highly cationic parts in LF interacts with porins and lipid-A component of lipopolysaccharide layer of Gram-negatives, hence LF-mediated LPS release resulted in disruption of bacterial cell wall integrity and permeability (Figure 1.10-a) (Ellison III, et al. 1988, Ellison III and Giehl 1991, Naidu 2003-a). However, exact action mechanism of lactoferrin has not been fully elucidated, yet. Antibacterial properties of LF for Gram-positive bacteria are still a challenge (Diarra, et al. 2002, Sanches and Watts 1998, Levay and Viljoen 1995, Appelmelk, et al. 1994). It is though that LF binds to positively charged molecules on bacterial surface of Gram positives such as lipoteichoic acid (Gonzalez-Chavez, et al. 2008) (Figure 1.10-b). Bovine-lactoferrin (bLF) is currently used by the food industry as a supplement in yoghurt, skim milk, infant formula and drinks. Recombinant human lactoferrin has been produced by using organisms such as *Saccharomyces* sp, *Aspergillus* sp (Lonnerdal and Iyer 1995), transgenic rice (Bethell and Huang 2004) and transgenic mice (Nuijens, et al. 1997).

Activated lactoferrin (ALF) is immobilized form of lactoferrin to a substrate via N-terminus region. Iron (Fe^{+3}) and various cellular targets biding capacity of LF is increased by immobilization to a food-grade molecule glycosaminoglycan (Naidu 2002). Thus, antimicrobial activity of LF is enhanced by this process (Figure 1. 8-b). The immobilization process of lactoferrin was developed and patented by A.S. Naidu

(Patent storm 2007) and there is no detailed information about ALF. This natural antimicrobial is found effective against bacteria, fungi, viruses and parasites (Gustilo, et al. 2003). Both milk derived LF and ALF are considered Generally Recognized as Safe (GRAS) status by United States Department of Agriculture (USDA) for use in meat industry and permitted up to the level of 65.2 mg/kg-beef by Food Drug Administration (FDA) (Wakabayashi, et al. 2006, Naidu, et al. 2003-b). It has been suggested that ALF can be used commercially in meat industry to extend shelf life and to reduce microbial load on carcasses surface (Naidu 2002). However, limited information has been documented on antimicrobial activity of ALF in food systems.

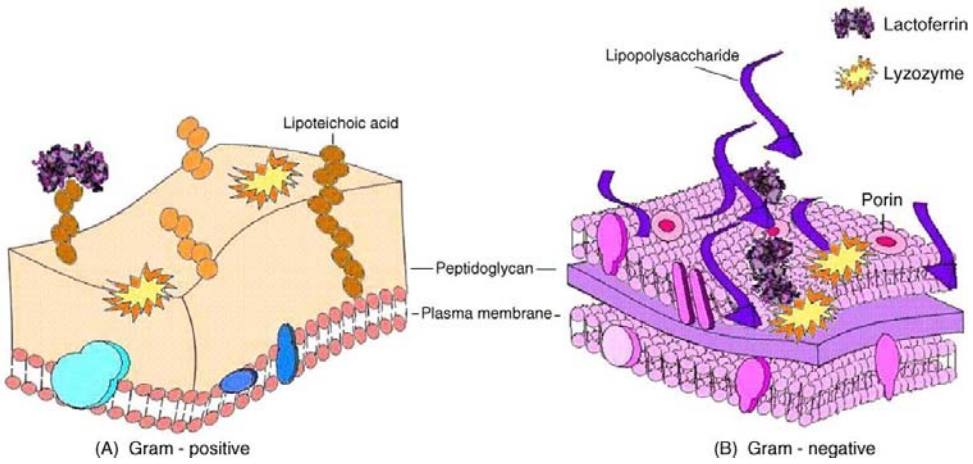


Figure 1. 10. Proposed mechanism of antibacterial action of lactoferrin (LF) a-) Gram-positive bacteria: LF is bound to negatively charged molecules of the cell membrane such as lipoteichoic acid, neutralising wall charge and allowing the action of other antibacterial compounds such as lysozyme b-) Gram-negative bacteria: LF can bind to lipid A of lipopolysaccharide, causing liberation of this lipid with consequent damage to the cell membrane (Source: Gonzalez-Chavez, et al. 2008)

1.2.2. Jenseniin-G

Propionibacterium spp. are Gram positive, anaerob and 'food grade' organisms. They can be isolated from cheese and milk products, soil, oils and silage fermentations. They produce propionic acid and CO_2; therefore, they give the unique nutty flavor and the eyes of Swiss cheese. Production of several bacteriocins such as Propionicin PLG-1 (Lyon and Glatz 1991), Jenseniin G (Grinstead and Barefoot 1992) and a bacteriocin produced by *P. jensenii* B1264 have been demonstrated up to now by this organism. Jenseniin G (JG) produced by *Propionibacterium thoenii* (*jensenii*) P126 is a small and a heat stable bacteriocin. JG inhibits the growth of Propionibacteria, some *Lactobacillus* spp., *Clostridium botulinum* spores type A, B, E, hence delay over acidification of yoghurt (Weinbrenner, et al. 1997). JG is stable to freezing, cold storage at 4 ^{o}C for 3 days, heat treatment at 100 ^{o}C for 15 minutes and a wide range of pH from 3 to 12 (Grinstead and Barefoot 1992). The features such as having antimicrobial activity, being stable to heat and cold, being food-grade and being nontoxic for human make JG promising as a preservative for food industry in future.

1.2.3. *Rosmarinus officinalis*

It is estimated that there are 250000 to 500000 plants on earth but only small percentage of these are used for food or medical purposes by humans (Cowan 1999). One of these plants is rosemary and it has been used since ancient times as a spice in foods and as a remedy for many illnesses. Rosemary (*Rosmarinus officinalis* L.) from Labiate family is an evergreen perennial shrub that has hard, woody gray bark and needle-shaped leaves, and sky blue flower (Figure 1.10). Herbs and spices have been

Figure 1.11. Leaves and flowers of *Rosmarinus officinalis*
(Source: VeggieHarvest 2008)

used in food industry and are considered GRAS (Skandamis, et al. 2001). Rosemary has been used in pharmaceuticals and folk medicine as antibacterial, antispasdomic in renal colic and dysmenorrheal, in relieving respiratory disorders, analgesic, anti-rheumatic, carminative, cholagogue, diuretic, expectorant and anti-epilectic (Arslan and Ozcan 2008, Soyal, et al. 2007, Bakirel, et al. 2007). Biological activity of rosemary extract is based on its ability to diminish oxidative damage of free radicals on DNA, proteins and membrane phospholipids. Although the mode of action of plant extracts are not known exactly, it is proposed that phenolic components of plant extract cause impairment of enzyme systems or give damages to membrane phospholipids, increase cell membrane permeability and essential intracellular components ribose, Na, glutamate, K^+ etc. leak (Cox, et al. 1998, Singh, et al. 2003, Moreira, et al. 2005). Some biologically active compounds of rosemary are given in Table 1.4. Rosemary has also displayed powerful antioxidant activity and has been widely used in food industry as an antioxidant agent (Tsai, et al. 2007). Besides having biological, antioxidant and antimicrobial activities (Riznar, et al. 2006, Fernandez-Lopez, et al. 2005), rosemary has good flavor and pleasant odor, thus it can be a good candidate for hurdle technology (Bozin, et al. 2007).

Table 1.4. Some biologically active compounds of rosemary
(Source:Ceylan and Fung 2004)

Anethole	Caryophyllene	Methyl eugenol
Apigenin	Chlorogenic acid	Niacin
Ascorbic acid	1,8-Cineole	Pinene
Borneol	p-Cymene	Rosmarinic acid
Bornyl acetate	Genkwanin	Safrole
Caffeic acid	Geraniol	Terpinene
Camphor	Glycolic acid	Thujone
Delta-3-Carene	Limonene	Ursolic acid
Carveol	Linalool	

1.2.4. Natamycin

Natamycin is a well known antimycotic agent since it was first discovered in 1955. The name of natamycin comes from producer culture *Streptomyces natalensis* and it is also known as 'Pimaricin'. Theoretical open formula of this polyene antifungal agent is $C_{33}H_{47}NO_{13}$ with molecular weight 665.7 KDa (Figure 1.11) (Thomas and Delves-Broughton 2003). Natamycin damages both mold spores and hyphae by binding cell membrane sterols irreversibly, especially ergasterol. This results in leakage of essential cellular constituents and finally cell lyses. It does not have activity against bacteria, because bacterial cell wall does not include sterols (Reps 2002, Pedersen 1992). Commercial application of natamycin in cheese and processed meat industry as a surface preservative is common around the World. The acceptable level is 0.3 mg kg^{-1} according to joint FAO/WHO Expert Committee on Food Additives (Var, et al. 2006). It has GRAS status and has properties such as high efficiency at low concentration, pH stability at the range of pH 3-9, chemical durability and prolonged effectiveness on foods surfaces and has no bad flavor and taste. Challenge for food industry is insolubility of natamycin in water and higher alcohols. However, several organic solvents like Glycerol, Glacial acetic acid and propylene glycol can be used to over come solubility problem.

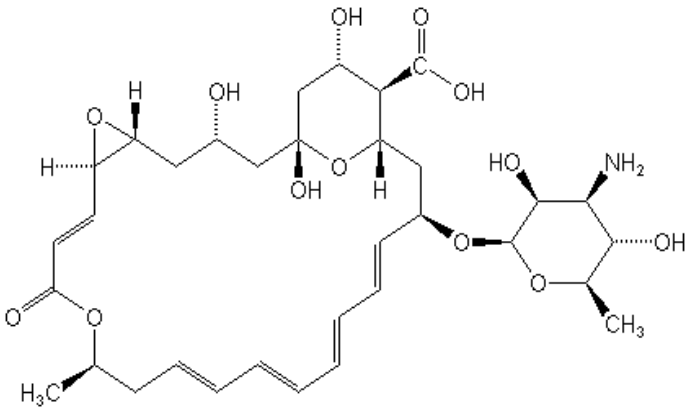


Figure 1.12. Open chemical structure of natamycin molecule.
(Source: Alanwood 2008)

CHAPTER 2

INTRODUCTION

Food-borne illnesses, generally called “food poisoning” in public, arise from consumption of different types of pathogens such as viruses, molds, parasites and bacteria rather than chemicals or natural toxins. There are more than 250 food-borne diseases transmitted with foods (CDC 2008-a). According to statistical data, food-borne pathogens cause approximately 76 million illnesses resulting in 325000 hospitalization and 5000 deaths in the United States each year (Mead, et al. 1999). In Turkey, 7875 discharged and 324 deaths in 2001, 8856 discharged and 91 deaths in 2002 were reported because of the food poisoning by Turkish Health Ministry (Figure 2.1) (Ozelhastaneler 2008). Overall, bacteria are responsible for 30% of all food-borne diseases and 72% of food-borne related deaths. Bacteria can multiply quickly and produce harmful substances or poisoning chemicals (toxins) on foods stored in abused conditions. Among pathogenic bacteria, in particular, *Listeria monocytogenes*, *E. coli* and *Salmonella* spp. are responsible for the largest number of outbreaks, cases and deaths (Oussalah, et al. 2007). It is believed that unreported cases are actually higher than reported numbers because ill persons recover from mild cases without taking medical care or can not be diagnosed (Mead, et al. 1999). Besides diseases and deaths, economical burden of food-borne illnesses to US was estimated as $6.9 billion due to only five major pathogens; *L. monocytogenes*, nontyphoidal *Salmonella*, *E. coli* O157:H7, *E. coli* non-O157:H7 and *Campylobacter* (Naidu, et al. 2003-b)

Numerous synthetic antimicrobial agents have been used in the food industry as additives to reduce food-borne illnesses and to extend the shelf life of foods. Such antimicrobials decrease the microbial load which is present on foods naturally or inhibiting the growth of dangerous pathogens that could contaminate foods during post processing. However, some of these synthetic agents sometimes might have toxic, allergenic, teratogenic or carcinogenic side effects on sensitive people (Sagdic and Ozcan 2003, Skandamis, et al. 2001). While consumers demand safer foods, having longer shelf life and more quality; they also prefer “natural foods” that is minimally processed containing no chemical additives (Abutbul, et al. 2004). Plants have been

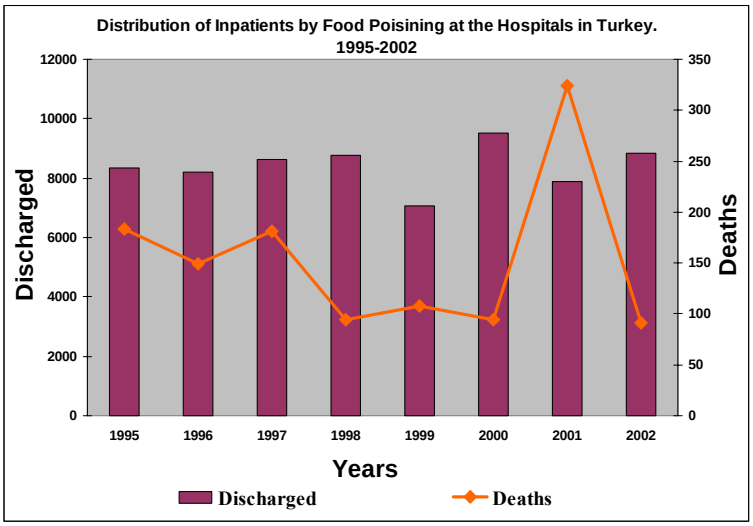


Figure 2.1. Distribution of inpatients by foodborne poisoning at the Hospital in Turkey. Bars show number of discharged patients and line show number of deaths between years 1995-2002 (Source: Ozelhastaneler 2008).

widely used in medicine at least 60000 years. After further development of antibiotics in the 1950's, they replaced plants (Cowan 1999). Many pathogens have ability to gain resistance rapidly against conventional antibiotics. Therefore, alternative natural microbial inhibitors such as plant extracts, bacteriocins, antimycotics and immune system proteins of mammalians have become increasingly more popular with consumers. *Rosmarinus officinalis* is an evergreen plant grown in Mediterranean region. Antimicrobial and antioxidant activity of rosemary extract is available in literature (Riznar, et al. 2006, Fernandez-Lopez, et al. 2005). JG is a bacteriocin isolated from *Propionibacterium thoenii* (*jensenii*) P126. Activated lactoferrin is an immobilized glycoprotein found in mammalian secretions and shows antimicrobial activity against many pathogens and viruses. Natamycin is a polyene macrolide antimycotic agent produced by *Streptomyces natalensis*. Biopreservatives from microorganisms, plants and complex organisms could be great alternative for extending the shelf life of foods, eliminating pathogen bacteria on foods and improving quality of foods. Use of natural antimicrobials in food preservation attracts both food producers and scientists as a promising area (Doyle 2006, Moreira, et al. 2005, Smith-Palmer, et al. 1998).

In vitro antimicrobial and antioxidant activities of natural agents have been demonstrated for many years using different testing methods such as disc diffusion assay, agar well diffusion technique and turbidimetric method. However, information about application of these agents on foods is limited. Investigation of decontamination

of meat and meat products with organic acids, hot water, electrolyzed water and natural based antimicrobials by dipping and spraying has great potential for researchers. The use of antimicrobials or additives as low as possible is desired, because treatment of foods at high concentration with these agents could change the appearance and the organoleptic properties of meat. For example, plant extracts could leave strong odor and flavor when added or applied to foods. A possible solution may be testing the combinational interaction of antimicrobials. Each antimicrobial, especially from different sources, has different targets on a bacterial cell. Herein, two antimicrobials could enhance the antimicrobial activity of each other which is called synergism. On the other hand, reduction of antimicrobial activity of agents due to negative interaction which is called antagonism is also possible. Before actual food application, testing of antimicrobials in combinations *in vitro* is more appropriate for determination of possible positive or negative interaction.

Therein, the purpose of this study is to determine individual minimum inhibitory concentration (MIC value) of activated lactoferrin, rosemary extract, Jenseniin-G and Natamycin against three food-borne pathogens: *Listeria monocytogenes, Escherichia coli* O157:H7 and *Salmonella* Enteritidis *in vitro* by microtiter plate method and disc diffusion assay and two spoilage molds: *Aspergillus niger* and *Penicillium roquefortii* by disc diffusion assay. It was also aimed to determine the activity of antimicrobials which was found effective in individual tests, in combinations, whether they would act synergistically or antagonistically. Finally, application of effective agents were also investigated on meat and kashar cheese samples by dipping assay. Results of microtiter plate assay indicated that ALF displayed powerful inhibitory activity against three pathogens. RE eliminated only growth of *L. monocytogenes* individually. However, none of the antimicrobials inhibited the pathogenic bacteria by disc diffusion assay. Natamycin was very active against both molds by disc diffusion assay and by dipping assay. Combinations of ALF and RE acted synergistically on *E. coli* O157:H7 in well. In real food tests on meat, ALF and RE decreased the load of *L. monocytogenes* by dipping application, remarkably while ALF displayed no reduction on *S.* Enteritidis. Natamycin were found very effective against *A. niger* and *P. roquefortii* by disc diffusion assay.

CHAPTER 3

MATERIALS AND METHODS

3.1. Maintenance and Preparation of Microbial Cultures

3.1.1. Bacterial Strains and Culture Conditions

All bacteria that were used in this study, *E. coli* O157:H7*, L. monocytogenes, S.* Enteritidis were obtained from National Culture Type of Collection (NCTC, United Kingdom). *E. coli* O157:H7 was grown in Lauria broth (LB) including yeast extract (Acumedia), tryptone (Fluka), sodium chloride (Riedel-deHaen) and on LB agar (Agar, Merck). *L. monocytogenes* was routinely grown in brain heart infusion broth (BHI, Fluka) and on BHI agar (Fluka). *S.* Enteritidis was grown in tryptic soy broth (TSB, Fluka) and on TSB agar (Agar, Merck). In meat applications PALCAM (Merck) agar for *L. monocytogenes*, xylose lysine deoxycholate (XLD, Merck) agar for *S.* Enteritidis and sorbitol Mac conkey (SMAC, Fluka) agar supplemented with cefixime tellurite (CT, Merck) for *E. coli* O157:H7 were used as selective agar medium.

Short term maintenance of all strains was on agar plates (LB, BHI and TSA). All bacteria were sub-cultured on appropriate agar plates every week and kept at 4 ^{o}C in order to maintain bacterial viability. All strains were maintained in the appropriate growth medium containing 20% glycerol and stored at -80 ^{o}C. They were refreshed from -80 ^{o}C stock once a month or as needed throughout the study. Gram staining, morphological and growth characteristics were used to confirm the purity of the strains from time to time.

3.1.2. Fungal Strains and Culture Conditions

Aspergillus niger was isolated from onion skin in Plant Protection Department of Mustafa Kemal University (Hatay, Turkey) and kindly provided by Dr. Gülsün Evrendilek. *Penicillium roquefortii* DBCI-1 was isolated from Danish blue cheese in

Food Engineering Department of Izmir Institute of Technology (Izmir, Turkey) and kindly provided by Dr. Handan Baysal. Both fungi were identified and confirmed by microscopy and morphological analysis. All fungal cultures were grown and maintained on Potato Dextrose Agar (PDA, Merck) slants at 30 ^{o}C throughout the study. The spores of both fungi were collected in 0.1% peptone water containing 20% glycerol and stored at -80 ^{o}C for long term maintenance.

In cheese applications dichloran rose bengal chloramphenicol agar (DRBC, Merck) were used as selective agar medium for both fungi.

3.1.3. Preparation of Bacterial Cultures

All cultures were propagated in the appropriate medium by inoculating a single colony from agar plates to test tubes containing 4 ml broth medium indicated in the section 3.1.1 for each bacterium. The incubation period at 37 ^{o}C without shaking was approximately 16 hours for all three bacteria. After the incubation period, optical density (OD) of bacterial cultures was adjusted to desired value at 600 nm wavelength with proper medium. This provided a 2% inoculum for cultures that was used in all antimicrobial tests. Then, cultures were incubated at 37 ^{o}C without shaking for approximately 5.5 hours for *L. monocytogenes*, 3 hours for *S.* Enteritidis and 2.5 hours for *E. coli* O157:H7 so that all bacterial cultures could be in mid-logarithmic phase (Figure B.1-a, B.1-b and B.1-c). At the end of the incubation time, cultures had approximately $1x10^{8}$, $5x10^{8}$, $5x10^{8}$ cfu/ml for *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes*, respectively. Immediately after incubation each culture was subjected to serial dilution with proper medium to desired inoculum level for the antimicrobial tests. The plate count method was used for enumeration of all cultures for confirmation of the bacterial loads in all antimicrobial tests.

