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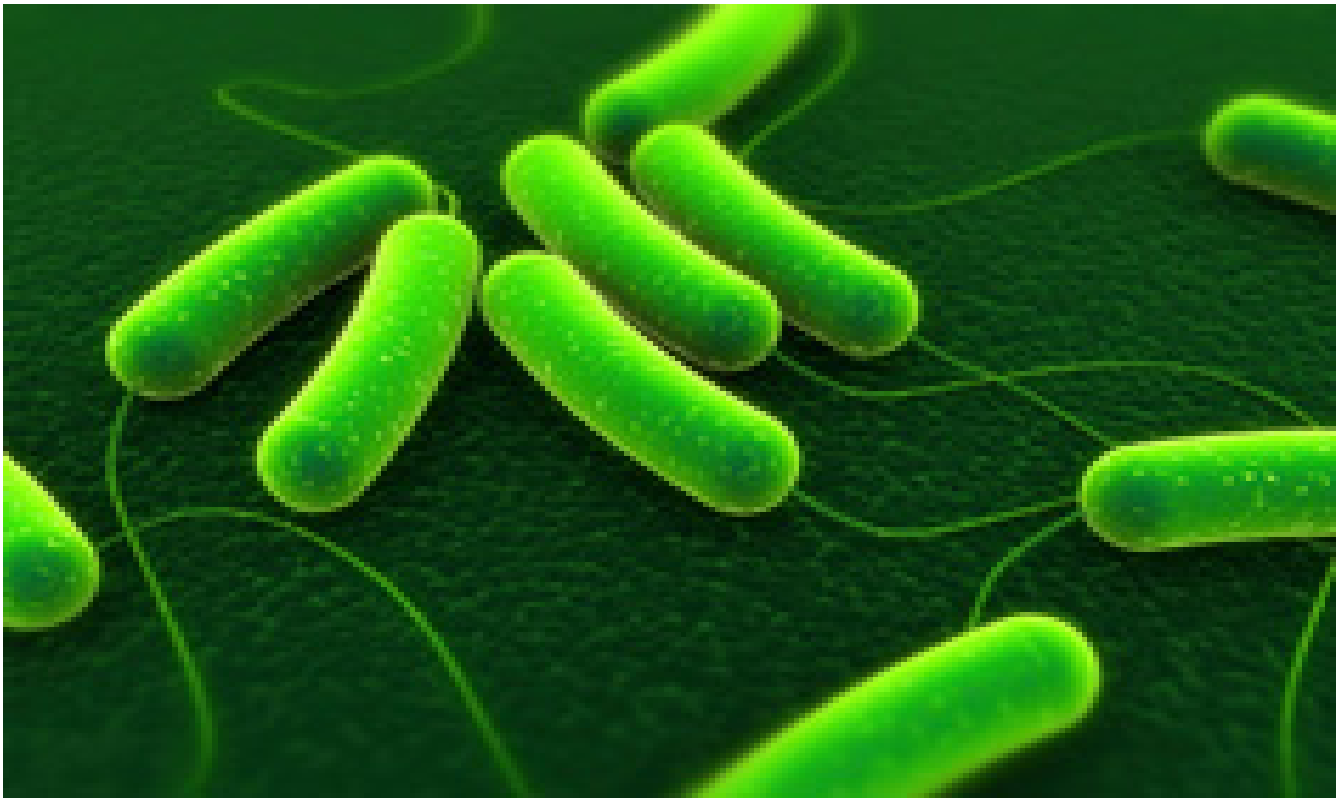
Effect of bacteriostatic agents on lactic acid bacteria and specific fish spoilage bacteria in a model system.



Lactic acid bacteria (www.google.com)

Master Thesis in Biological Chemistry

Prepared By: Aparna Saha

Year : 2012

Abstract

Lactic acid bacteria (LAB) are gram positive bacteria, which are the dominant microflora in lightly preserved fish products (LPFP) and many LAB species can spoil LPFP. To increase the shelf-life of LPFP, it is necessary to inhibit the growth of LAB using preservatives. The aim of the study was i) to examine the effect of different concentration of preserving agents (PURAC; Purasal Opti.Form PPA Plus and liquid smoke; Arosmoke P-50) on the growth of *Photobacterium phosphoreum*, *Pseudomonas putida*, *Vibrio vulnificus*, *Listeria innocua* (as a *L. monocytogenes* non-pathogenic substitute) and LAB (*Carnobacterium inhibens, Carnobacterium maltaromaticum, Lactococcus lactis, Enterococcus faecalis and Lactobacillus curvatus*) at 20 ºC by using spectroscopic techniques (Bioscreen C) ii) to compare the effect of different concentrations of natural salt (NaCl), potassium lactate and potassium acetate on the growth of *V. vulnificus* at 20 ºC. Another objective of this study was to evaluate a panel of candidate reference genes for their potential use for normalization of gene expression in bacteria under food processing relevant conditions.