3.1.4. Preparation of Fungal Cultures

Both fungi were grown on PDA slants at 30 ^{o}C and displayed different growth characteristic, texture and sporulation time. *A. niger* was incubated for 3-5 days and spores of cultures older than 5 days were not used in the experiments. *P. roquefortii* was

incubated for 5-7 days and spores of cultures older than 7 days were not used in the experiments.

Our preliminary studies indicated that $1x10^4$ spore/ml for *A. niger* and $1x10^6$ spore/ml for *P. roquefortii* were needed to spread on PDA for a good lawn formation in disc diffusion assays. Also, incubation time for zone measurements was 3 days for *A. niger* whereas this period was 6 days for *P. roquefortii.*

Fungal cultures were propagated on PDA slants when required and incubated at 30 ^oC for the time required for sporulation. The spores were collected by sterilized 0.1% peptone water by shaking vigorously. The collected spores were enumerated via spore count on Thoma slide. The spore loads were adjusted by diluting with 0.1% sterile peptone water if needed. $1x10^4$ spore/ml for *A. niger* and $1x10^6$ spore/ml for *P. roquefortii* were used in the disc diffusion assay. Spore solution containing $1x10^5$ spore/ml were prepared for dipping assays of cheese for both fungi.

3.2. Bacterial Growth Curves

Each organism displays their own growth characteristics depending on their physiological and environmental needs. The growth curves for each bacterium were determined to observe the growth characteristics of the organisms used in this study. Therefore, it was possible to conduct all antimicrobial tests at early exponential phase of the cultures.

A single colony of each strain was inoculated into 4 ml appropriate medium and left to incubation at 37 ^oC for 16 hours in an incubator. Optical density (OD) of bacterial culture was measured at 600 nm wavelength and OD value was adjusted to a determined value with the proper medium. A 2% inoculum was used from adjusted culture and transferred to twelve tubes containing 4 ml proper medium. All were incubated at 37 ^oC without shaking. Then, a tube was removed from incubator every 2 hours for bacterial enumeration and OD measurement. At the end of 24^{th} hour sampling period, growth curves were plotted as time versus number (log) and time versus OD values. The bacterial cultures used in the experiments were prepared according to data from the growth curves.

3.3. Preparation of Natural Compounds

In this work, a plant material (RE), a bacteriocin (JG), an immobilized glycoprotein (ALF) and antimycotic agent natamycin from natural sources were tested against three food-borne pathogens and two spoilage molds. All agents were prepared prior to use and further dilutions were made by using sterile de-ionized water.

3.3.1. Activated Lactoferrin

ALF was donated by DMV International Nutritionals, USA. To prepare stock solution, recipe recommended by manufacturer was followed. For a 4% ALF (wt/vol) solution, 0.54 g ALF was dissolved in 10 ml sterilized de-ionized water (containing low iron or divalent cations). Prepared stock solution was serially diluted with sterilized de-ionized water to appropriate concentrations in tubes (Table 3.1).

3.3.2. Rosemary Extract

Extraction procedure was modified from Madsen et al. (1998). The rosemary samples were collected from Izmir Institute of Technology area and washed with de-ionized water. After leaves of rosemary were removed from their stems, 70 ml absolute ethanol per 12 g of leaves was used for homogenization procedure (Heidolph Silent Crusher M Homogenizer, Germany) at 26000 rpm for 5 minutes. Homogenizate was stirred for 30 minutes in a dark bottle and then centrifuged at 5000 rpm (Nuve NF 615, Turkey) for 5 minutes. The aqueous phase was transferred into a new bottle and 30 ml absolute ethanol was added to precipitate. The same procedure (stirring, centrifugation, collecting of the supernatant and adding ethanol to precipitate) was repeated two more times by using 30 ml and 20 ml ethanol, respectively. At last, collected supernatant was mixed with 44 ml de-ionized water and this final volume was evaporated at 40 ^{o}C for approximately 1 hour by a rotary evaporator under vacuum (Heidolph Laborato 4000, Germany). After evaporation, the solution obtained was subjected to vacuum filtration by using a 5.0 μm pore size cellulose nitrate filter (Sartorius). The prepared rosemary extract was accepted as 100% and serially diluted to required concentrations with sterile de-ionized water (RE% vol/vol) (Table 3.1).

3.3.3. Jenseniin-G

Production of Jenseniin-G was carried out at Suleyman Demirel University in Isparta and kindly provided by Dr. Yesim Ekinci. Samples were transported to our laboratory in insulated box containing dry ice and ice packs. Concentration of the bacteriocin was determined as activity unit (AU/ml) by producer via a bio-assay and the supplied JG was serially diluted with sterilized de-ionized water to prepare different concentrations before use in the antimicrobial tests (Table 3.1).

3.3.4. Natamycin

Pimalac®, containing 50% NA and 50% lactose, was used as natamycin and stored in dark in a refrigerator until used. Natamycin solution was prepared by dissolving natamycin powder in one part ethanol and one part glycerol (1:1 vol/vol) solution. After well mixing, it was serially diluted with sterile d-water to required concentration.

3.4. Antimicrobial Activity Tests

Antimicrobial activities of natural compounds -ALF, RE and JG- alone and in combinations were investigated against three food-borne pathogens by using both microtiter plate assay and disc diffusion assay. Antifungal activities of ALF, RE, JG and NA were tested against two molds by the disc diffusion assay only. All tests were performed in duplicates and repeated at least twice.

3.4.1. Individual Effects of Antimicrobials by Microtiter Plate Assay

The microtiter plate growth assay was performed with some modifications from Dufour et al. (2003). The stock solutions of ALF, RE and JG were diluted serially in test tubes to those concentrations given in Table 3.1, with sterile de-ionized water. From each concentration 100 µl volumes was dispensed into a well of flat bottom 96-well microtiter plate (Bio-Grainer, Germany). Prepared cultures of the test organisms were

diluted in proper medium to approximately $1x10^4$ cfu/ml and 100 µl volumes added to each well. Thus, final antimicrobial agent concentrations were decreased to half in each well (Table 3.2). All tests were conducted with appropriate controls. Then, the microtiter plates were incubated at 37 ^oC for 24 hours and optical density (OD) of the plates were measured at 600 nm by Thermo Multiscan Spectra Reader (Finland) at every 2 hours.

Table 3.1 Concentrations of ALF, RE and JG in dilution tubes

Antimicrobial agents	ALF (%)	RE (%)	JG (AU/ml JG)
Concentration range of antimicrobial compounds in dilution tubes	4	60	8000
	3	40	6000
	2.5	30	4000
	2	20	2000
	1	10	1000
	0.5	-	-

Table 3.2 Concentrations of ALF, RE and JG in the wells

Antimicrobial agents	ALF (%)	RE (%)	JG (AU/ml JG)
Concentration range of antimicrobial compounds in well	2	30	4000
	1.5	20	3000
	1.25	15	2000
	1	10	1000
	0.5	5	500
	0.25	-	-

3.4.2. Combinational Effects of Antimicrobials by Microtiter Plate Assay

Antimicrobials, found effective against pathogens in individual tests, were tested in combinations in order to determine their synergistic activities. Similar procedure, described above for individual activities, was applied. Prepared cultures of the test organisms were diluted in proper medium to approximately $1x10^4$ cfu/ml and 100 µl volumes added to each well. The antimicrobial agents were prepared four times

concentrated what was required in the wells and 50 µl volume was dispensed into each well. Microtiter plates were incubated at 37 ^{o}C for 24 hours and OD was measured at 600 nm every 2 hours. Experiments for each agent were performed in duplicate and repeated twice. Combinations of concentrations of antimicrobial agents in wells were given in Table 3.3.

3.4.3. Activity of ALF in Double Strength Medium by Microtiter Plate Assay

Preparation procedure of bacterial culture was the same as in section 3.1.2. Twice as much amount of medium powder was dissolved in the amount of water needed for regular (1x) medium to prepare the double strength (2x) BHI, LB and TSB. Bacterial cultures were diluted in 2x medium to $1x10^4$ cfu/ml before dispensing into each well. ALF concentrations used in this experiment presented in Table 3.4. A 100 µl volume of bacterial culture in 2x medium and 100 µl volume of ALF solution were dispensed into the same well of flat bottom 96-well plate. 100 µl sterile de-ionized water and 100 µl of bacterial culture were deposited into well to observe bacterial growth in a well as control. The same test was repeated as duplicate and replicate. The plates were incubated at 37 ^{o}C for 24 hours and the optical density (OD) of the plates was measured every 2 hours.

Table 3.3 ALF concentration ranges in test tubes and in wells for three food borne pathogens for testing effectiveness of double strength medium on ALF

Pathogens	ALF concentration (%)	
	In tubes	In well
L. monocytogenes	0.5-0.1-0.2-0.5-1	0.025-0.05-0.1-0.25-0.5
***E. coli* O157:H7**	0.2-0.5-1-2-3	0.1-0.25-0.5-1-1.5
***S.* Enteritidis**	0.5-1-2-3-4	0.25-0.5-1-1.5-2

Table 3.4. Concentrations of ALF and RE in microtiter plate wells for synergistic activity

Bacteria	Concentration intervals of natural compounds	
	ALF	Rosemary Extract
Listeria monocytogenes	0.075%	10%
		7.5%
		5%
	0.05%	10%
		7.5%
		5%
	0.025%	10%
		7.5%
		5%
Salmonella Enteritidis	0.5%	20%
		15%
		10%
	0.3%	20%
		15%
		10%
	0.1%	20%
		15%
		10%
Escherichia coli O157:H7	0.25%	20%
		15%
		10%
	0.15%	20%
		15%
		10%
	0.05%	20%
		15%
		10%

3.4.4. Individual Effects of Antimicrobials against Bacterial Strains by Disc Diffusion Assay

The antimicrobial agents were diluted in sterile de-ionized water to following concentrations: ALF: 4, 3, 2, 1.5, 1 %; RE: 60, 40, 30, 20, 10 %; JG: 4000, 3000, 2000, 1000, 500 AU/ml. 20 µl of each antimicrobial agent for each concentration was pipeted onto the 1 cm^2 squared sterile-whatman paper and placed at the centre of the appropriate agar plates that was seeded with 100 µl of $1x10^6$ cfu/ml bacterial suspension. The plates were incubated at 37 ^oC for 24 hours and zone formation was measured if formed around the paper pieces. The measured zone diameter contained the paper pieces.

3.4.5. Combinational Effects of Antimicrobials against Bacterial Strains by Disc Diffusion Assay

ALF and RE were diluted to obtain final concentrations of 50% RE+2% ALF, 25% RE+3% ALF, and 35% RE+1 % ALF. 100 µl of bacterial culture containing $1x10^6$ cfu/ml was spread onto agar medium. Then, 20 µl mixed antimicrobial solutions was deposited onto 1 cm^2 squared sterile whatman paper and placed at the centre of agar plates. Whatman paper, impregnated with 20 µl de-ionized water, was also tested as control. After 10 hours of incubation at 37 ^oC, the plates were checked for any zone formation at every two hours.

3.4.6. Individual and Combinational Effects of Antimicrobials against Molds by Disc Diffusion Assay

Individual antifungal activities of ALF, RE, JG and NA were tested against *A. niger* and *P. roquefortii* by disc diffusion assay as the same procedure as explained in section 3.4.4. The same concentration ranges were utilized for ALF, RE and JG as explained above. In addition to those, NA concentrations were applied between 250-5000 ppm for individual tests. NA+RE concentration ranges were 25-2500 ppm NA+50% RE for combinational tests. 100 µl of $1x10^4$ spore/ ml *A. niger* or 100 µl of $1x10^6$ spore/ml *P. roquefortii* spore solution was spread onto PDA to form fungal lawn.

Paper pieces, impregnated with antimicrobial agents, were placed onto PDA and agar plates were left to incubation for 3 days for *A. niger* or for 6 days for *P. roquefortii* at 30 ^{o}C. Control groups of d-water, 0.1% peptone water, glycerol, ethanol and glycerol + ethanol were used in the same experiment. Zones including paper pieces were measured with hand micrometer (SHAN Electronic, China) at the end of the defined incubation time.

3.5. Food Applications of Antimicrobial Agents

Antimicrobials which had activity against tested microorganisms were applied onto food samples by dipping to observe changes in microbial load. All bacterial experiments were carried out on meat samples. For all mold applications kashar cheese was used.

3.5.1. Dipping Applications of Meat Cuts Inoculated with Bacteria

An entire sirloin or round of beef in vacuum packages were purchased from Tanet slaughterhouse, located at Buca-Izmir, Turkey and transported immediately to laboratory in an insulated box containing ice packs. Sirloin steak was used with no treatment for experiments on *L. monocytogenes*. Round of beef was used for experiments on *E. coli* O157:H7 and *S.* Enteritidis. A thin layer skinned around round of beef with sterile knife to minimize the initial number of microflora. Meat block was cut into pieces about 2 cm in thickness and 20 ± 1 g with a sterile knife (Figure 3.1-a). Then, they were separately immersed into 30 ml of 1x10^{5} cfu/ml bacterial culture for 2 minutes and left in ClassII biohazard safety cabinet (Esco, Singapore) to allow

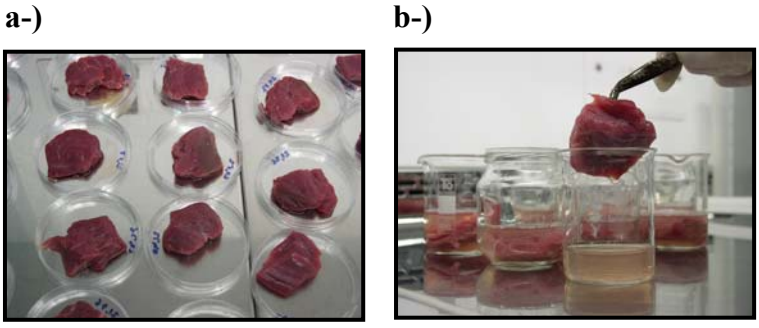


Figure 3.1. a-) Meat cuts b-) Dipping application.

attachment of the bacteria for 15 minutes for each surface and total of 30 minutes. The inoculated samples were dipped into beakers containing 30 ml of antimicrobial solution for 15 minutes (Figure 3.1-b). Similarly, a group of samples were dipped into 30 ml of sterile de-ionized water instead of antimicrobial solution as a control. Two control groups were used in the test. First group which was marked as C-1 was not-treated meat samples. Second group which was marked as C-2 was inoculated with the tested bacteria and dipped in sterile de-ionized water on meats. All samples were first placed onto sterile petri dishes, they were packaged in zipper plastic bags and stored at 4 ^{o}C for *L. monocytogenes* and 10 ^{o}C for *E. coli* O157:H7 in a refrigerator for 9 days. Incubation time was 8 days for *S.* Enteritidis at 10 ^{o}C.

3.5.2. Dipping Applications of Cheese Cuts Inoculated with Molds

A block of kashar cheese was purchased from a local market in Izmir. Cheese bar (1 kg) was cut into 20± 1 g circular pieces (2 cm diameter, 1 cm thickness) with sterile knife (Figure 3.2-a). Probable microbial load of cheese samples were reduced by UV (U.V.P., USA) treatment of each surface at a distance of 15 cm for 10 minutes.

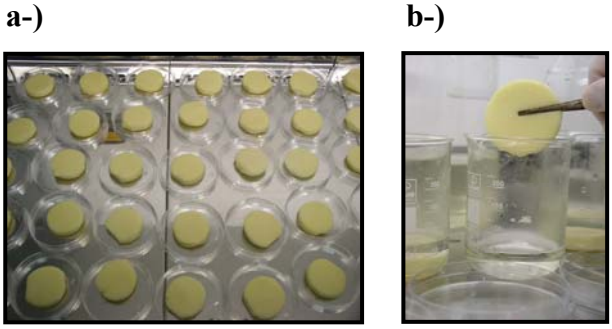


Figure 3.2. a-) Cheese cuts in circular shape b-) Dipping application.

Samples were inoculated with fungus by dipping into 50 ml of fungal solution containing 1x10^{5} spore/ml for 2 minutes. Inoculated cheese cuts were left in laminar hood to allow fungal attachment for 30 minutes (15 minutes for each surface). Inoculated and dried 8 samples for each treatment group were dipped into beaker containing 50 ml of natamycin solution for 15 minutes (Figure 3.2-b). Control groups were also used in the experiment. Growth control was dipped into 50 ml d-water instead of natamycin solution after inoculated with fungal spore and kept under hood. After all

cheese samples were placed in the sterile petri dishes, these plates were packed in a zipper bag and incubated at 10 ^{o}C in refrigerator for 30 days.

3.5.3. Microbiological Analysis

Sampling of meat cuts were conducted at 0, 3, 6 and 9th days for *L. monocytogenes*, *E. coli* O157:H7 and at 0, 2, 4, 6, 8th days for *S.* Enteritidis. Meat pieces of approximately 20 g were homogenized in 180 ml sterile 0.1% peptone water for 2 minutes with a stomacher (BagMixer, France). 1 ml of the homogenized sample was serially diluted with 9 ml of 0.1% sterile peptone water and 0.1 ml sample was spread onto appropriate selective agar medium that is specific for tested bacteria (PALCAM for *L. monocytogenes*, XLD for *S.* Enteritidis and CT-SMAC for *E. coli* O157:H7). After incubation at 37 ^{o}C until being visible, bacterial colonies were checked and counted.

Microbiological analysis of the cheese samples were performed at 0, 10, 20 and 30th days. Before the sampling on day 0, all groups were kept for 2 hours to allow penetration of antimicrobial into cheese. The cheese samples (approximately 20 g) were homogenized in 180 ml of 0.1% sterile peptone water for 2 minutes with a stomacher. All tests were performed in duplicate. The homogenized samples were serially diluted with 0.1% of sterile peptone water. Sampling solution (0.1 ml) was dispersed onto DRBC agar in duplicate. The agar plates were incubated at 30 ^{o}C until mold colonies observed. Visible colonies were numbered and mold loads were calculated on the cheese samples as cfu/g.

3.6. Atomic Force Microscopy (AFM) Analysis

AFM (MMSPM Nanoscope IV) analysis was conducted to observe how natural antimicrobials affect the bacterial cell wall, appendages, flagella or appearance at Izmir Institute of Technology Material Research Centre. Tested bacteria were prepared as explained in 3.1.3. Three times more concentrated solution of antimicrobial agent than MIC value was used in AFM analysis (3% ALF for *E. coli* O157:H7, 1.5% ALF for *S.* Enteritidis and 0.5% ALF for *L. monocytogenes*). 1 ml of inoculum without any dilutions, 1 ml of appropriate medium and 1 ml of antimicrobial agents were mixed in a

sterile tube before incubation at 37 ^{o}C for 2 hours. At the end of treatment time, 3 ml of the mixture was divided into eppendorf tubes and centrifuged at 10.000 rpm (Sigma, Germany) for 3 min. The supernatant was removed, and bacterial pellet accumulated at the bottom of the tubes was suspended in 1 ml of 0.1% peptone. The same procedure was repeated twice to remove unwanted components coming from medium ingredients, cell debris and antimicrobial agent. After last centrifugation, the bacterial cells were collected in another tube. 40 µl of suspension was spread onto a microscope slide and left to dry in the laminar flow hood. The slide was shattered and nearly 1 cm^2 slide piece was analyzed with AFM.

3.7. Statistical Analysis

Data of microtiter plate assays at 12th, 18th and 24th hours were analyzed using one way analysis of variance (ANOVA) with MINITAB (version 13.20). Tukey test was used to compare means at 95% confidence level. Results of combinational tests were analyzed in a model with MODDE 7 (Version 7.0.0.1 Umetrics, Sweden). Bacterial numbers and number of spores were transformed into $\log_{10}$ in dipping applications. All results were reported as means and standard deviations of means were indicated in all graphs.

CHAPTER 4

RESULTS AND DISCUSSIONS

In this study, the individual and combinational microbial activities of four antimicrobials which are promising for usage in food were examined. Concentrations found effective in microtiter plate and disc diffusion assays were applied to meat and cheese samples by dipping method. However, it appears that a standardized test and test conditions have not been developed to evaluate antimicrobial activity of agents against food-borne pathogens, yet. Because some parameters such as inoculum size, incubation time and temperature, culture medium and growth characteristics affect the antimicrobial test results (Burt 2004).

4.1. Individual and Combinational Effects of ALF RE and JG by Microtiter Plate Assay

Activities of ALF, RE and JG and combinations of ALF and RE against three food-borne pathogens, *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* were investigated. The MICs were determined as the lowest concentration of natural antimicrobials that inhibited the growth of bacteria during 24 hours incubation period at 37 ^{o}C in a microtiter plate. Concentrations of each compound were analyzed with one way ANOVA and means compared with Tukey test. Similarities and differences of means at 12^{th}, 18^{th} and 24^{th} hours were indicated with letters ($^{a-d}$) in Table 4.1. All ANOVA analyses were significant at $P>0.05$.

4.1.1. Antimicrobial Activity of ALF

MIC values of ALF were determined as 0.1%, 0.5% and 1% against *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7, respectively (Figure 4.1-a, 4.1-b and 4.1-c). A significant retardation was observed in *E. coli* O157:H7 growth when

0.25-0.75% ALF were used. While 0.05% ALF elongated only the lag phase of *L. monocytogenes*, 0.25% ALF both retarded and decreased the growth of *S.* Enteritidis, markedly. Naidu (2002) also determined the MIC value of ALF as 1% against *E. coli* O157:H7 (4-log) by optical density measurement after 24 hours incubation period.

It was also reported that ALF exhibited antimicrobial activity against *E. coli* O157:H7, *Bacillus* spp., *Pseudomonas* spp., *Klebsiella* spp., multi drug-resistant *Salmonella* Typhimurium DT104, vancomycin-resistant *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus*, radiation-resistant bacteria such as *Brochothrix thermospacta*, *Deinococcus radiopugnans*, *Deinococcus radiodurans*, *Acinebacter radioresistens* and *Methylobacterium radiotolerans*. Therefore, use of ALF could be promising for the decontamination of beef, poultry and ready to eat foods (Naidu, et al. 2003-b). It has been proved that lactoferrin has restricted activity while ALF, immobilized form of lactoferrin, showed broader and stronger activity against pathogens (Naidu, et al. 2003-a). Antimicrobial activity of lactoferrin individually and in combination with RE was also tested against all three bacteria. *E. coli* O157:H7 that is the only bacterium RE and ALF showed powerful synergistic activity. According to our preliminary results, 1% LF did not affect the growth of *E. coli* O157:H7 (data not shown) while ALF inhibited the growth the same concentration. In addition to that, RE and LF did not exerted any synergism against the same bacteria (data not shown).

4.1.2. Antimicrobial Activity of ALF in Double Strength Medium

One of the proposed mechanism of antimicrobial action of ALF is associated with iron binding properties of lactoferrin molecule. The antimicrobial activities of molecules are directly related with medium component. Change in effectiveness of ALF in 2x strength medium (BHI for *L. monocytogenes*, LB for *E. coli* O157:H7 and TSB for *S.* Enteritidis) was tested instead of in 1x strength medium explained in section 3.4.1. MIC value of ALF in 1x and 2x strength medium was found to be as 0.1% and 0.5% against *L. monocytogenes*, respectively (Figure 4.1-a and Figure 4.2-a). Therefore, it is concluded that the MIC of ALF is increased about five fold when nutrient amount is doubled. Findings on *E. coli* O157:H7 and *S.* Enteritidis also supported this result (Figure 4.1-b, 4.2-b and Figure 4.1-c, 4.2-c, respectively). Growth inhibition of ALF observed for *E. coli* O157:H7 and *S.* Enteritidis in 1x medium decreased drastically in

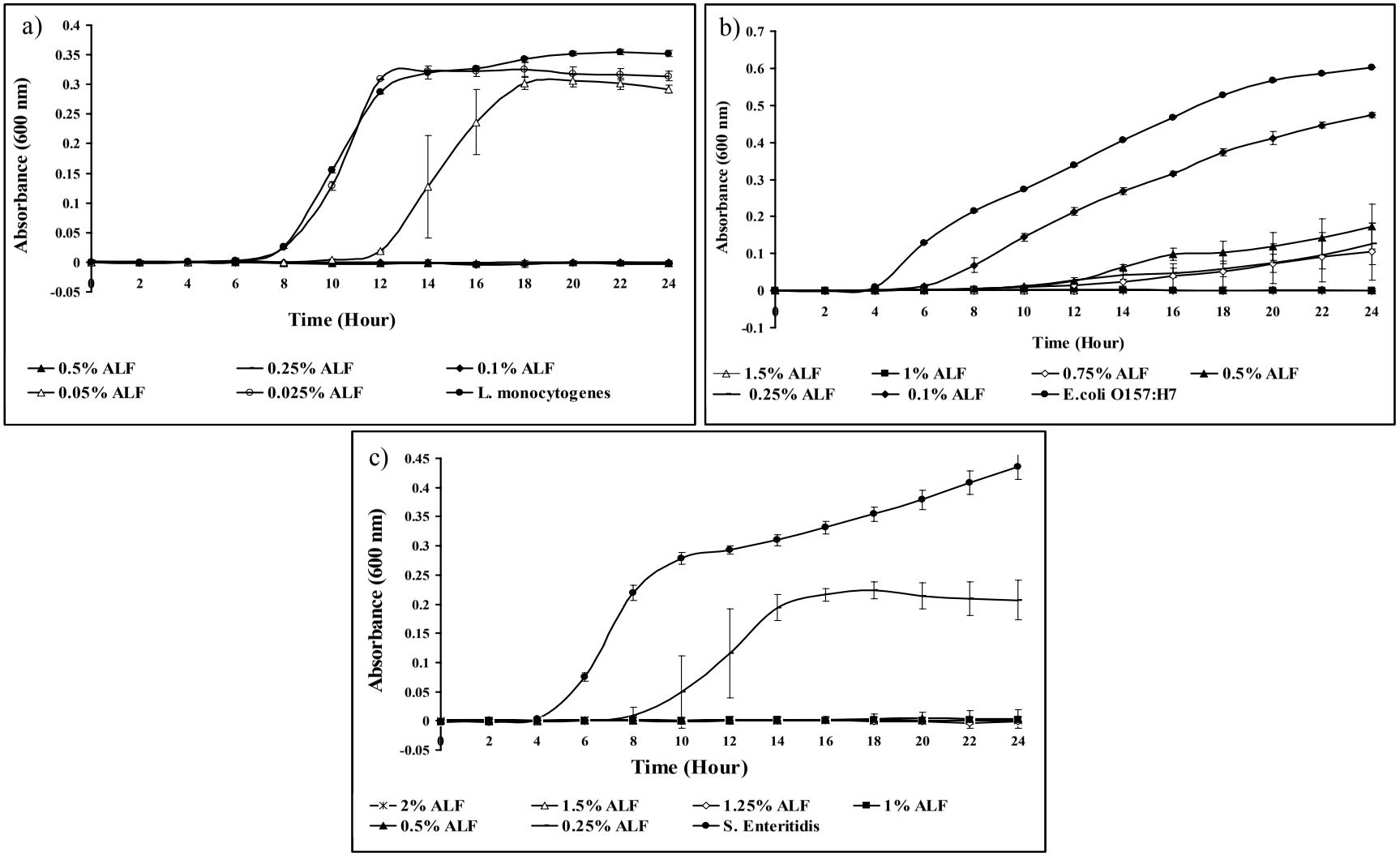


Figure 4.1. Antimicrobial activity of ALF on the growth of three foodborne pathogens: a-) *Listeria monocytogenes*, b-) E*scherichia coli* O157:H7 and c-) S*almonella* Enteritidis. Bars indicate standard error of mean.

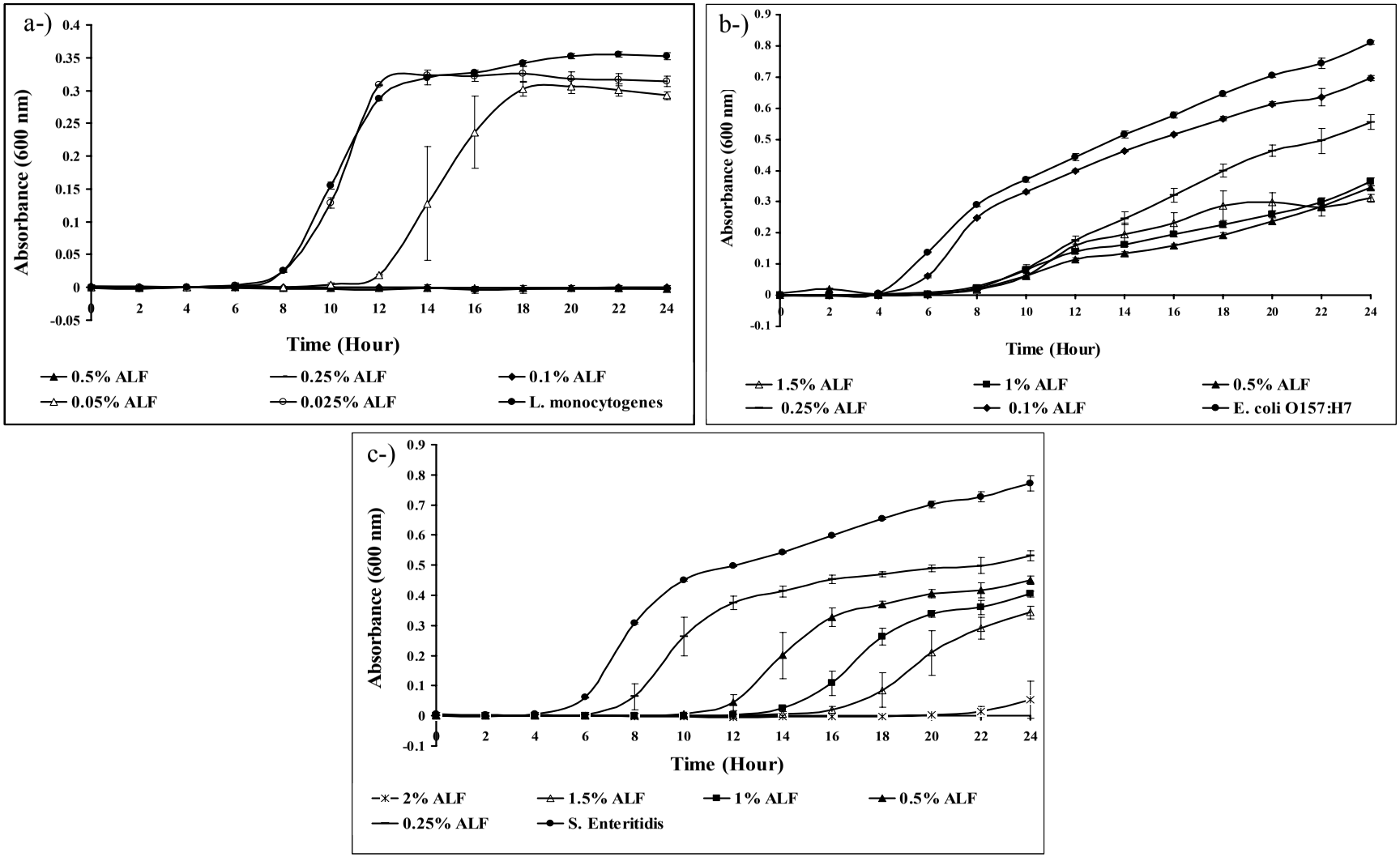


Figure 4.2. Antimicrobial activity of ALF in double strength medium on the growth of three food borne pathogens: a-) *Listeria monocytogenes*, b-) E*scherichia coli* O157:H7 and c-) S*almonella* Enteritidis. Bars indicate standard error of mean.

2x strength medium. ALF most likely saturated with iron ions or other metal atoms, consequently lost its binding ability to bacterial wall components. Another reasonable explanation is that bacteria show more resistant due to high nutrient amount they required for growth. ALF is a new and promising natural antimicrobial agent for food industry. Although it exhibits powerful antibacterial activity, application on food industry may be restricted by limited activity in nutritionally complex and rich environments. Ellison III and Giehl (1991) tested the activity of lysozyme and lactoferrin combination in a medium supplemented with iron, calcium, magnesium, sodium, potassium, chloride to detect whether bactericidal effect would be resulted from level of medium components or osmolarity. They concluded that medium osmolarity was directly in correlation with bactericidal activity according to transmission electron microscopy analysis. One reasonable explanation is that lactoferrin chelates the iron in medium and restricts the bacterial growth. Iron supplementation to medium saturates the LF and the antimicrobial activity of lactoferrin is weakened against *Staphylococcus aureus* (Aguila, et al. 2001). In another study, iron-saturated LF had weak antimicrobial activity than apo-LF against *E. coli*. This result was attributed to decrease in binding ability of ALF to *E. coli* cells because of iron saturation (Recio and Visser, 2000). In contrary, addition of iron in medium did not affect the inhibitory activity of lactoferrin against ß-lactam-resistant *S. aureus*, so iron-biding ability is not the only reason for bacterial growth inhibition (Diarra, et al. 2002). Antimicrobial activity of LF markedly depends on milieu conditions such as pH, iron excess, citrate/bicarbonate ratio and ionic environment adjusted with calcium or phosphate (Naidu 2002).

4.1.3. Antimicrobial Activity of JG

JG displayed no antimicrobial activity against three tested pathogens. Only high concentrations of JG (4000 AU/ml) caused little decrease in the growth of *L. monocytogenes*. Interestingly, growth of *E. coli* O157:H7 and *S.* Enteritidis were promoted by JG (Figure 4.3-a, 4.3-b and 4.3-c).

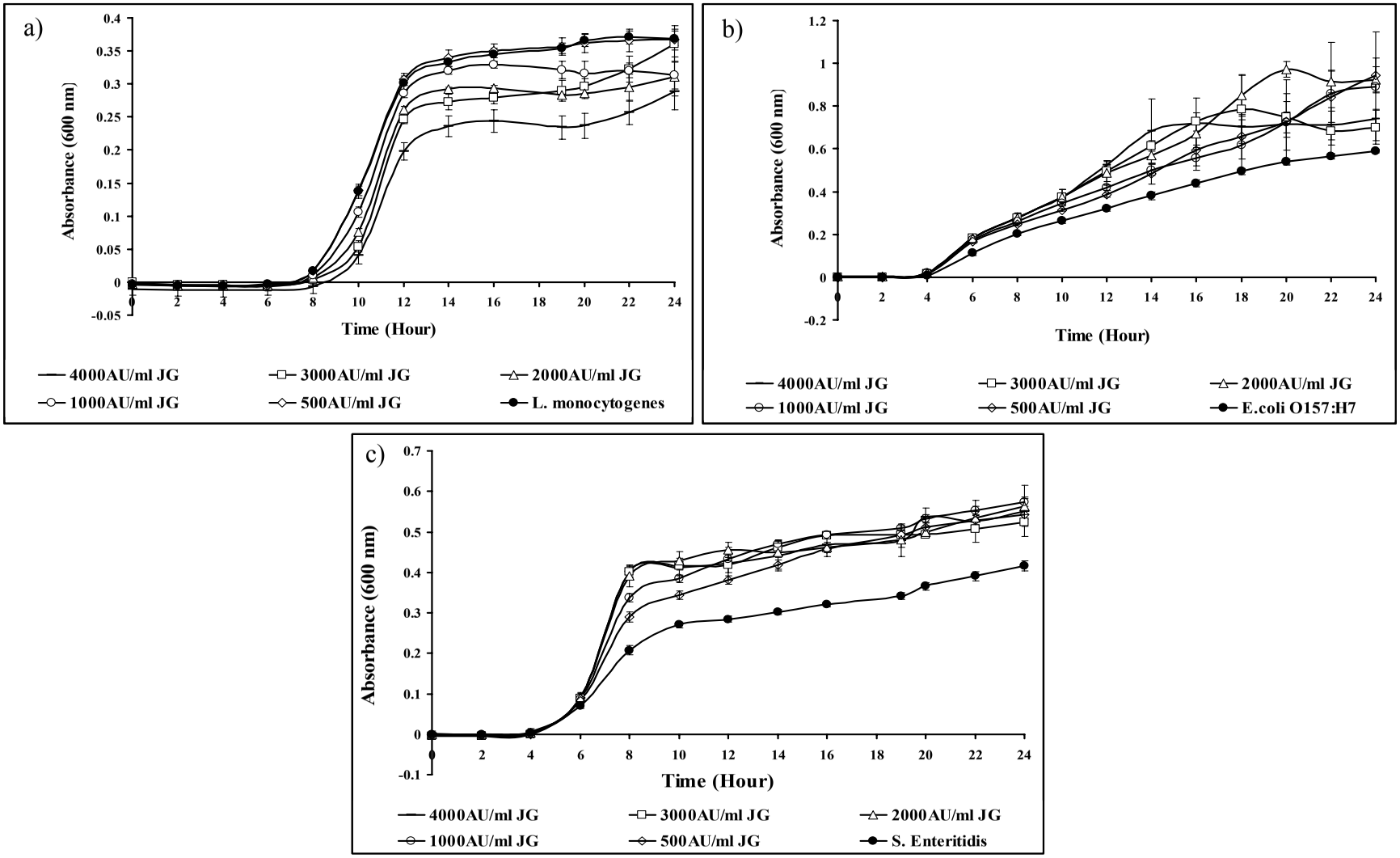


Figure 4.3. Antimicrobial activity of JG on the growth of three foodborne pathogens a-) *Listeria monocytogenes*, b-) E*scherichia coli* O157:H7 and c-) S*almonella* Enteritidis. Bars indicate standard error of mean.

In the previous studies in literature, JG was applied on some pathogens after stress exposure. Baker et al. (2004) tested the antibacterial activity of JG against several pathogens under adverse conditions such as acidic, cold, heat and salt stress. When JG was applied after the heat stress, *L. monocytogenes* and *S. aureus* were the most affected species. On the other hand, *E. coli*, *S.* Typhimurium and *Shigella flexneri* were more sensitive to JG when applied after cold stress. *L. monocytogenes* and *Enterococcus faecalis* showed more sensitivity when exposed to JG under acidic condition. In general, JG exhibited activity against all tested bacteria under stress conditions like other bacteriocins produced by Gram-positive bacteria. In another study, JG were insensitive against indicator cultures including *A. niger*, *Penicillium* spp., *E. coli* ATCC 25922, *E. coli* O:24 B:17, *L. monocytogenes* and *S.* Typhimurium ATCC 14028 (Grinstead and Barefoot 1992).

4.1.4. Antimicrobial Activity of RE

RE in the concentration range of 5-30% did not totally eliminate the growth of *E. coli* O157:H7 and *S.* Enteritidis. However, the higher concentrations of RE caused more inhibition of the growth of these organisms (Figure 4.4-b, 4.4-c). The highest concentration of RE, 30% (vol/vol), used in this study, reduced the growth and extended the lag phases of *E. coli* O157:H7 and *S.* Enteritidis the most. MIC value of RE against *L. monocytogenes* was 15% (vol/vol) RE and 10% (vol/vol) also retarded and decreased significantly the growth of *L. monocytogenes* (Figure 4.4-a). Although MIC value for RE against *L. monocytogenes* seems very high (15% vol/vol RE) at first glance, RE used in this study was not in lyophilized (powder) form. The final volume of extract was approximately 33 ml solution and volume of the extract was accepted as 100% that was further diluted up to the concentration of 15% with de-ionized water.

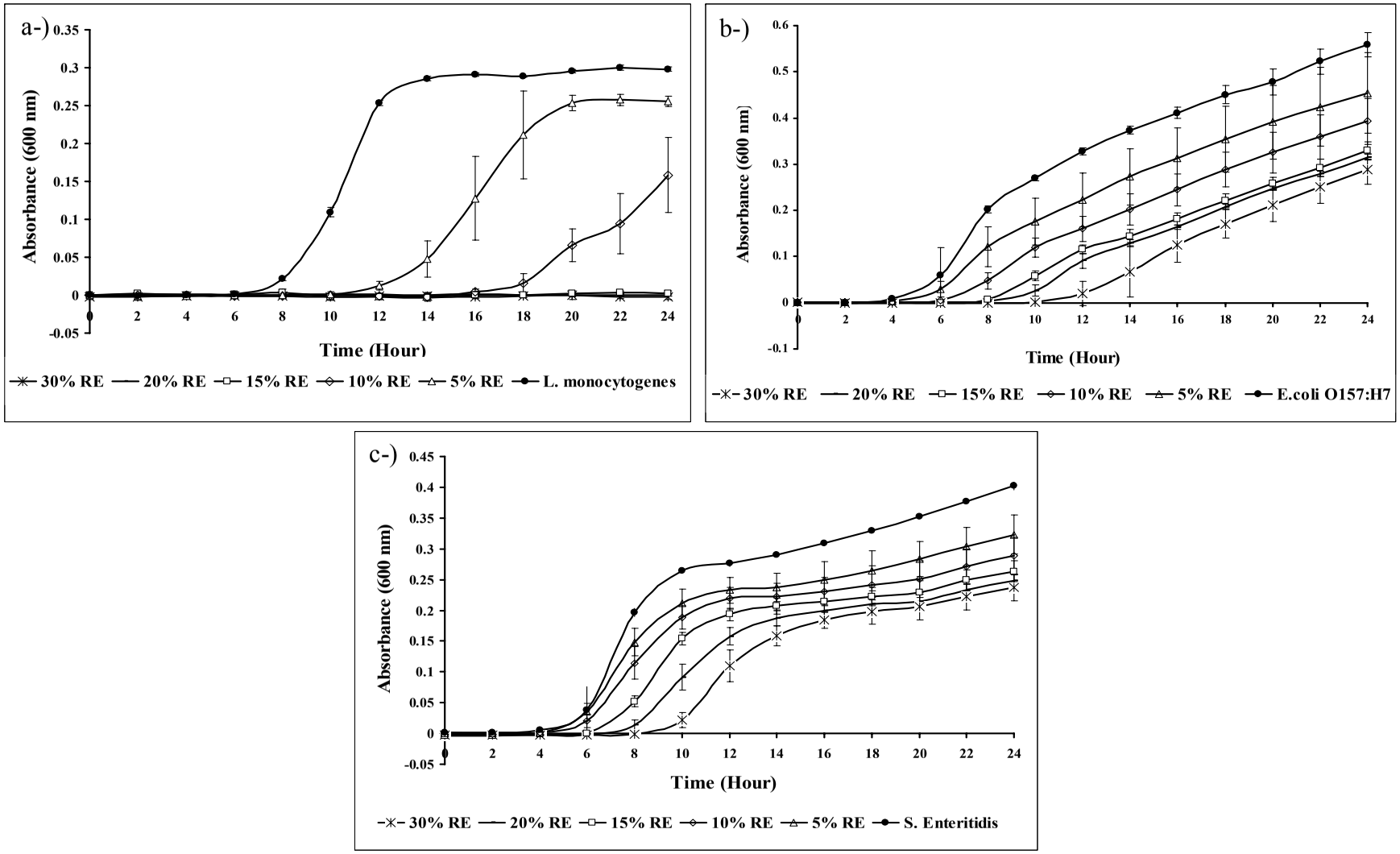


Figure 4.4. Antimicrobial activity of RE on the growth of three foodborne pathogens: a-) *Listeria monocytogenes*, b-) E*scherichia coli* O157:H7 and c-) S*almonella* Enteritidis. Bars indicate the standard error of mean.