The above mentioned bacteria were inoculated as mono-cultures in tryptic soy broth with yeast extract (TSBYE) supplemented with 8 different combination of preservatives; i) 3% PURAC (Potassium lactate +Potassium acetate), ii) 3% PURAC, iii) 0.07% liquid smoke (LS), iv) 0.14% LS, v) 3% PURAC + 0.07% LS, vi) 3% PURAC + 0.14% LS solution, vii) 6% PURAC + 0.07% LS and viii) 6% PURAC + 0.14% LS. Bacterial growth at 20 °C was measured for up to one week by recording absorbance at 600 nm every 10 minutes using a microplate incubator and reader (Bioscreen C). The treatments had varying effects on growth depending on species. The most detrimental was the effect of PURAC solution on the growth of *L. curvatus*. Interestingly, growth of this species appeared to be enhanced by the supplement of LS, and it was able to grow in the presence of 6% PURAC + 0.07% LS, but not when 6% PURAC was the sole preservative. However, the apparent lag time in this situation (106 h) was threefold longer compared to 0.07% LS alone. In general, the isolated effect of LS was minor compared to the effect of PURAC, but in most cases, a combinatorial effect of the two preservatives was observed. Another interesting result was the inhibiting effect of PURAC on the growth of *V. vulnificus*. This bacterium is recognized as a halophilic species by many authors but was not able to grow in any combination

with 6% PURAC (all other species where), and was significantly inhibited by the lower PURAC concentration (3%).

Eight candidate reference genes and one gene of interest were used in gene expression analysis. Among eight reference genes, six candidate reference genes were stably expressed under the experimental conditions (different temperature). Two genes were sufficient for normalization of gene expression analysis found from geNorm analysis. The expression of the gene of interest (*hsp60*), was induced significantly when inoculated of high temperature (40 ºC) for 8 hours.

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1 Introduction

1.1 Background

Fish and seafood is one of the main sources of protein for human and have high nutritional food value. In Norway, fish production is one of the major economic activities. Norwegian salmon, known scientifically as *Salmo salar* (Atlantic salmon), is a well-known fish product from Norway. Two species of salmonid fish; Atlantic salmon and rainbow trout are extensively cultured in Norway. The production of Norwegian farmed salmon and trout has increased dramatically during the past decades, from a few thousand tonnes in 1980 to around 1.4 million tonnes in 2009 (Larsen and Asche, 2011).

Raw fish can be contaminated by microorganisms. Fish skin, gills and gut contains significant amounts of bacteria and the composition and quantity of it varies according to the fish species, temperature and salinity of the water, level of dissolved oxygen, etc. (Leroi, 2010). Therefore, proper preservation, processing and storage are essential in the fish industry.

Modern consumers demand a great variety of convenient foods; many of them are ready to eat products including lightly preserved fish products (LPFP). LPFP are generally uncooked or mildly heat treated food products, preserved by low levels of preservatives (NaCl <6% water phase, corresponding approximately to water activity (a_w) <0.96, and pH >5) and stored at chilled temperature (≤5º C) under vacuum or modified atmosphere to extend shelf-life. LPFP are usually produced from raw fish and further processing involves one or more steps that increases the risk of re-contamination (Leroi, 2010).

Lactic acid bacteria (LAB) are gram positive bacteria, which are the dominating microflora in LPFP (Leroi, 2010) because they can adapt to the conditions of preservation. They can easily reach 10^7-10^8 colony forming units (cfu)/gram at the end of the shelf-life of these products. Among LAB species, *Carnobacterium* spp. has been found as the dominating bacterial species at the beginning of the storage time, whereas lactobacilli are dominating at the end of the shelf-life of cold smoked salmon (CSS). Some LAB species (*Lactobacillius sakei*) have been found to be able to produce off-odors in spoiled vacuum packaged CSS. Additionally, LAB can produce acid

and create bitter taste by degrading of proteins. Truelstrup Hansen et al. (1995) reported that in vacuum-packaged cold-smoked salmon at the time of sensory rejection, three different types of microfloras were present; (1) LAB, (2) LAB and *Enterobacteriaceae*, and (3) *Photobacterium phosphoreum* with high levels of LAB. Lactobacilli and Carnobacteria are able to spoil vacuum-packaged cold-smoked gravad rainbow trout. Lyhs et al. (2008) found that *Lactobacillius sakei* (*L. sakei*) and *L. curvatus* were the main LAB species associated with spoiled maatjes herring when stored in air and under modified atmosphere. However, the role of this flora is not clear as many authors have reported that there is no correlation between shelf-life and LAB count, or any other bacterial number (Hansen *et al.*, 1995; Huss *et al.*, 1995; Leroi *et al.*, 1998). But still it is essential to decrease their growth to extend the shelf-life of products by using appropriate preservatives and storage conditions.