Table 4.1. Means of different concentrations of compounds at 12th, 18th and 24th hour were compared with Tukey test. [a-d] the same letters show that there is no statistical difference between different ALF, RE and JG levels at P>0.05

Listeria monocytogenes

ALF (%)			RE (%)			JG (AU/ml)		
12h.	**18h.**	**24h.**	**12h.**	**18h.**	**24h.**	**12h.**	**18h.**	**24h.**
0.5[ab]	0.5[a]	0.5[a]	30[a]	30[a]	30[a]	4000[a]	4000[a]	4000[a]
0.25[a]	0.25[a]	0.25[a]	20[a]	20[a]	20[a]	3000[b]	3000[b]	3000[bc]
0.1[b]	0.1[a]	0.1[a*]	15[a]	15[a]	15[a*]	2000[b]	2000[b]	2000[ab]
0.05[c]	0.05[b]	0.05[b]	10[a]	10[a]	10[b]	1000[c]	1000[c]	1000[ab]
0.025[d]	0.025[c]	0.025[c]	5[b]	5[b]	5[c]	500[d]	500[d]	500[c]
0[e]	0[c]	0[d]	0[c]	0[c]	0[c]	0[cd]	0[d]	0[c]

***Escherichia coli* O157:H7**

ALF (%)			RE (%)			JG (AU/ml)		
12h.	**18h.**	**24h.**	**12h.**	**18h.**	**24h.**	**12h.**	**18h.**	**24h.**
1.5[a]	1.5[a]	1.5[a]	30[a]	30[a]	30[a]	4000[a]	4000[abc]	4000[ab]
1[a]	1[a]	1[a*]	20[b]	20[ab]	20[ab]	3000[a]	3000[ab]	3000[ab]
0.75[ab]	0.75[ab]	0.75[b]	15[bc]	15[ab]	15[ab]	2000[ab]	2000[a]	2000[a]
0.5[b]	0.5[b]	0.5[b]	10[cd]	10[bc]	10[bc]	1000[bc]	1000[bc]	1000[a]
0.25[b]	0.25[ab]	0.25[b]	5[d]	5[c]	5[c]	500[cd]	500[abc]	500[a]
0.1[c]	0.1[c]	0.1[c]	0[e]	0[d]	0[d]	0[d]	0[c]	0[b]
0[d]	0[d]	0[d]	-	-	-	-	-	-

***Salmonella* Enteritidis**

ALF (%)			RE (%)			JG (AU/ml)		
12h.	**18h.**	**24h.**	**12h.**	**18h.**	**24h.**	**12h.**	**18h.**	**24h.**
2[a]	2[a]	2[a]	30[a]	30[a]	30[a]	4000[ab]	4000[a]	4000[a]
1.5[a]	1.5[a]	1.5[a]	20[b]	20[a]	20[ab]	3000[ab]	3000[a]	3000[a]
1.25[a]	1.25[a]	1.25[a]	15[bc]	15[ab]	15[ab]	2000[a]	2000[a]	2000[a]
1[a]	1[a]	1[a]	10[cd]	10[ab]	10[bc]	1000[a]	1000[a]	1000[a]
0.5[a]	0.5[a]	0.5[a*]	5[d]	5[b]	5[c]	500[b]	500[a]	500[a]
0.25[b]	0.25[b]	0.25[b]	0[e]	0[c]	0[d]	0[c]	0[b]	0[b]
0[c]	0[c]	0[c]	-	-	-	-	-	-

* MIC value at 24 hours

Various extraction procedure of rosemary in different solvents exhibit different active component profiles. Methanol, ethanol, water, ether, chloroform are possible solvents for plant extractions (Cowan 1999). Cervenka et al. (2006) studied antibacterial activity of rosemary that was extracted in methanol and chloroform to elucidate which extract was more active against *Arcobacter butzleri*. It was found that chloroform extract was more effective than methanol extract; however, other tested species in these solvents acted adversely. Tsai et al. (2007) also studied antibacterial activity of both aqueous and methanolic extracts of rosemary against *Streptococcus sobrinus*. The ethanolic extract of rosemary showed significantly higher antibacterial activity than aqueous extract. In a similar study conducted by Sagdic and Ozcan (2003), hydrosols of rosemary against fifteen bacterial species were not effective. This outcome is more likely originated from different chemical composition of extracts as a result of different solvents used in extraction. Therein, in this study, ethanol was preferred since it is good solvent and safe for food application.

Comparisons of our data about antimicrobial activity of rosemary with previously published data may give misleading results. In the literature, different extraction methods and various antimicrobial testing methods are used. Trivial modifications of same testing method can produce incredibly unlike results or composition of the same plant collected from different environments, climates and seasons would be highly variable (Oussalah, et al. 2006, Celiktas, et al. 2005, Hammer, et al. 1999).

In microtiter assay, antimicrobial agent directly interacts with bacteria in broth medium. As a result, microtiter plate assay has more advantages than disc diffusion or well diffusion assay which are based on diffusion rate of tested agent. Rosemary extraction was carried out according to a modified procedure of Madsen et al. (1998). When tested using disc diffusion assay, RE (20 $\mu l/cm^2$ squared paper disc) did not have any antimicrobial activity. Celiktas et al. (2005) also demonstrated similar result: methanol extract of rosemary did not inhibit growth of a panel of bacteria except for *S. aureus* by disc diffusion technique. However, in another study employed by Del Campo et al. (2000), MIC value of ethanolic solution of commercial rosemary extract was 0.5% for *L. monocytogenes* while Gram negative bacteria, *E. coli* and *S.* Enteritidis, were not affected up to 1% ethanolic extract.

The antimicrobial compounds in herbs and spices are generally essential oils and phenolic compounds. In the literature, it was demonstrated that fractions of essential

oils exhibited antimicrobial activity *in vitro*. (Oussalah, et al. 2006, Oussalah, et al. 2007, Smith-Palmer, et al. 1998, Moreira, et al. 2005, Singh, et al. 2003, Burt and Reinders 2003). However, there is evidence that total plant extract shows stronger antimicrobial activity. Because different components of plant may have different targets on cell and synergistic interactions among them is possible. (Oussalah, et al. 2006). For this reason, total extract of rosemary was preferred in this study.

4.1.5. Combinational Activity of Antimicrobials

In view of the preliminary experiments, ALF and RE was determined as the agents that can be screened for interactive activity. The concentration ranges were chosen between the MIC values and a lower concentration for two agents. ALF and RE together acted synergistically against *E. coli* O157:H7 while they did not exhibit any interaction when tested on *L. monocytogenes* and *S.* Enteritidis (Figure 4.5-a, 4.5-b and 4.5-c). MIC value of 1% ALF was found for *E. coli* O157:H7; however all combinations of RE (20-15-10%) with 0.5 and 0.3% ALF repressed the growth of this bacterium. However, retardation of growth depends on RE concentration at 0.1% ALF level. (Figure 4.5-b). For *L. monocytogenes* and *S.* Enteritidis, MIC value was not determined but growth of *L. monocytogenes* was reduced in high concentration of ALF (Figure 4.5-a) and no synergistic activity was observed for *S.* Enteritidis (Figure 4.5-c).

Synergistic activity of ALF and RE was analyzed statistically. Prediction plots revealed that concentrations of both ALF and RE were significant against *L. monocytogenes* and *S.* Enteritidis through 24 hours (Figure 4.6-a, 4.6-b). ALF was the main agent that directly reduced the number of *E. coli* O157:H7 while RE had slight effect. RE played an enhancer role for the activity of ALF. ALF was significant through 24 hours but RE lost slightly their activity after 18th hour. (Figure 4.6-c). Overall, ALF was main agent which inhibited significantly the growth of three pathogens through 24 hours.

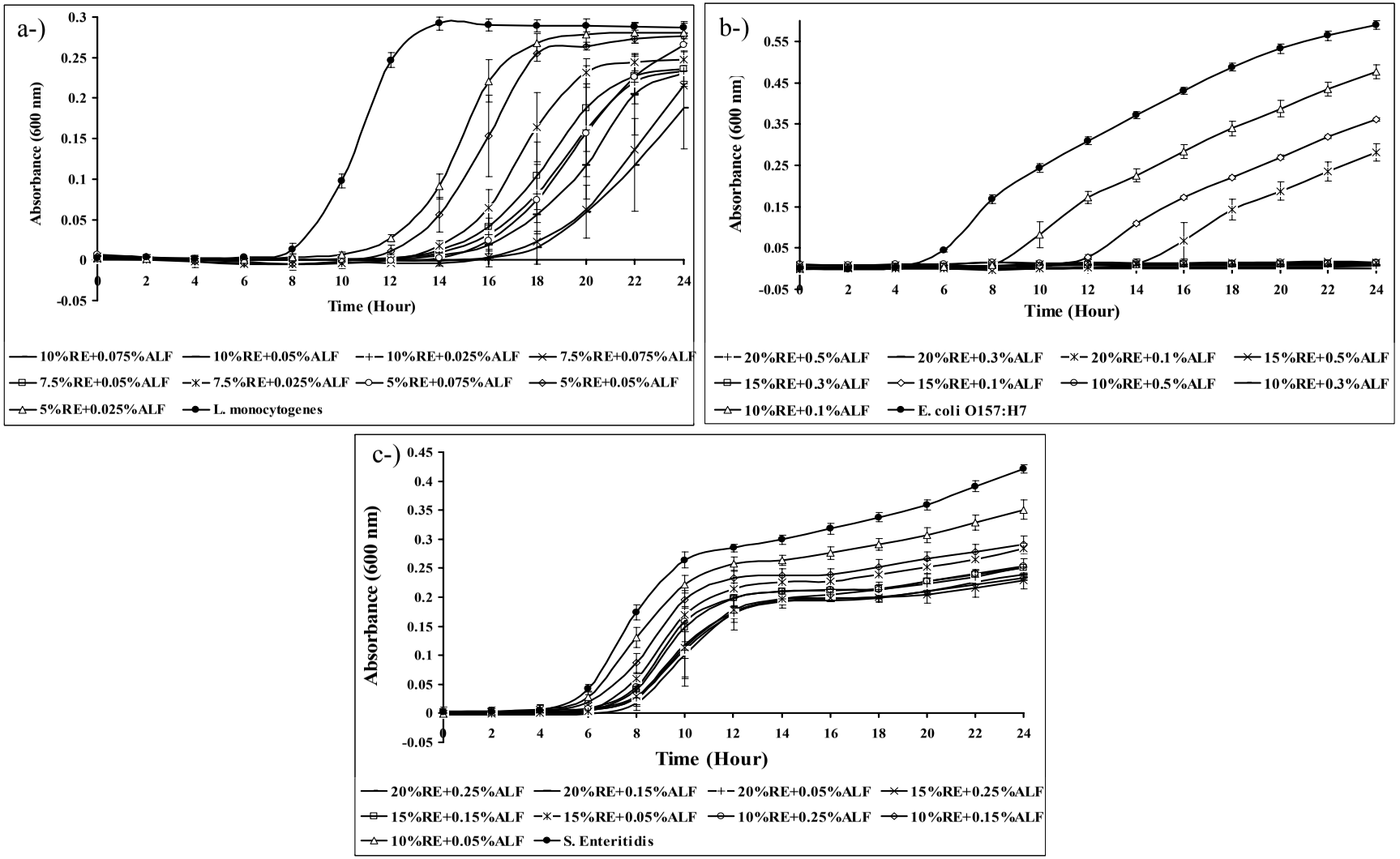


Figure 4.5. Effect of combinations of ALF and RE on the growth of three foodborne pathogens: a-) *Listeria monocytogenes*, b-) E*scherichia coli* O157:H7 and c-) S*almonella* Enteritidis. Bars indicate standard error of mean.

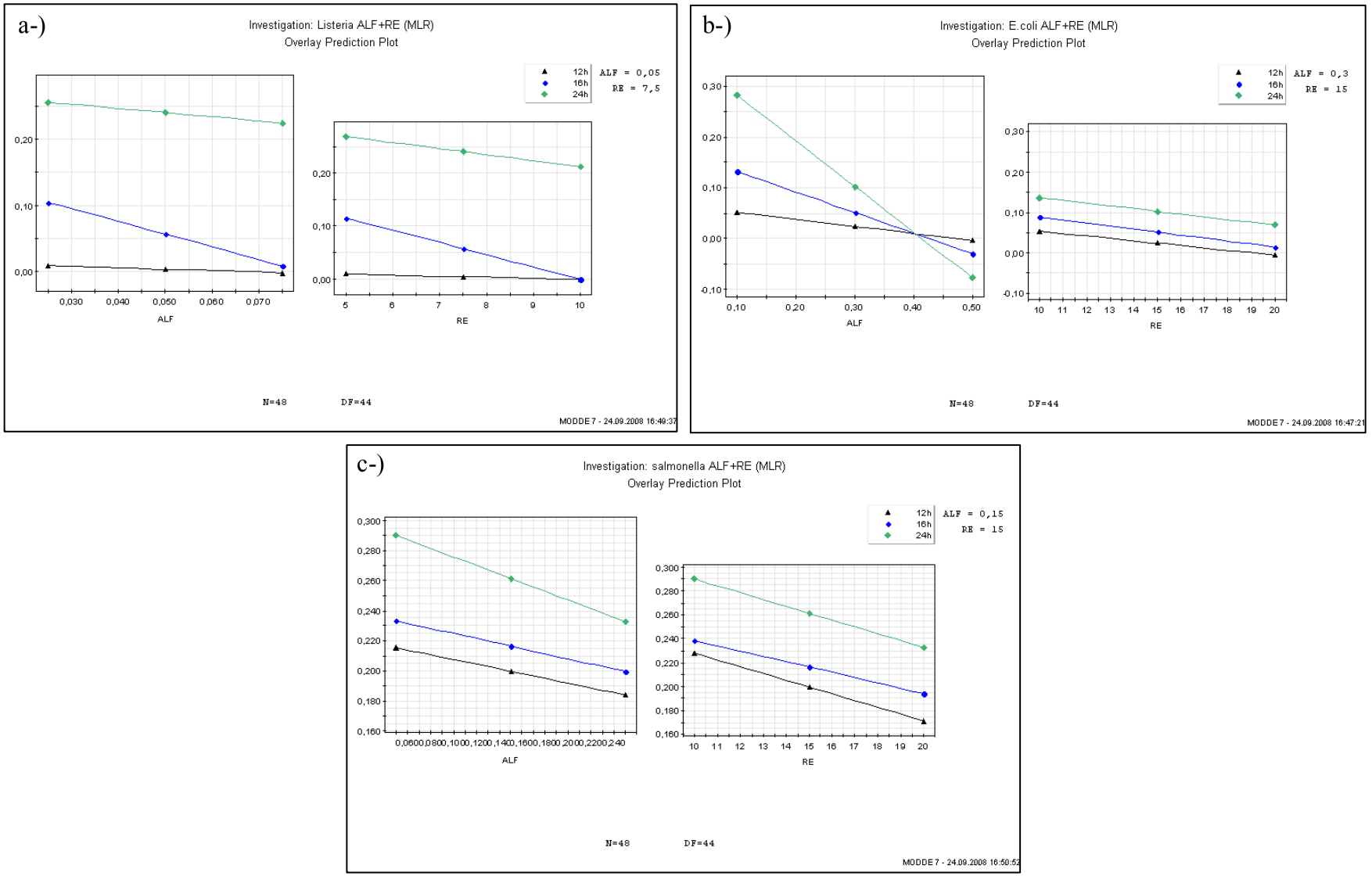


Figure 4.6. Prediction plots at 12th, 16th and 24th h. for a-) *Listeria monocytogenes*, b-) E*scherichia coli* O157:H7 and c-) S*almonella* Enteritidis.

Synergistic interaction between lactoferrin and some antimicrobials was also demonstrated in literature by many researchers. Cleveland and Tchikindas (2001) reported the synergistic activity between nisin and lactoferrin against *E. coli* O157:H7 and *L. monocytogenes* Scott A. Similarly, combination of LF and nisin, and again combination of LF and monolaurin exhibited enhanced antimicrobial activity against *L. monocytogenes* and *E. coli* O157:H7, respectively (Branen and Davidson 2004). In another study, it was shown that lysozyme enhanced the activity of lactoferrin against *Vibrio cholerae*, *Salmonella* Typhimurium and *E. coli*. Although these agents are bacteriostatic when they are used individually, they had bacteriocidal activity together (Ellison III and Giehl 1991). Combinational treatment of lactoferrin with fluconazole increased the activity of lactoferrin against clinical *Candida* isolates (Kuipers, et al. 1999). Similarly, Diarra et al. (2002) reported that the use of lactoferrin and penicillin in combinations also acted synergistically against *S. aureus*. Enhanced activity of lactoferrin against pathogens with certain antibiotics was also observed by Naidu and Arnold (1994). Solomakos et al. (2008) showed a good example of synergistic activity between a plant compound and a polypeptide bacteriocin. Combination of thyme essential oil with nisin exerted synergistic activity against *L. monocytogenes*.

Generally, peptide antimicrobials have more powerful activity than plant extract and essential oils. Our results also supported that idea, for example MIC value of ALF was 0.1% while it was 15% for RE on *L. monocytogenes*. Plant extracts and essential oils include a large number of components which have different targets in the bacterial cell. It is more reasonable to use them with peptide antimicrobials or antibiotics to screen possible synergistic activity. It was elucidated from our data that activity of ALF against *E. coli* O157:H7 was enhanced nearly three fold when applied in combination with RE in this study.

4.2. Individual and Combinational Effects of Antimicrobials Tested by Disc Diffusion Assay

Disc diffusion assay was also performed to observe the antimicrobial activity of natural agents against the same pathogens used in microtiter plate assay.

4.2.1. Antibacterial Properties of ALF, RE and JG

None of the all concentrations (ALF: 4, 3, 2, 1.5, 1% [wt/vol]; RE: 60, 40, 30, 20, 10% [vol/vol]; JG: 4000, 3000, 2000, 1000, 500 AU/ml [vol/vol]) of tested antimicrobial agents were found effective against *L. monocytogenes*, *E. coli* O157:H7 and *S.* Enteritidis when disc diffusion assay were employed. In other words, no zone formation was observed around the paper discs, impregnated with antimicrobial agents, for any of the concentrations (Figure A.1, A.2 and A.3).

Combinations of ALF with RE (25% RE + 3% ALF, 35% RE + 1% ALF, 50% RE+ 2% ALF) were also displayed no synergistic activity against *L. monocytogenes*, *E. coli* O157:H7 and *S.* Enteritidis by disc diffusion assays (Figure A.4).

ALF showed strong antimicrobial properties against the same food-borne pathogens when tested with microtiter plate assay. Similar results were not achieved with the disc diffusion assay, although tested concentrations were increased more than two fold of what was used in microtiter plate. The reason of this may be due to high molecular weight of ALF. The suitable substrate that lactoferrin was immobilized is a big food-grade molecule glycosaminoglycan such as galactose rich polysaccharide or carrageenan (Naidu 2002). Disc diffusion assay depends not only on power of antimicrobial substance but also on diffusion rate of antimicrobial agent in the solid agar medium.

Gram-positive bacteria have been reported to be more susceptible to antibacterial agents and plant extracts than Gram-negatives (Bozin, et al. 2007, Oussalah, et al. 2006, Lopez, et al. 2005, Burt 2004, Pintore, et al. 2002, Smith-Palmer, et al. 1998). Our findings are in agreement with this general assumption. When all tests for each antimicrobial agent were considered, *L. monocytogenes* was the only strain susceptible to RE with MIC value of 15% RE (vol/vol) via microtiter plate assay. In addition, it had the lowest MIC value of 0.1% for ALF and only its growth was inhibited slightly by JG.

4.2.2. Antifungal Properties of NA, RE, ALF and JG

NA was clearly showed antifungal activity against tested molds: *A. niger* and *P. roquefortii* by disc diffusion assay. The MIC value was determined as 750 ppm for both

molds tested and the inhibition zone diameters were 1.5 and 3.02 cm for *A. niger* and *P. roquefortii*, respectively. In higher concentrations, zone diameter was higher and reached up to 3.97 cm against *A. niger* and 5.32 cm against *P. roquefortii* at 5000 ppm. Growth of both fungi were not affected by any concentrations of ALF, RE and JG. Also combinations of any concentrations of NA and RE did not show any synergistic or antagonistic activity against both molds (Ture, et al. 2008); although in previous studies several plant extracts were very effective against molds and yeasts (Stark 2003).

Table 4.2.General view: MIC value and effectiveness of natural antimicrobials against tested food-borne pathogens and molds by microtiter plate and disc diffusion assay

	Microtiter Plate Assay				Disc Diffusion Assay			
Pathogens	**JG (AU/ml)**	**RE (%)**	**ALF (%)**	**NA (ppm)**	**JG (AU/ml)**	**RE (%)**	**ALF (%)**	**NA (ppm)**
L. monocytogenes	Reduce growth	15	0.1	Not applied	No activity	No activity	No activity	Not applied
E. coli O157:H7	Promote growth	Reduce growth	1	Not applied	No activity	No activity	No activity	Not applied
S. Enteritidis	Promote growth	Reduce growth	0.5	Not applied	No activity	No activity	No activity	Not applied
A. niger	Not applied	Not applied	Not applied	Not applied	No activity	No activity	No activity	750
P. roquefortii	Not applied	Not applied	Not applied	Not applied	No activity	No activity	No activity	750

4.3. Application of Antimicrobials on Meat by Dipping

When applied individually, both 0.5% ALF and 45% RE reduced the number of *L. monocytogenes* on meat by 2 logs (Figure 4.7-a and 4.7-b). The 30% RE also showed 1 log reduction in *L. monocytogenes* numbers. There was no log reduction in 0.5-1.5% ALF concentrations against *S.* Enteritidis on meat (Figure 4.7-c). However, the highest concentration of ALF (2.5%) reduced the growth of this bacterium approximately 0.8 log. When ALF at the range of 1-3-5% applied on meat inoculated with *E. coli* O157:H7, 3 and 5% ALF caused 2 log reduction on this bacterium at the end of the 9 days incubation at 10 ^{o}C. When the graphs are examined carefully, it is clear that *L. monocytogenes* grows slower than other two pathogens on meat due to lower incubation temperature. The growth of *E. coli* O157:H7 was much faster compare to other two organisms when applied on meat. Solomakos et al. (2008) demonstrated that inhibitory

activity of nisin, a polypeptide bacteriocin, against *L. monocytogenes* in minced beef. Antimicrobial activity was depending on the supplementation level of nisin, the storage temperature and the strain tested. They also showed synergistic activity against *L. monocytogenes* in minced beef by addition of thyme essential oil and nisin.

Chiu and Kuo (2007) demonstrated that lactoferrin exhibits antioxidative and antimicrobial properties in hot-boned ground pork with addition of 80 mg/kg. They suggested that addition of LF extend the shelf life of ground pork. Naidu et al. (2003-b) reported that 2% ALF reduced the number of radiolabeled *E. coli* O157:H7 on beef surface by spraying. They also reported in the same study that ALF blocked the attachment of S. Typhimurium on broiler skin surface when sprayed onto. Natural extract was reported for their antimicrobial activity on meat in literature is LactiSAL®, lipoprotein based dairy extract. Spraying application of LactiSAL® reduced the number of both *E. coli* O157:H7 and *Salmonella* Typhimurium inoculated on meat (Pearce and Bolton 2007). Tu and Mustapha (2002) dipped the fresh beef cubes inoculated with *Brothotrix thermosphacta* or *Salmonella* Typhimurium into nisin or nisin/EDTA solution. They found that neither treatment inhibited significantly the growth of *Salmonella* Typhimurium. However both treatments increased the shelf life of beefs by inhibiting completely the growth of *Brothotrix thermosphacta*. Anang et al. (2007) tested antimicrobial activity of lauricidin against *L. monocytogenes*, *S. enteritidis* and *E. coli* O157:H7 on chicken breast by dipping application. *L. monocytogenes* was the most susceptible bacteria than *S. enteritidis* and *E. coli* O157:H7 by dipping of chicken breast into lauricidin solution. Spraying of mixture of rosemary extract and vitamin-C onto beef samples showed antioxidant activity as well as reducing the microbial load and extending the shelf life of beef samples from 10 to 20 days.

To prevent the growth or reduce the number of pathogens on foods, combinations of intervention applications such as hot or cold water washing, organic acid spraying and steam treatment are more effective than single or 2-treatment combinations in multi hurdle technology (Delmore, et al. 1998, Koohmaraie, et al. 2005). Application of natural antimicrobials can also be used individually or in combinations with other treatments in this technology. Naidu et al. (2003-b) treated beef sample with hot water (180 ^{o}F for 30 sec.), cold water (10 sec.) and 2% lactic acid to decontaminate *E. coli* O157:H7 from beef. 1 %ALF was sprayed onto beef (10 sec.) that is inoculated with *E. coli* O157:H7 between hot water and cold water treatments and this application was successful 72.2% for detachment of *E. coli* O157:H7 cells from

beef tissue. If 2% ALF sprayed, the efficacy was almost 100% *E. coli* O157:H7 detachment/g of beef tissue. Hot water and acid treatment reduced the *E. coli* O157:H7 cells but debris of this bacterium was still firmly attached to beef tissue; however 2%ALF treatment removed these attached cell debris and residues. In our findings on *E. coli* O157:H7 cells *in vitro* supported this result. According to atomic force microscopy analysis shown in Figure 4.8, ALF damages the fimbria of *E. coli* O157:H7 cells in broth medium. Fimbria play main role for bacterial attachment to epithelial surface and to collagen surface on beef tissue. In this sense, ALF with no odor, no flavor, no color and no alternation of treated meats is excellent candidate for multi-hurdle approach. When ALF applied onto carcass surfaces, it gives damage to cell surface structures such as fimbria that is responsible for bacterial attachment to tissue surface and prevents the attachment of bacteria during slaughtering (Naidu 2002).

In our study, one, three and five folds of MIC concentrations of antimicrobial agents were required to reduce the bacterial number on meat surface. Moreira et al. (2007) used also two three or four times the MIC value of tea tree essential oils in actual food applications to reduce pathogen number. In general, antimicrobials lost their activity in food systems due to the interaction between antimicrobial agents and food components (proteins, fats and salts), solubilization of antimicrobial on food surface or protection of pathogens from antimicrobials by sheltering to rough surface of foods (Moreira, et al. 2007, Ceylan and Fung 2004, Singh, et al. 2003, Dawson, et al. 2002, Smith-Palmer, et al. 2001, Hao, et al. 1998, Shelef 1983). Thereof, usage of high amount of plant extracts as an antimicrobial agent in food industry can lead to undesirable organoleptic properties and, loss of labor and money. However, these extracts, showing antimicrobial properties, in combination with other peptide antimicrobials can control pathogens by minimizing unwanted organoleptic properties. Extracts which are effective in low concentrations can help to preserve foods without affecting sensory quality. One more advantage of plant extracts is their strong antioxidant activity. The more promising application of plant extracts is combination with other preservation techniques such as heat, cold, pressure, low O_2 or chemical preservatives treatment (Singh, et al. 2003).

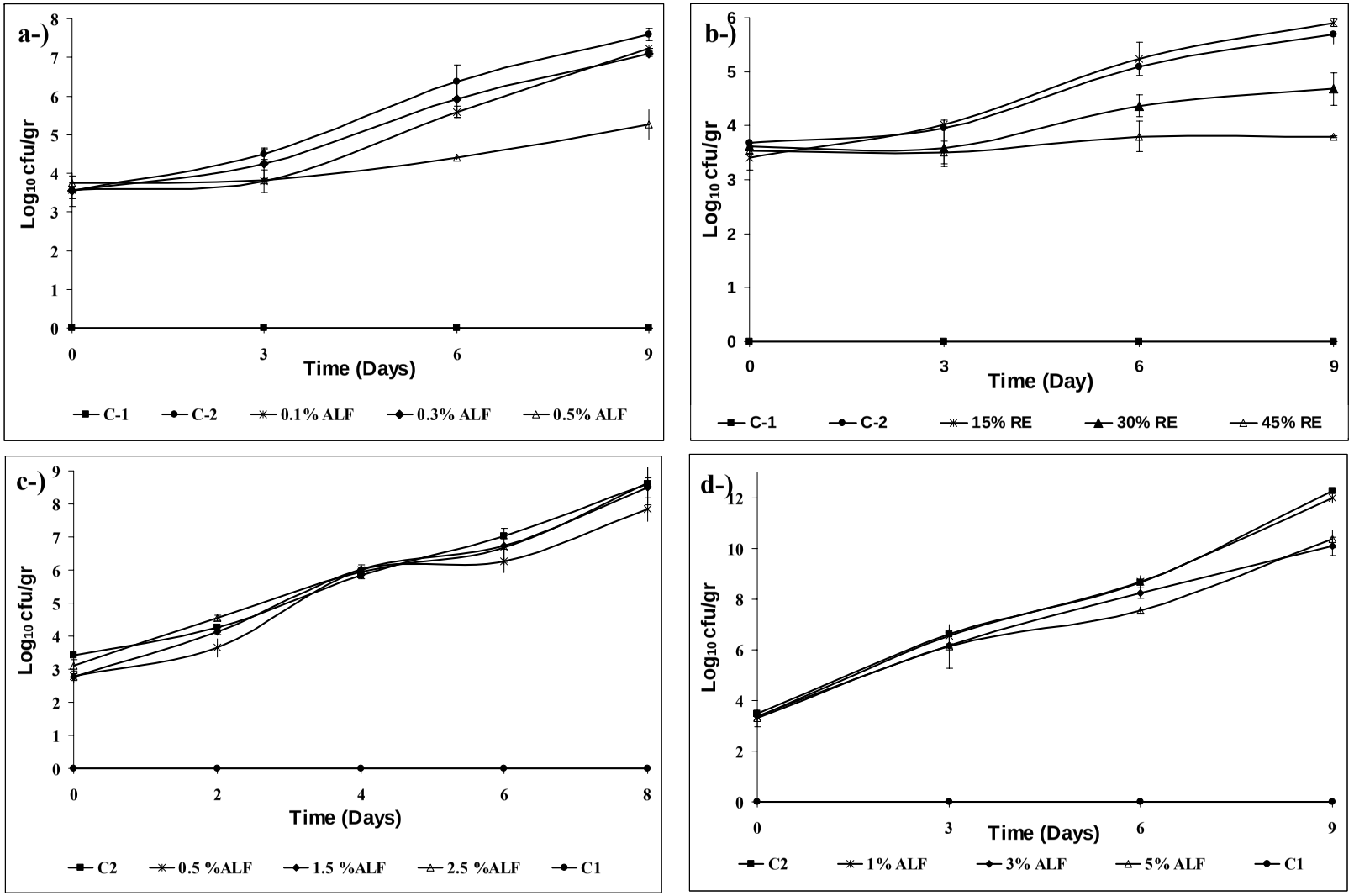


Figure 4.7. Activity of antimicrobials by dipping application of meat samples a-) ALF against *L. monocytogenes* b-) RE against *L. monocytogenes* c-) ALF against *S.* Enteritidis d-) ALF against *E. coli* O157:H7.

4.4. AFM Results

The surface structure of pathogen cells were screened under AFM microscopy to enlighten morphological or structural changes of cells if available. AFM analysis revealed that ALF targets appendages of *E. coli* O157:H7 cells. ALF caused shedding of fimbria (hair like structure) when compared with control group (Figure 4.8-a and 4.8-b). Fimbria are responsible for bacterial attachment and colonization on mucosal cell surface in colon. Naidu et al. (2002) reported similar findings in a study on *E. coli* O157:H7.

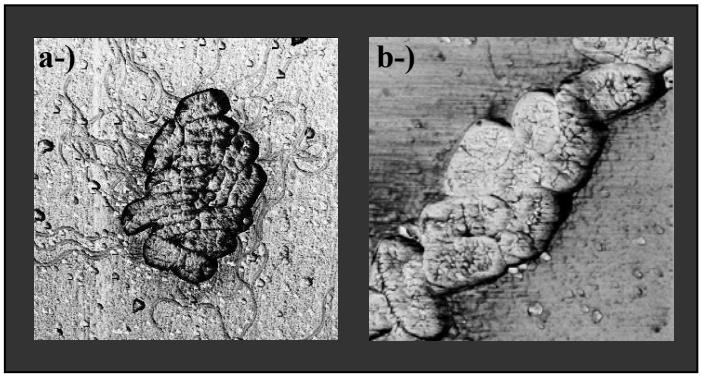


Figure 4.8. AFM analysis showing the effect of ALF on *E. coli* O157:H7 cells a-) No ALF treatment b-) 3% ALF treated bacteria

When 1.5% ALF applied to *S.* Enteritidis, the organism did not show any significant morphological or structural changes. As seen in Figure 4.9-a and 4.9-b, appendages of *S.* Enteritidis were similar for both ALF treated and non-treated groups.

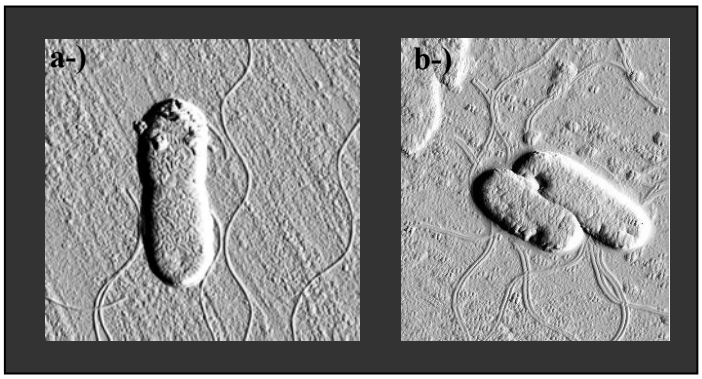


Figure 4.9. AFM analysis showing the effect of ALF on *S.* Enteritidis cells a-) No ALF treatment b-) 1.5% ALF treated bacteria

Similarly, when 0.5% ALF was applied to *L. monocytogenes*, the cells also did not show any significant morphological or structural change (Figure 4.10-a and 4.10-b).

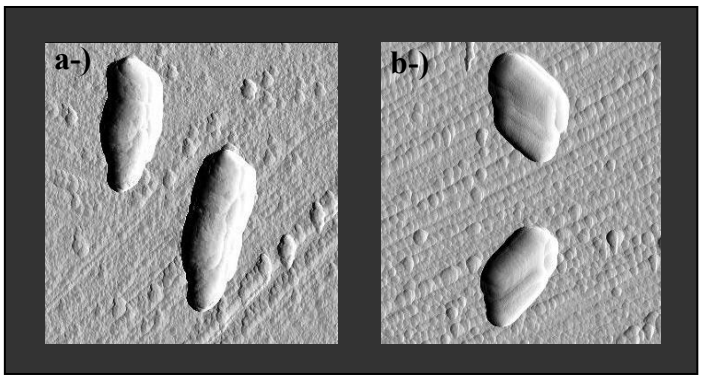


Figure 4.10. AFM analysis showing the effect of ALF on *L. monocytogenes* cells a-) No ALF treatment b-) 0.5% ALF treated bacteria

The mode of action of LF is still under investigation with several putative mechanisms by other researchers also. Some researchers claim that LF has ability to chelate iron that is a vital element for bacterial growth (Bullen 1981). *In vivo* iron level is regulated by binding of iron by unsaturated LF and transferring. Iron restriction is a natural resistance mechanism to bacterial infection in vivo (Bullen, et al. 2004). Another slight probability is insertion of highly cationic LF molecule, particularly the N-terminal domain, into anionic cell membrane (Ellison, et al. 1988); therefore, the membrane permeability is increased (Nibbering, et al. 2000). The other researchers also claimed that LF interacts with bacterial cell wall (Dionysius, et al. 1993, Kalmar and Arnold 1988, Ellison, et al. 1988, Bortner, et al. 1986). Dionysius et al. (1993) tested the activity of iron-free LF (apo-LF), Zn-saturated-LF and iron-saturated LF (holo-LF) *in vitro*. They reported inhibitory effect of apo and Zn-saturated form of LF whereas iron-saturated LF did not inhibit the growth of two *E. coli* strains. Zn-saturated and apo-LF were similar structurally but holo-form was dissimilar. Conformational change resulting with reduced interaction occurred on structure of LF by binding with Fe. Therefore, inhibitory mechanism of LF depends on bacterial cell interaction. Similar results were demonstrated by Bortner et al. (1986). Iron-free LF was bactericidal against *Legionella pneumophila* but iron-saturated LF had no activity. Arnold et al. (1980) and Ellison et al. (1988) also showed that saturation of LF with iron alleviates bactericidal activity of LF due to the conformational change. All results would support the idea that LF

interacts with bacterial cell wall. Although interaction of LF with lipopolysaccharide (LPS) layer of Gram negatives seems more reasonable, other explanations should not be ignored.

4.5. Application of Natamycin on Kashar Cheese by Dipping

NA reduced significantly the growth of both molds on cheese stored at refrigerator (10 ^{o}C) by dipping application. Growth control groups, inoculated with spore culture, were completely covered with *P. roquefortii* while growth was like pin head on NA treated groups (Figure 12-a). In another word, *P. roquefortii* could not expand on NA treated cheese sample. Similarly, no *A. niger* growth was observed on cheese cuts treated with NA when compared with control group (Figure 12-b). Microbiological sampling also confirmed the appearance of cheese sample. There was approximately 4 log difference between growth control group and treatment groups for *A. niger* (Figure 11-a). NA again reduced log number of *P. roquefortii* spores by dipping application (Figure 11-b). When the growth of these two molds compared, it was noticed that *P. roquefortii* grows faster than *A. niger* on kashar cheese. Because, *A. niger* covered partially the cheese surface at the end of 30 day. However, *P. roquefortii* covered completely the cheese surface before sampling day of 10th. The fungal spore load of growth control group was same at 10th, 20th and 30th sampling day. As stated in disc diffusion assay, *P. roquefortii* needed more incubation time (day) on solid medium (PDA agar). In conclusion, kashar cheese is very suitable environment for the growth of *P. roquefortii*.

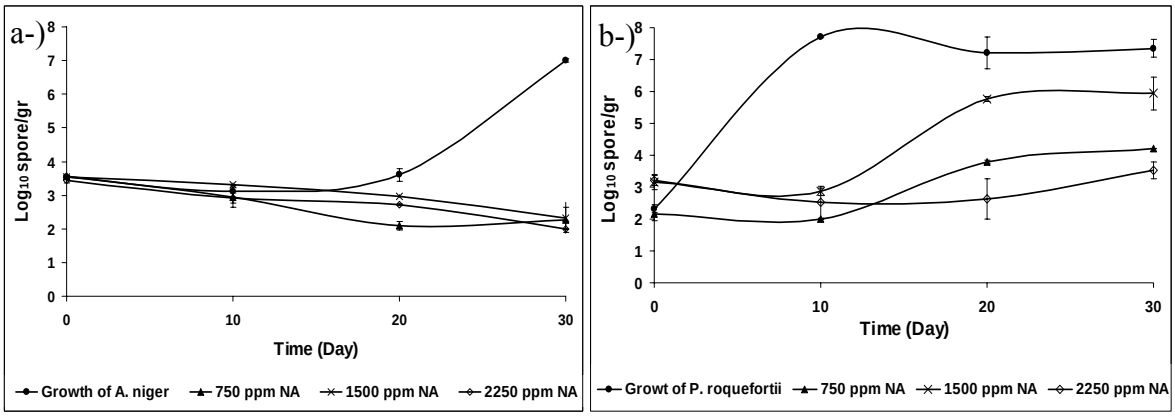


Figure 4.11. Microbiological analysis results of growth of a-) *A. niger* b-) *P. roquefortii* at 0, 10th, 20th and 30th days.