Listeria monocytogenes is a human pathogenic bacterium that may cause listeriosis. The primary disease signs of listeriosis are meningitis, abortion and prenatal septicaemia. The estimated annual incidence of foodborne listeriosis in the United States is 1850 cases and 425 deaths (Anonymous, 2001a). Although foodborne listeriosis is rare, the mortality rate among infected is as high as 20 % in risk groups (Anonymous, 2001a). Studies have shown that CSS can be a source of food-borne listeriosis. About 13.6% of cold-smoked salmon imported into Switzerland was contaminated with *L. monocytogenes* (Jemmi, 1993), while a Norwegian study showed that 11% of vacuum-packed smoked salmon contained *L. monocytogenes* (Rørvik *et al.*, 1991). According to Jørgensen and Huss, (1998) the highest prevalence was found in cold-smoked fish (34-60%), while the lowest was found in heat-treated and cured seafood (4-12%). The mild temperatures (20-30 ºC) applied at cold smoking processing, is far from sufficient to inhibit *Listeria monocytogenes* (Hwang *et al.*, 2009).

To increase the shelf-life of CSS, it is important to use appropriate volumes of preservatives (salt and smoke/ liquid smoke) at appropriate storage temperature, and, hence decrease the growth of LAB and other fish spoilage bacteria (FSB) *and L. monocytogenes.* As a preservative, organic acid salts have the ability to control microbial growth, improve sensory properties and extend the shelf-life of fish products. Liquid smoke (LS) flavourings, which have replaced wood smoking over recent years in fish and meat products, are well known for potential antimicrobial properties (Thurette *et al.*, 1998). PURAC solution contains organic acid salts (potassium lactate and

potassium acetate), and liquid smoke (Aro-smoke P-50) contains mostly phenol besides carbohydrates and organic acids that can be used to preserve fish products. These two preservatives have been found to be able to control the growth of *L. monocytogenes* (Kin *et al.*, 2011; Vitt *et al.*, 2001) and some LAB species (Stekelenburg and Kant-Muermans, 2001).

The mechanisms behind the inhibition effect of various salts and temperature on the growth of bacteria may be studied by means of gene expression analysis. Some genes of LAB may be differentially expressed under different conditions, initiated by temperature and salt. Real-time quantitative PCR has been used as a powerful tool for gene expression analysis in bacteria (Reiter *et al.*, 2011; Zhao *et al.*, 2011). For accurate and reliable gene expression analysis, normalization of gene expression data using one or more reference genes is essential. One of the goals for this time-limited master exercise was to find stable reference genes to normalize gene expression data. Among LAB, lactobacilli species have been found in many LPFP such as cold-smoked salmon, marinated herring, gravad rainbow trout etc.(as reviewed by Leroi, 2010). Therefore *L. curvatus* was chosen for this first approach gene expression analysis.

1.2 Scope of the study

LAB are the dominant microflora in LPFP and many LAB species can spoil LPFP. To increase the shelf-life of LPFP, it is necessary to inhibit the growth of LAB using preservatives. PURAC and LS has already been known as good preservatives (Guilbaud *et al.*, 2008; Kin *et al.*, 2011). TSBYE is an appropriate model substitute for smoked salmon (Hwang, 2009). This medium contains preservatives (PURAC and LS) which can be used to monitor quantitative effect on growth of LAB, *L. innocua* and specific fish spoilage bacteria at 20 ºC through measuring optical density.

This master thesis is closely linked to the project 'LABLiPS; Lactic Acid Bacteria in Lightly Preserved Seafood', financially supported by the foundation NORCONSERV.

The objectives of this study were;

1) Examine the effect of different concentrations of preserving agents (PURAC and liquid smoke) on the growth of *Photobacterium phosphoreum*, *Pseudomonas putida*, *Vibrio vulnificus*, *Listeria innocua* (as a *L. monocytogenes* non-pathogenic substitute) and LAB (*Carnobacterium inhibens, Carnobacterium maltaromaticum, Lactococcus lactis, Enterococcus faecalis, Lactobaccilius curvatus*) at 20 ºC by using spectroscopic techniques (Bioscreen C).
2) Compare the effect of different concentrations of natural salt (NaCl), potassium lactate (K-L) and potassium acetate (K-A) on the growth of *V.vulnificus* at 20 ºC.
3) To evaluate a panel of candidate reference genes for their potential use for normalization of gene expression in bacteria under food processing relevant conditions.