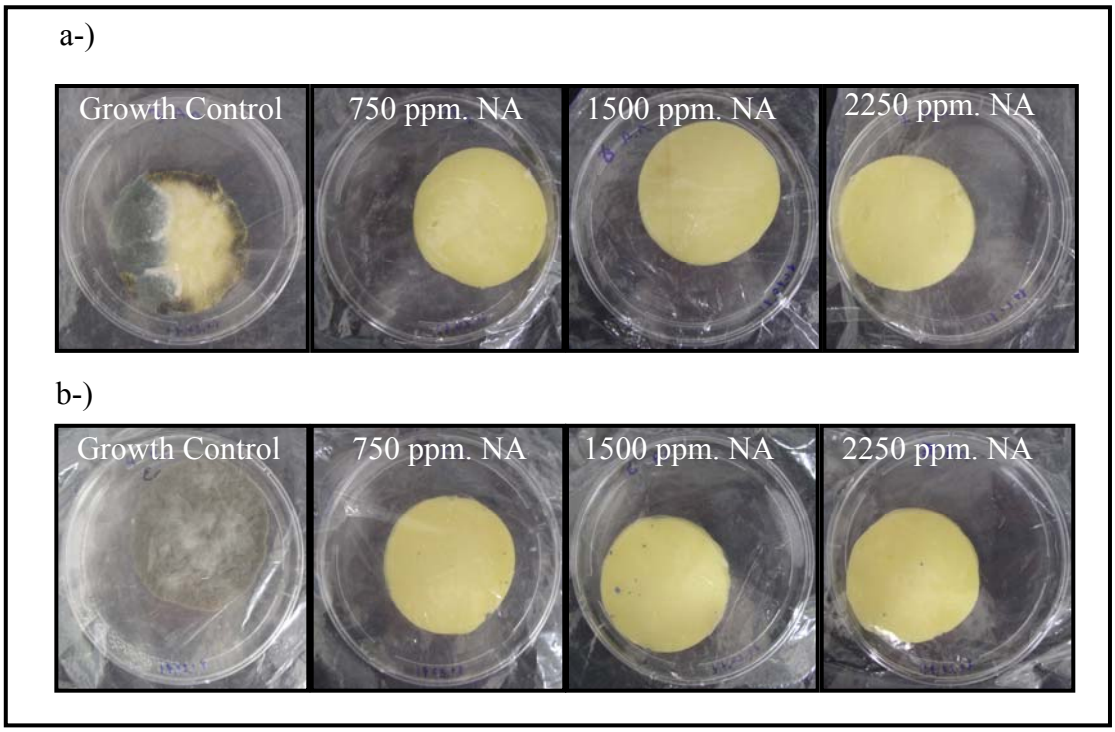


Figure 4.12. Growth of a-) *A. niger* b-) *P. roquefortii* on cheese sample treated with or non-treated with NA.

In the study performed in our laboratory, cellulose and protein based packaging materials incorporated with natamycin inhibited significantly the growth of same molds on kashar cheese (Ture 2008). Yildirim et al. (2006) demonstrated that natamycin both alone by dipping treatment and in casein coating reduce the visible growth of molds in ripening period of kashar cheese for three weeks and a month, respectively. In a similar study conducted in ripening period of kashar cheese, natamycin alone and combined with packaging material prevented mold formation for two months (Var, et al. 2006). According to FDA food additive regulations, natamycin can be applied on foods by dipping or spraying using an aqueous solution containing 200-300 ppm natamycin (FDA 2001). In our study, the lowest concentration for dipping application was 750 ppm which was determined as MIC value in vitro. This concentration seems to be too high; however commercial powder (Pimalac®) contains 50% NA and 50% lactose. In our study, 750 ppm natamycin displayed powerful antifungal activity and good visible appearance, but lower concentrations can be tested.

4.6. Future Studies and Needs

More research, especially molecular approach, is needed in this field in order to enlighten the action mechanism of natural antimicrobials on these and different pathogens. Also, number of tested microorganism can be expanded to other pathogenic bacteria, fungi and viruses. Antibacterial activity of phenolic fractions of rosemary can be tested separately both *in vitro* and on food samples. Then, sensory analysis can be applied on food treated with antimicrobial agents to determine potential usage of antimicrobials in the food industry. Physical conditional changes such as pH and heat can be used together with natural antimicrobials to investigate their synergistic effect on microbial inhibition.

CHAPTER 5

CONCLUSION

Main goal of this study was to determine antimicrobial activity of bio-based agents (activated lactoferrin, rosemary extract Jenseniin-G and Natamycin) individually and in combinations against *L. monocytogenes*, *E. coli* O157:H7, *S.* Enteritidis, *A. niger* and *P. roquefortii*. Two antimicrobial tests -microtiter plate assay and disc diffusion assay-were employed to determine the method for application studies.

None of the agents displayed antibacterial activity on agar plate by disc diffusion assay. However, these agents showed strong antibacterial activity when tested by microtiter plate assay. Activated lactoferrin was found as a powerful antimicrobial agent against tested three food-borne pathogens. RE inhibited the growth of *L. monocytogenes* totally at concentrations above 15%, while the growth of *E. coli* O157:H7, *S.* Enteritidis was inhibited partially at the highest concentration of 30%. In general, JG promoted the growth of pathogens instead of inhibiting. RE enhanced the activity of ALF 2-3 folds when tested against *E. coli* O157:H7.

Actual food applications also indicated that some of these natural agents can be good choice to use in meat industry. Dipping application of ALF and RE reduced the growth of *L. monocytogenes* on meats about two log.

On the contrary to bacterial experiments none of the ALF, JG and RE had any antifungal activity against *A. niger* and *P. roquefortii*. However, NA had significant antifungal activity against both fungi in disc diffusion assays. Dipping applications of cheese also indicated that NA can be a candidate in cheese industry as a powerful antifungal agent.

The next step of this research could be *in vitro* and *in vivo* investigation of natural antimicrobials on other pathogens like viruses, parasites. Active compounds of plant extract should be determined and tested with peptide antimicrobials in combinations to diminish pungent flavor and to compete with synthetic agents in food industry. In addition to all, the results of researches on natural antimicrobials can be expanded to other markets such as cosmetic and drug industry.

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APPENDIX-A

DISC DIFFUSION ASSAY RESULTS AGAINST FOOD-BORNE PATHOGENS

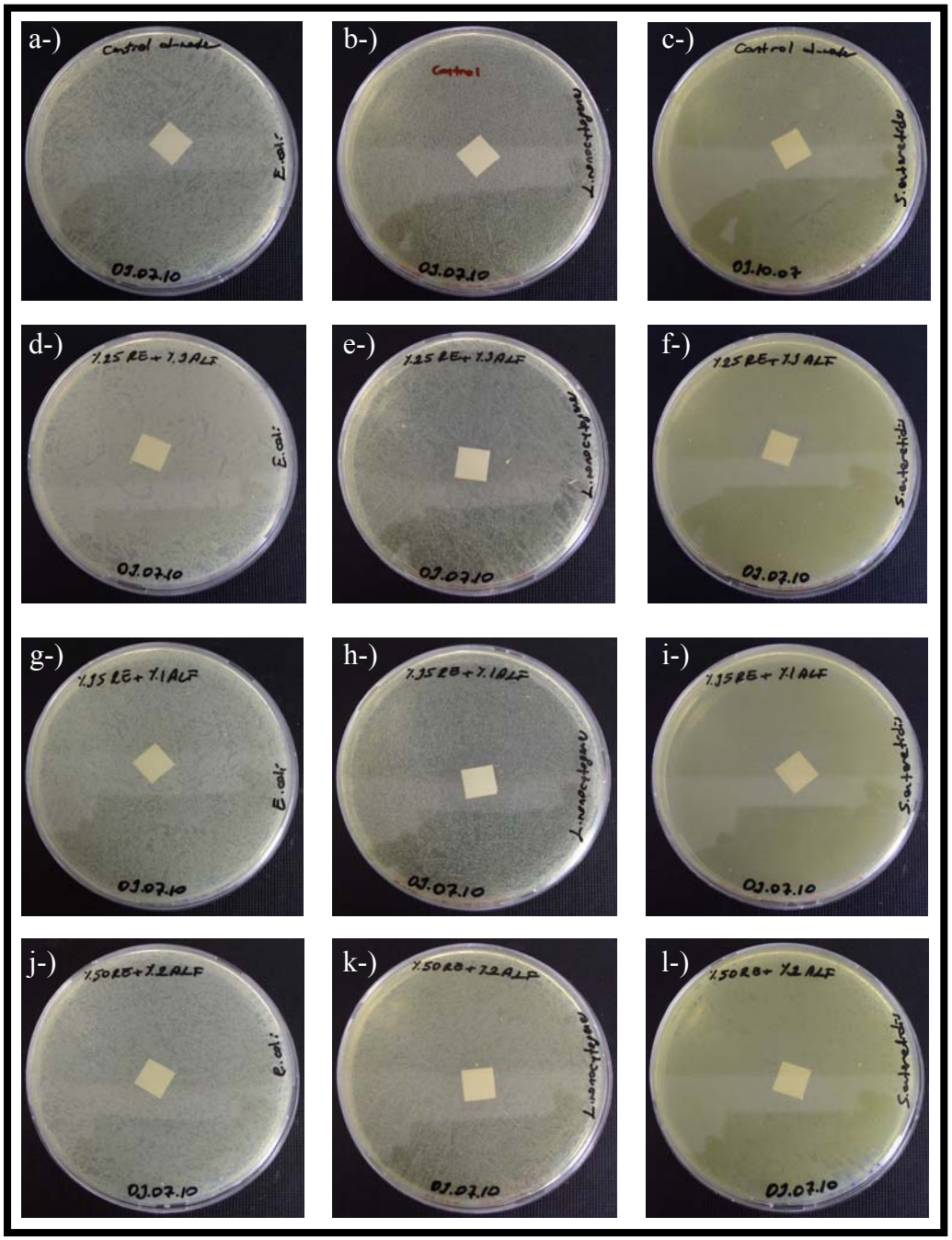


Figure A.1. Effect of RE and ALF in combinations on food borne pathogenic bacteria by disc diffusion assay a,b,c-) Control (sterilized d-water), d,e,f-) 25%RE+3%ALF, g,h,i-) 35%RE+1%ALF, j,k,l-) 50%RE+2%ALF on *E. coli* O157:H7, *L. monocytogenes* and *S.* Enteritidis, respectively.

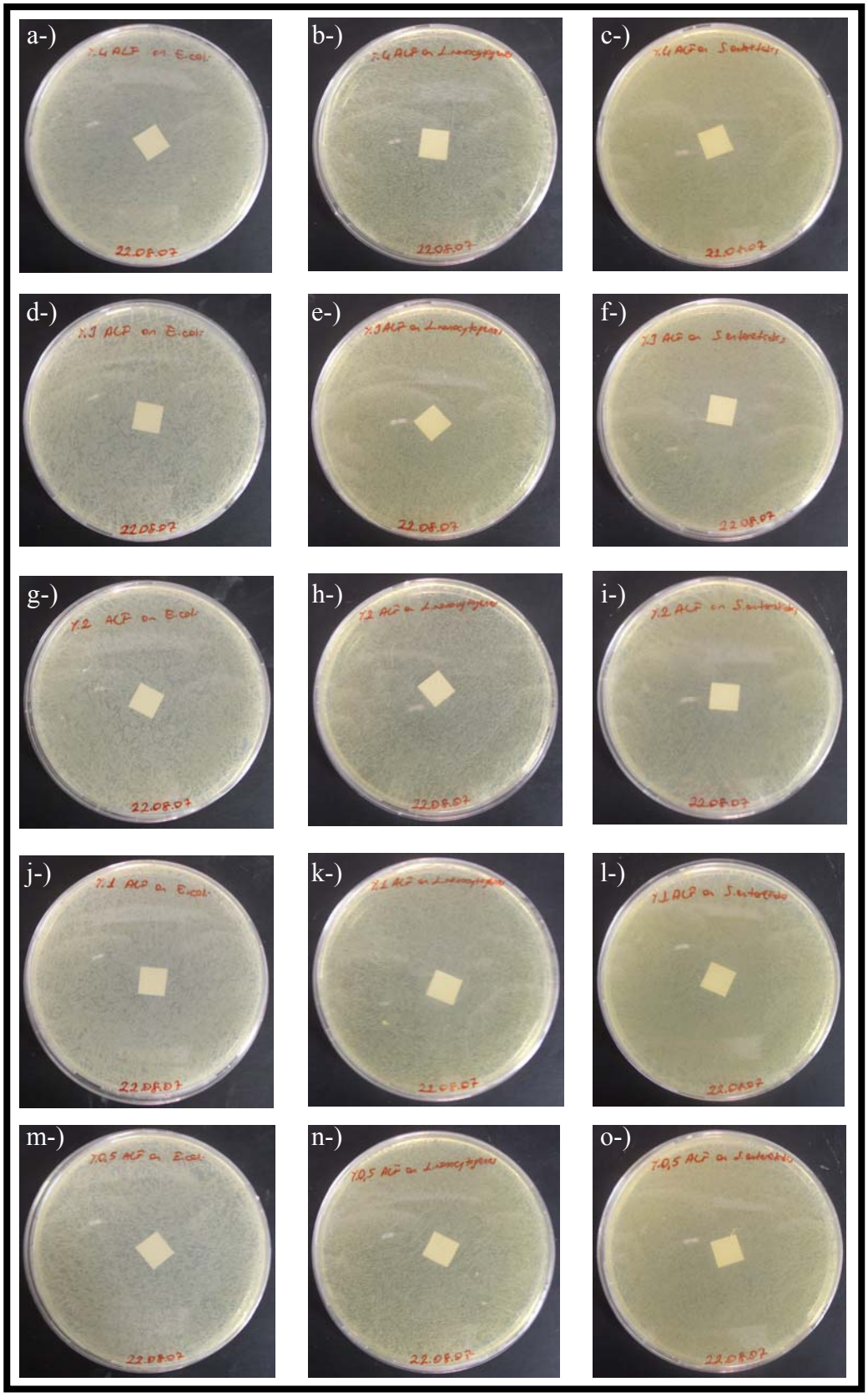


Figure A.2. Effect of ALF by disc diffusion assay a,b,c-) 4% ALF d,e,f-) 3% ALF, g,h,i-) 2% ALF, j,k,l-) 1% ALF, m,n,o-) 0.5% ALF on *E. coli* O157:H7, *L. monocytogenes* and *S.* Enteritidis, respectively.

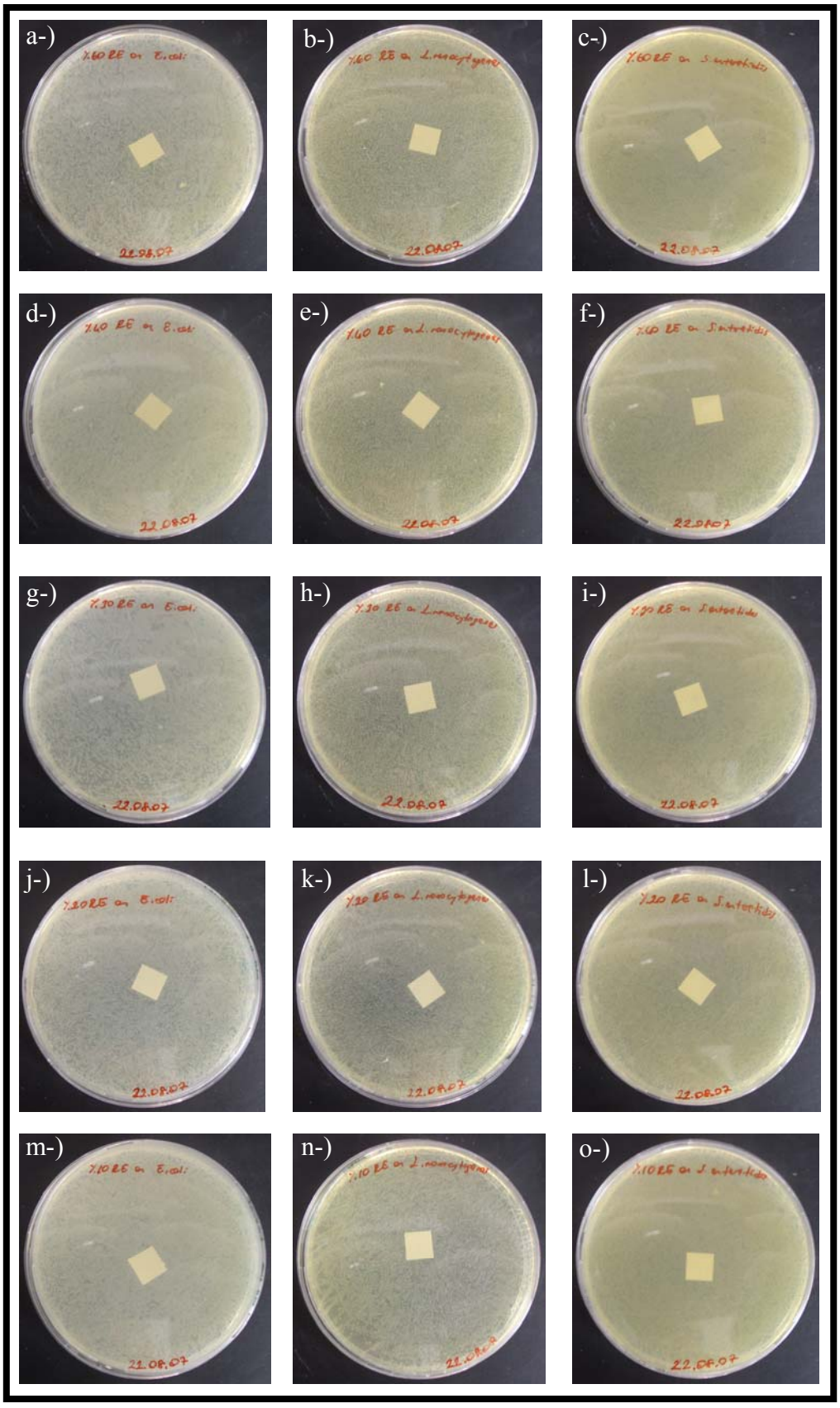


Figure A.3. Effect of RE by disc diffusion assay a,b,c-) 60% RE d,e,f-) 40% RE, g,h,i-) 30% RE, j,k,l-) 20% RE, m,n,o-) 10% RE on *E. coli* O157:H7, *L. monocytogenes* and *S.* Enteritidis, respectively.

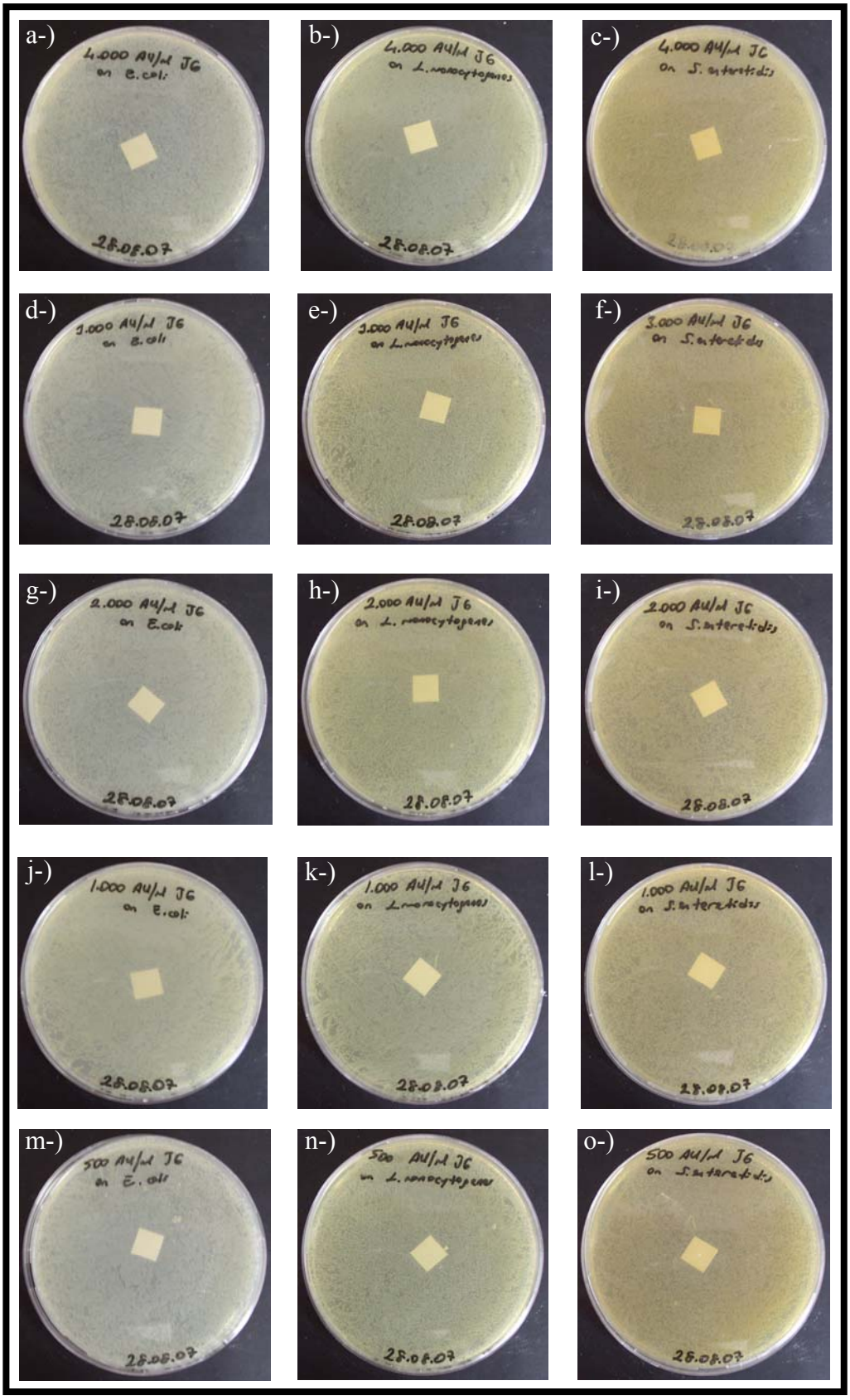


Figure A.4. Effect of JG by disc diffusion assay a,b,c-) 4000 AU/ml JG d,e,f-) 3000 AU/ml JG, g,h,i-) 2000AU/ml JG, j,k,l-) 1000 AU/ml JG, m,n,o-) 500 AU/ml JG on *E. coli* O157:H7, *L. monocytogenes* and *S.* Enteritidis, respectively.

APPENDIX-B

GROWTH CURVES OF THREE FOOD-BORNE PATHOGENS

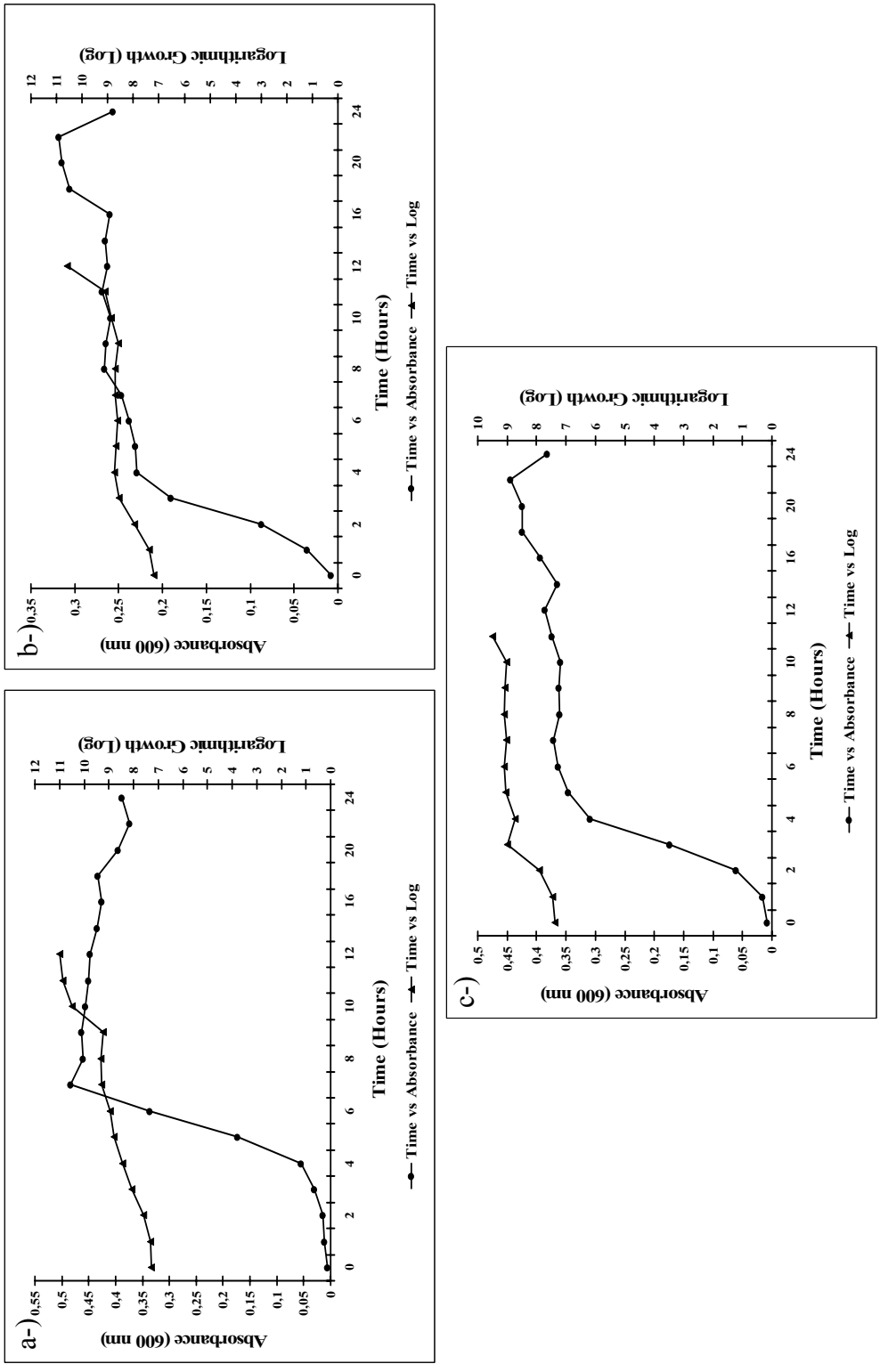


Figure B.1. Growth curves of (absorbance vs time) a-) *Listeria monocytogenes*, b-) E*scherichia coli O157:H7* and c-) *Salmonella* Enteritidis.

APPENDIX-C

RSKK CULTURES

C.1. RSSK Cultures

Gram negatives; *Escherichia coli* O157:H7, *Salmonella* Enteritidis and Gram positive; *Listeria monocytogenes* were purchased from RSKK as pathogen cultures on March, 2007. After Gram staining in our laboratories, it was realized that Gram(+) basil (Figure C.1-b) *Listeria monocytogenes* appeared as Gram(+) coccus under light microscopy examination (Figure C.1-a). It was confirmed by numerous Gram staining

a-) **b-)**

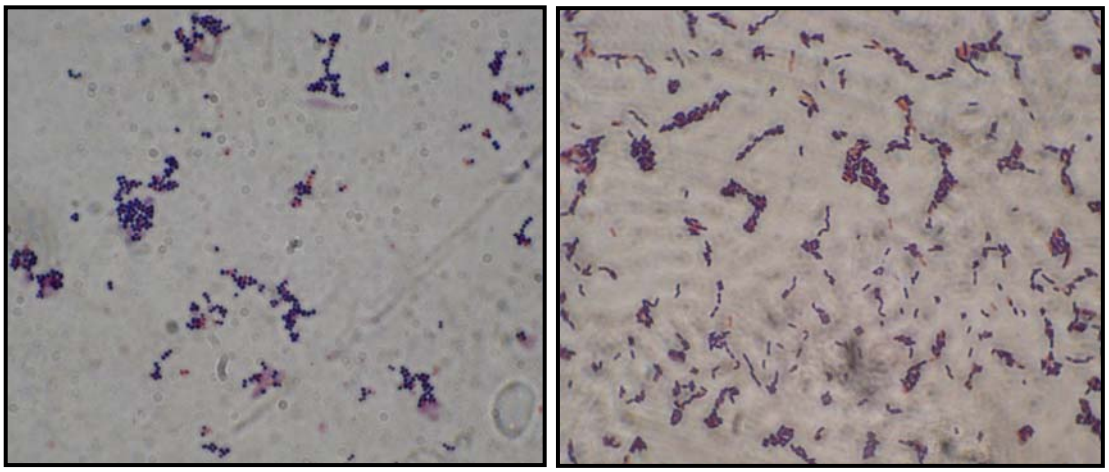
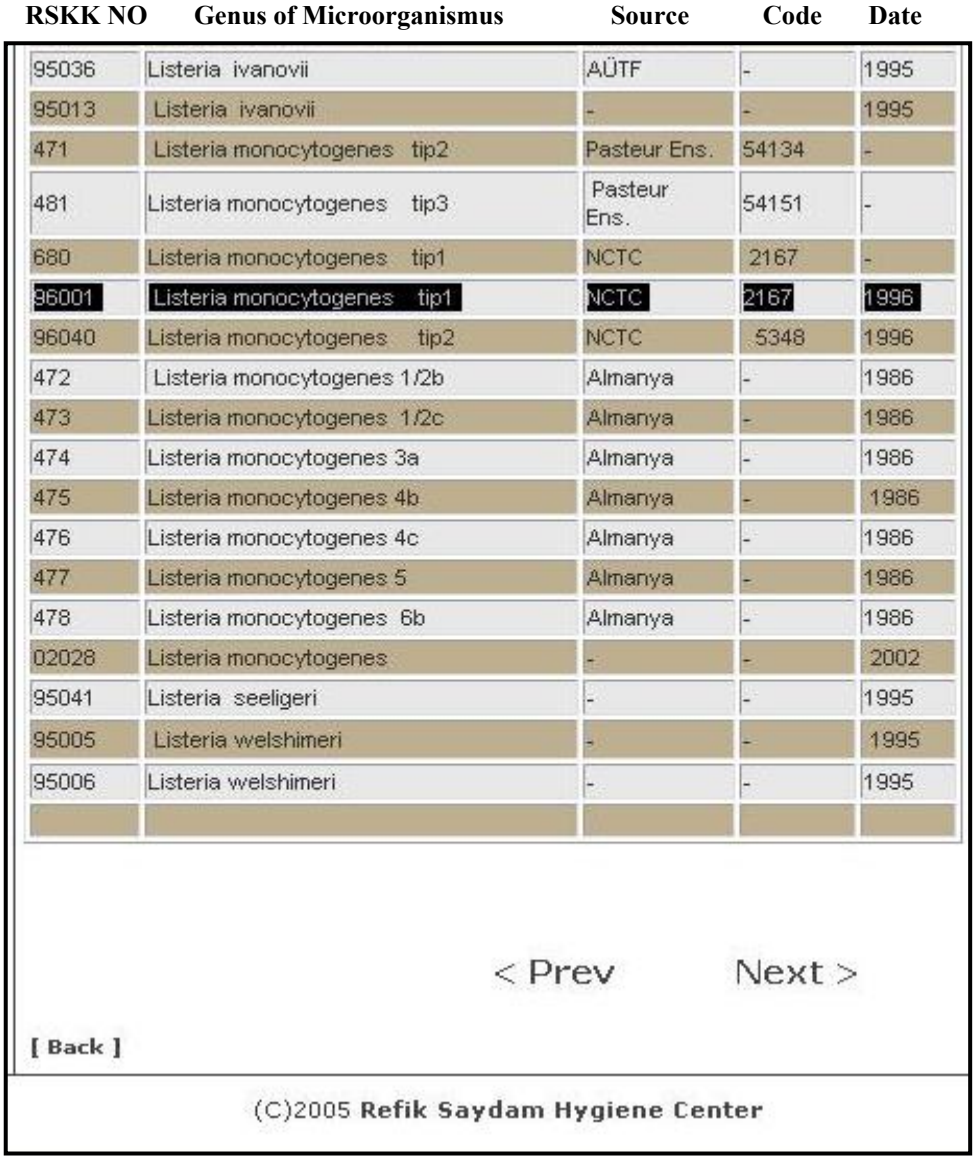


Figure C.1. a-) Gram staining of bacterium purchased from RSKK as *L. monocytogenes*. Gram(+) coccus morphology is clear. b-) Gram staining of *L. monocytogenes* (Gram(+) basil bacterium) purchased from NCTC.

and light microscopy examinations in our laboratory. The source and catalog number of these bacteria was traced on the web site of RSKK. The number and source for *Listeria monocytogenes* was 2167 and NCTC on RSKK's page (Figure C.2). Then, *Listeria monocytogenes* was searched as a keyword on NCTC's search engine and also the catalog number (2167) given by RSKK was searched through NCTC's website. All catalog numbers of NCTC for *Listeria monocytogenes* did not contain the number 2167. It was also found no record for number 2167 on NCTC catalogue (Figure C.3-a, C.3-b). Gram staining results and web sites images were reported to RSKK. After API test conducted by RSKK, it was declared that there was a mistake in the collection and

Listeria monocytogenes cultures was mislabeled by RSKK. According to API test, this strain was, a Gram(+) coccus, *Streptococcus* spp. After that, RSKK revised their web page and subtracted *L. monocytogenes* supplied from NCTC with 2167 NCTC catalog number (Figure C.4). Subtracted part after revision was demonstrated in Figure C.2 with highlights. Another bacterium purchased from RSKK as *Escherichia coli* O157:H7 (RSKK No: 03018) was not serotyped. It was *Escherichia coli* but not serotype O157:H7 (Figure C.5). Thereupon, All experiments worked with RSKK cultures were not taken in an account. All pathogen strains were purchased from NCTC and experiments were repeated with NCTC cultures.



RSKK NO	Genus of Microorganismus	Source	Code	Date
95036	Listeria ivanovii	AÜTF	-	1995
95013	Listeria ivanovii	-	-	1995
471	Listeria monocytogenes tip2	Pasteur Ens.	54134	-
481	Listeria monocytogenes tip3	Pasteur Ens.	54151	-
680	Listeria monocytogenes tip1	NCTC	2167	-
96001	Listeria monocytogenes tip1	NCTC	2167	1996
96040	Listeria monocytogenes tip2	NCTC	5348	1996
472	Listeria monocytogenes 1/2b	Almanya	-	1986
473	Listeria monocytogenes 1/2c	Almanya	-	1986
474	Listeria monocytogenes 3a	Almanya	-	1986
475	Listeria monocytogenes 4b	Almanya	-	1986
476	Listeria monocytogenes 4c	Almanya	-	1986
477	Listeria monocytogenes 5	Almanya	-	1986
478	Listeria monocytogenes 6b	Almanya	-	1986
02028	Listeria monocytogenes	-	-	2002
95041	Listeria seeligeri	-	-	1995
95005	Listeria welshimeri	-	-	1995
95006	Listeria welshimeri	-	-	1995

Figure C 2. The web page of Refik Saydam Hıfzısıhha Center's national type culture collection of bacterial strains before the revision. The purchased, *L. monocytogenes*, strain was highlighted (Source: RSHM 2008-a)

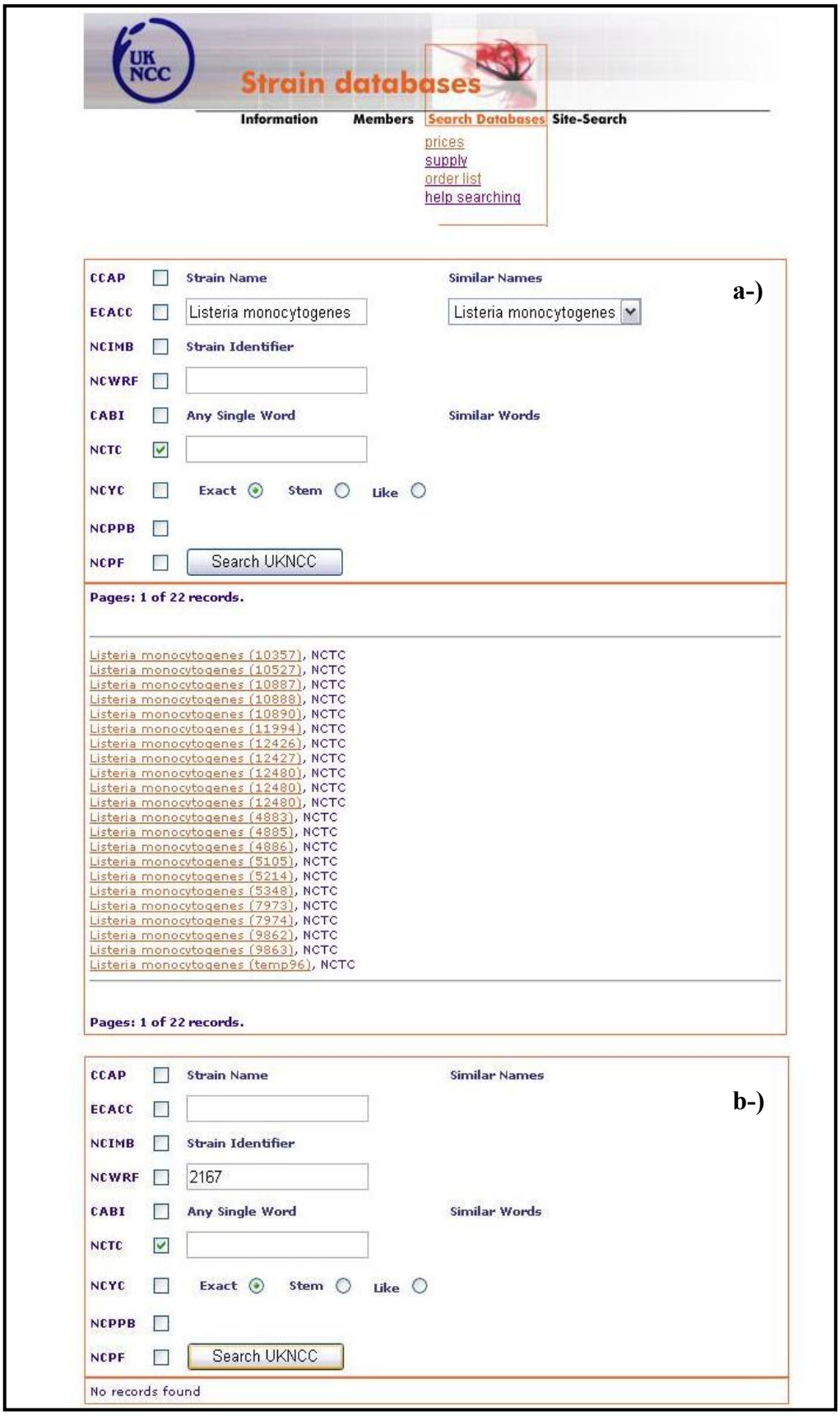
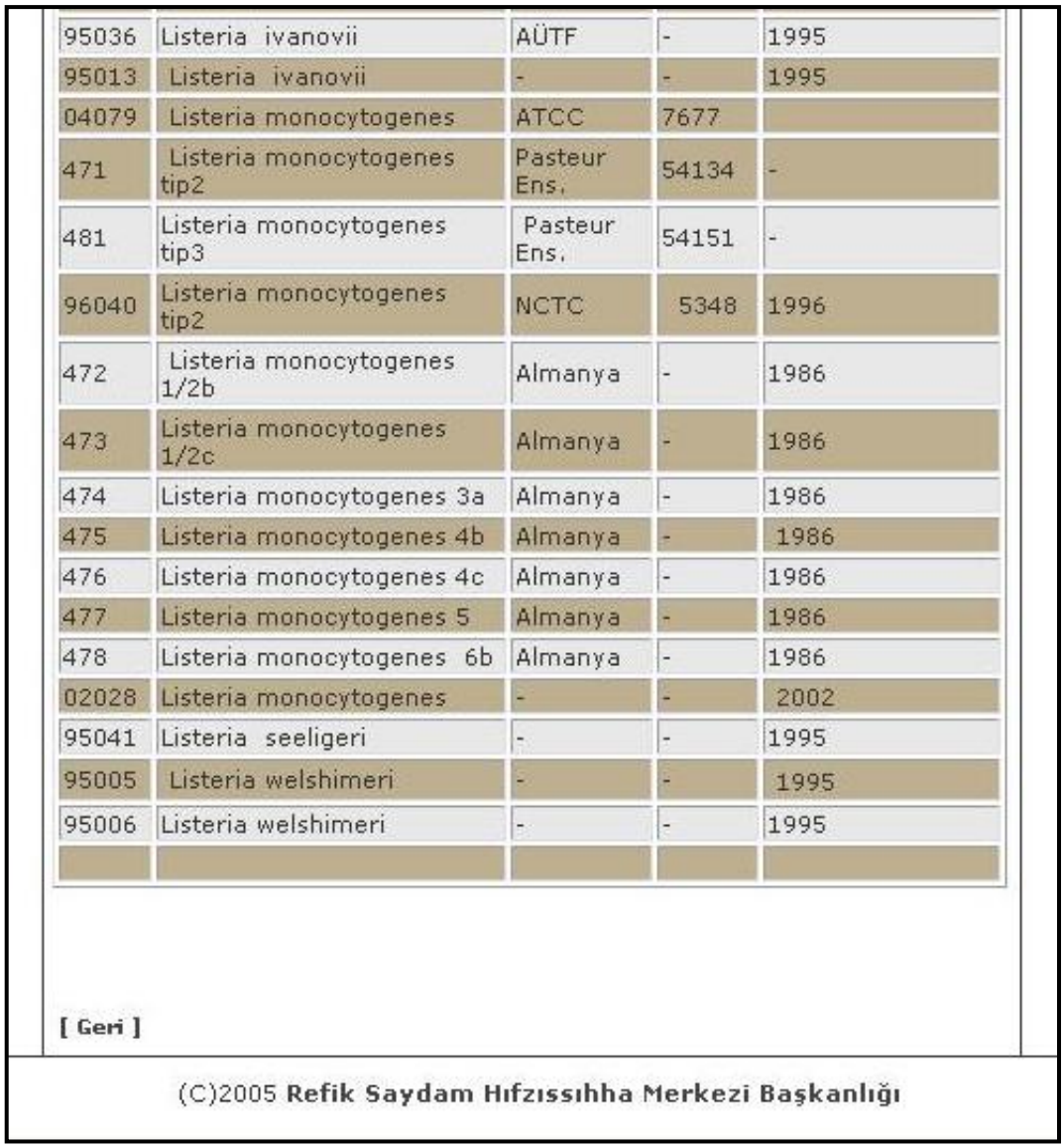


Figure C 3. a-) Database search of bacterium *Listeria monocytogenes* on NCTC search engine b-) Database search of NCTC catalog number given by RSKK (2167) on NCTC's search engine (Source:UKNCC 2008).



RSKK NO	Genus of Microorganismus	Source	Code	Date
95036	Listeria ivanovii	AÜTF	-	1995
95013	Listeria ivanovii	-	-	1995
04079	Listeria monocytogenes	ATCC	7677	
471	Listeria monocytogenes tip2	Pasteur Ens.	54134	-
481	Listeria monocytogenes tip3	Pasteur Ens.	54151	-
96040	Listeria monocytogenes tip2	NCTC	5348	1996
472	Listeria monocytogenes 1/2b	Almanya	-	1986
473	Listeria monocytogenes 1/2c	Almanya	-	1986
474	Listeria monocytogenes 3a	Almanya	-	1986
475	Listeria monocytogenes 4b	Almanya	-	1986
476	Listeria monocytogenes 4c	Almanya	-	1986
477	Listeria monocytogenes 5	Almanya	-	1986
478	Listeria monocytogenes 6b	Almanya	-	1986
02028	Listeria monocytogenes	-	-	2002
95041	Listeria seeligeri	-	-	1995
95005	Listeria welshimeri	-	-	1995
95006	Listeria welshimeri	-	-	1995

Figure C 4. The web page of Refik Saydam Hıfzısıhha Center's national type culture collection of bacterial strains after revision. *Listeria monocytogenes* with 2167 NCTC's number was taken out from RSKK's page. (Source: RSHM 2008-b)

RSKK NO	Genus of Microorganismus	Source	Code	Date
234	Escherichia coli	Pasteur Ens.		1968
1004	Escherichia coli	ATCC	26	
281	Escherichia coli	ATCC	3509	
05010	Escherichia coli	ATCC	25922	2005
03018	Escherichia coli	ATCC	35218	2003
96091	Escherichia coli	ATCC	35211	1996
97021	Escherichia coli	ATCC	12798	1997
283	Escherichia coli	ATCC	1553	
285	Escherichia coli	ATCC	997	1984
06015	Escherichia coli	ATCC	11229	2006
291	Escherichia coli Wildetip	-	-	-
888	Escherichia coli	ABD	-	-
889	Escherichia coli	ABD	-	-
890	Escherichia coli	ABD	-	-
891	Escherichia coli	ABD	-	-
* Bu suşlar için Laboratuvar ile direkt görüşülmesi gerekmektedir				
892	Escherichia coli	ABD	-	-
893	Escherichia coli	ABD	-	-
894	Escherichia coli	ABD	-	-
895	Escherichia coli	ABD	-	-
896	Escherichia coli	ABD	-	-
899	Escherichia coli	ABD	-	-

Figure C.5. The web page of Refik Saydam Hıfzısıhha Center's national type culture collection of *Escherichia coli* (Source: RSHM 2008-c).

C.2. Activity of Natural Antimicrobials on RSKK Cultures

Antimicrobial activity of natural antimicrobial agents: ALF, RE, JG and their combinations were tested on cultures supplied from RSKK by microtiter assay (Figure C.6, C.7, C.8 and C.9). Preparation of antimicrobials, preparation of cultures, media and microtiter assay were same as explained in Chapter 3. The only difference in microtiter assay was the control groups: growth control (100 µl m.o. + 100 µl Medium), sterility control (200 µl Medium).

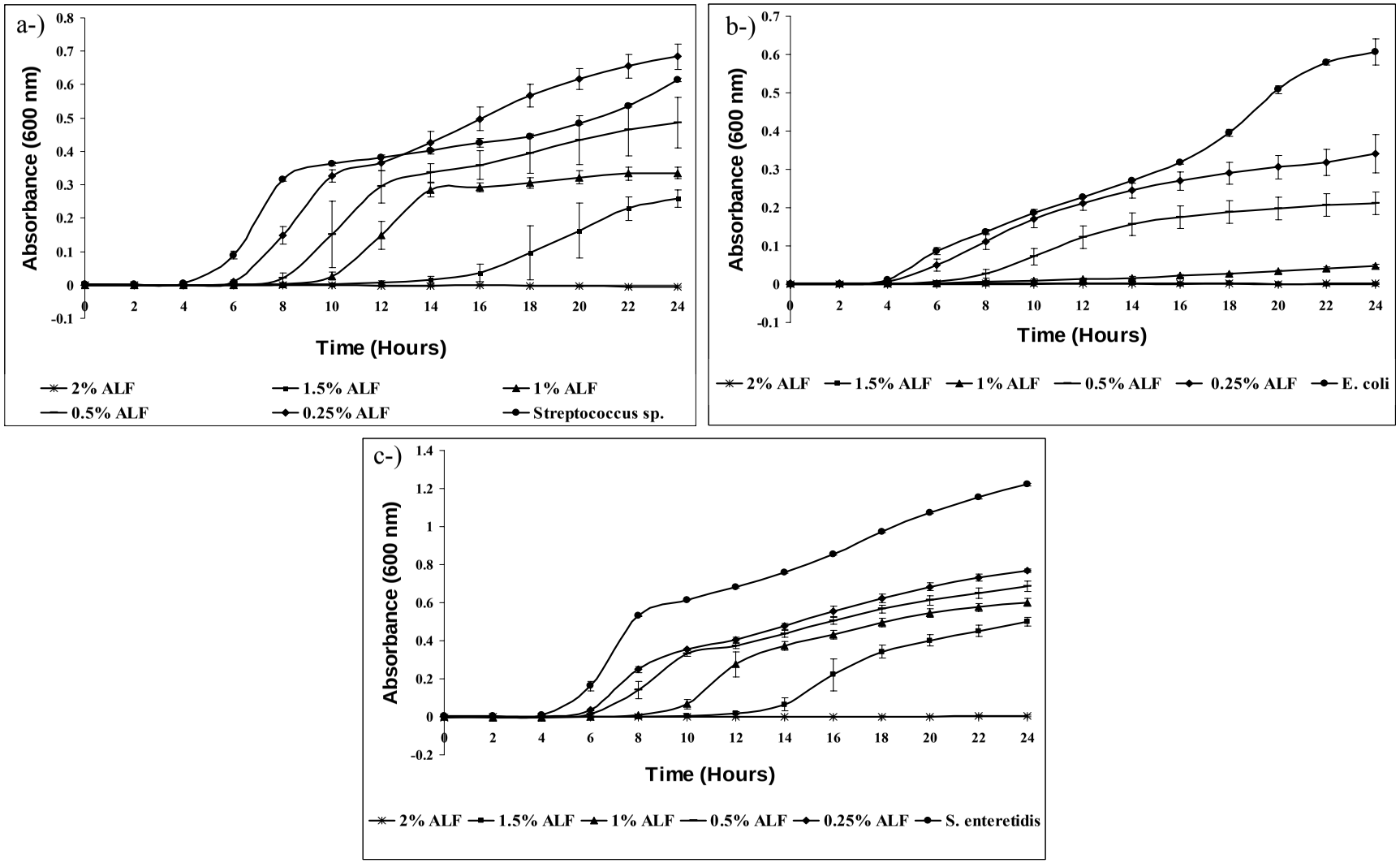


Figure C.6. Effect of ALF on the growth of three bacteria supplied from RSKK: a-) *Streptococcus* spp. (as *L. monocytogenes*), b-) E*scherichia coli* and c-) S*almonella* Enteritidis. Bars indicate standard error of mean.

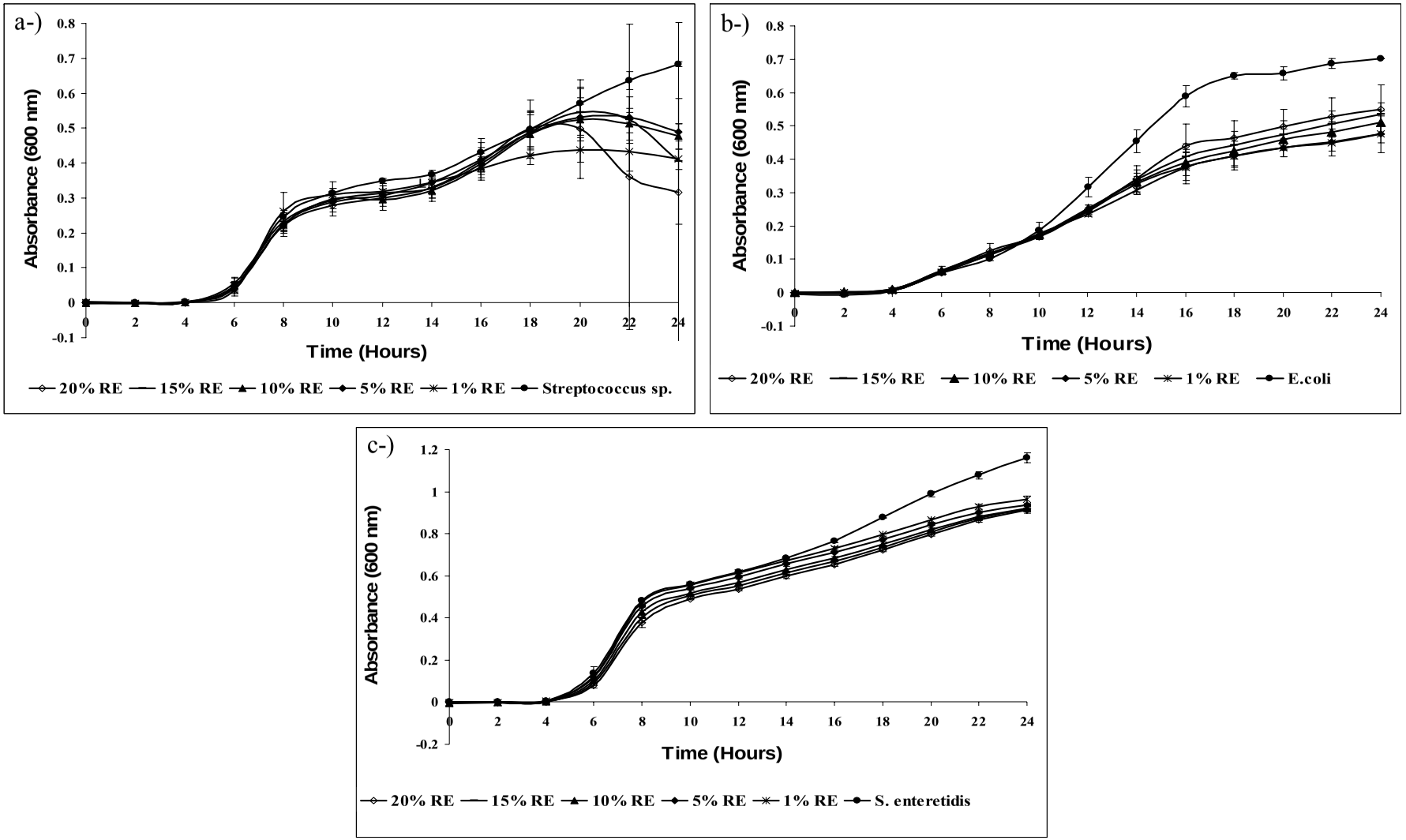


Figure C.7. Effect of RE on the growth of three bacteria supplied from RSKK: a-) *Streptococcus* spp. (as *L. monocytogenes*), b-) E*scherichia coli* and c-) S*almonella* Enteritidis. Bars indicate standard error of mean.

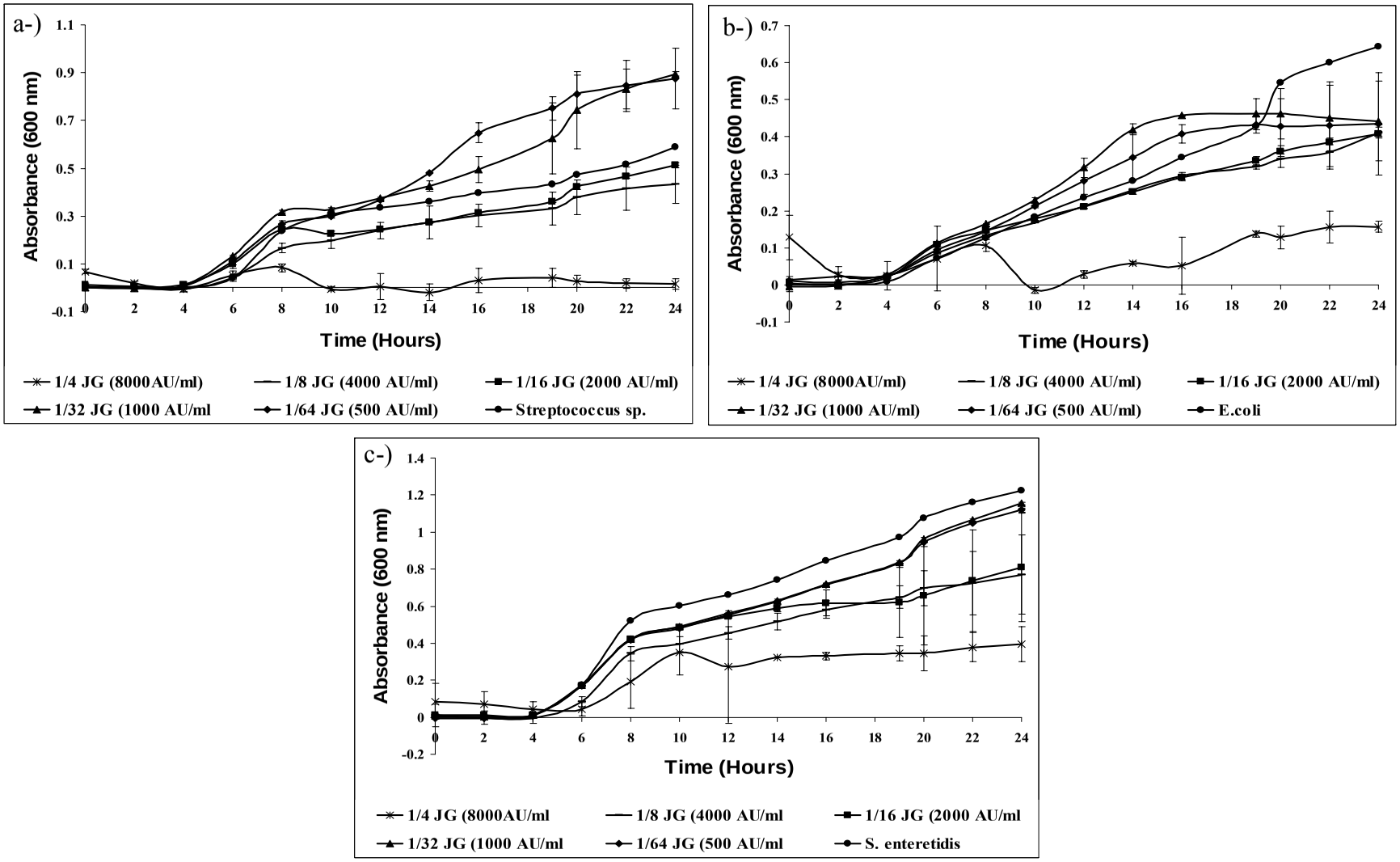


Figure C.8. Effect of JG on the growth of three bacteria supplied from RSKK: a-) *Streptococcus* spp. (as *L. monocytogenes*), b-) E*scherichia coli* and c-) S*almonella* Enteritidis. Bars indicate standard error of mean.

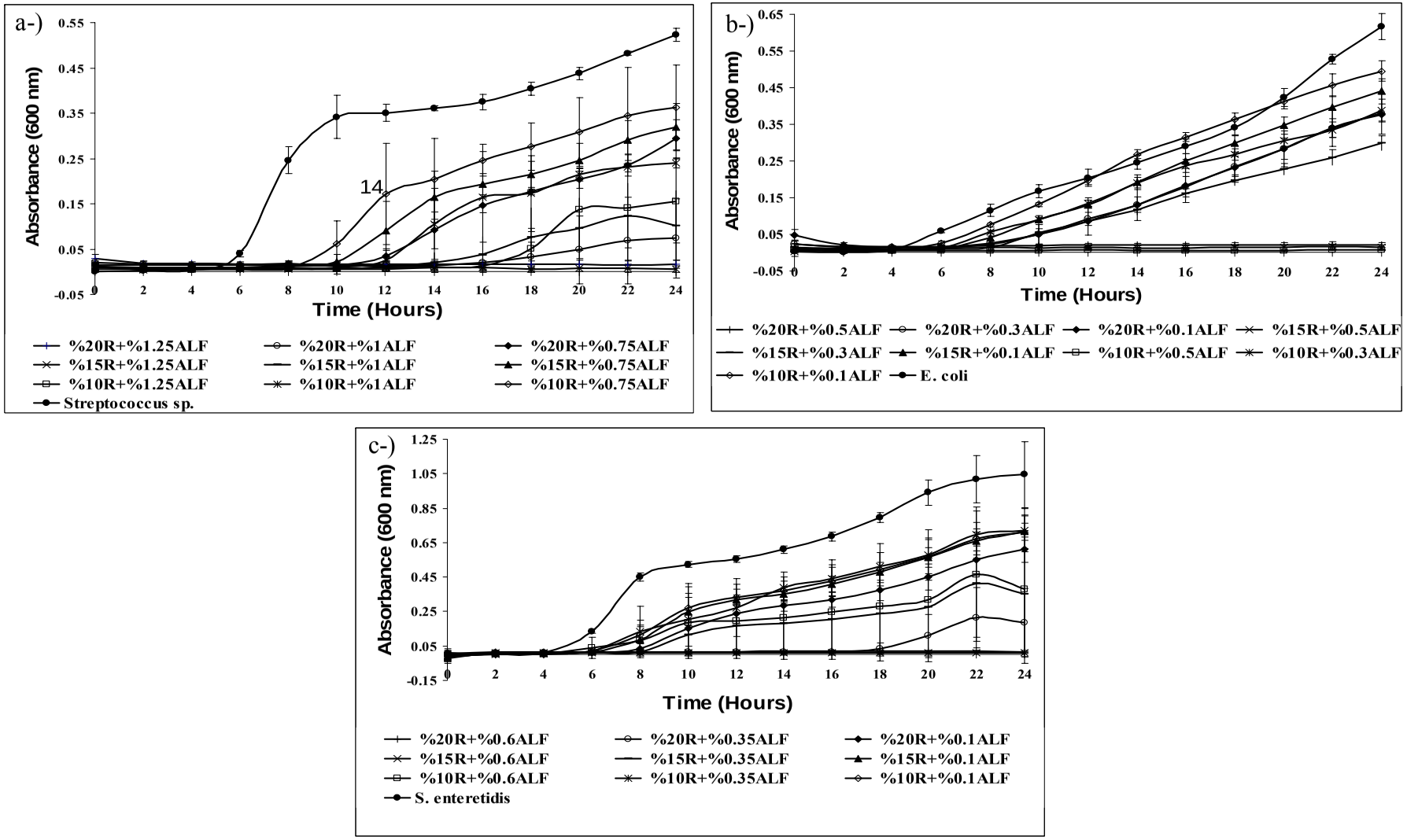


Figure C.9. Effect of ALF+RE on the growth of three bacteria supplied from RSKK: a-) *Streptococcus* spp. (as *L. monocytogenes*), b-) E*scherichia coli* and c-) S*almonella* Enteritidis. Bars indicate standard error of mean.