The effects of preservatives on growth of LAB, *L. innocua* and FSB are to be examined at 20 ºC, this temperature is suitable for bacterial growth in different concentration of additives in short experimental period (one week). The inhibition effect on bacterial growth may differ from 20 ºC to refrigerated temperature. At low temperature, bacteria may need longer time to grow than at high temperature (20 ºC). However, the main purpose of this experiment is to find inhibitory effect on growth of bacteria by different concentrations of additives.

2 Theoretical background

2.1 Lactic acid bacteria (LAB)

LAB are a group of Gram-positive, non-spore-forming, fermentative bacteria producing lactic acid as the major end product of sugar fermentation. They are anaero-aerotolerant and generally have complex nutritional requirements especially for amino acid and vitamins. LAB are catalase and oxidize negative rods and cocci (Leroi, 2010).Two major hexose fermentations are used to classify LAB genera. Glycolysis (Embden-Meyer pathway) results in almost exclusively lactic acid as the end product (homofermentation). The 6-phosphogluconate/phosphoketolase pathway results in other end products, such as ethanol, acetic acid and CO_2 in addition to lactic acid (heterofermentative) (Schlegel, 1992c).

LABS are among the most important groups of microorganisms used in food fermentations. They contribute to the taste and texture of fermented products and inhibit food spoilage bacteria by producing growth-inhibiting substances and large amounts of lactic acid. As agents of fermentation, LAB are involved in making yogurt, cheese, cultured butter, sour cream, sausage, cucumber pickles, olives and sauerkraut. But some species may spoil beer, wine and processed meats. Beside food products, these bacteria are widespread in nature, as well as the genital, intestinal and oral cavities of animal and human (Leroi, 2010).

2.1.1 LAB in fresh fish

Among LAB species, certain species (i.e. *Carnobacterium, Vagococcus, Lactobacillus, Enterococcus, Lactococcus*) have been found in freshwater fish and their surrounding environment (Austin and Austin, 1992). Carnobacteria, *Carnobacteria maltaromaticum* (previously *pisicola*) and *C. divergens* have been reported as a part of the intestinal microbial population of many fish species, for example, Atlantic salmon (*Salmon salar*), wild pike (*Esox Lucius*) and wild brown trout (*Salmon trutta*) (Ringø and Gatesoupe, 1998; Gonzalez et al., 1999; Gonzalez et al., 2000; Ringø et al., 2000). Additionally, *C.maltaromaticum* and *Lactobacillus* sp. have been associated with fish diseases. *C. maltaromaticum* appears to have a low virulence to fish and it seems to be most susceptible when the fish is exposed to stress conditions, such as handling (Starliper *et al.*, 1992). Furthermore, some strains of *C. maltaromaticum* are pathogenic

for several fish species, including Australian salmonids, carp, rainbow trout and salmon (Leisner *et al.*, 1994).

2.1.2 LAB in fish products

LAB have been found to constitute the major part of the microflora in many fish products such as vacuum-packaged CSS, smoked herring, cold-smoked rainbow trout, gravad fish products and maatjes herring (as reviewed by Leroi, 2010). They have also been found to be potential spoilage organisms in meat products (Leroi *et al.*, 1998). LAB are dominating the microflora in vacuum-packaged CSS at the end of the shelf-life (Muller *et al.*, 1998). Leroi et al. (1998, 2000) observed that in vacuum-packaged cold-smoked salmon, *Carnobacterium* spp. dominated the flora during the first 2-3 weeks of storage, and *Lactobacillus* spp. at the end of the storage. Among *Lactobacillus* spp., *L. curvatus* and *L. sakei* have been found the major species associated with spoiled maatjes herring stored in air and under modified atmosphere at 4 or 10 ºC (Lyhs and Bjorkroth, 2008). Jeppesen and Huss (1993) reported that LAB as a dominant microbial group in vacuum- packaged gravad salmon (content of 3-6% salt and pH>5) stored 2-4 weeks at 5 and 10 ºC. Knøchel (1983) found that the microflora of this product dominated by lactobacilli after 2 weeks storage at 4 ºC in few samples. Leisner (1994) found the same results after 18 days of storage at 5 ºC. Carnobacteria play a role in the spoilage of cooked MAP shrimp as they are part of the dominant microbiota at the time of sensory rejection (Leisner *et al*., 1994).

Some LAB strains were found to be able to produce some characteristics off-odours (sour, cabbagey, sulphurous) associated with the spoilage of cold-smoked salmon. Furthermore, a strain of *L. sakei* produced H_2S during growth on cold-smoked salmon (Gram and Huss, 1996). In vacuum-packaged meat, LAB are the main spoilage bacteria, producing lactic and acetic acid from sugar, responsible for typical sour and acid odours and flavours (Leroi *et al.*, 1998).

2.2 *Listeria monocytogenes*

Listeria monocytogenes is one of the species of the genus *Listeria,* which includes *L. ivanovii, L. innocua, L. welshimeri, L. denitrificans, L. murrayi* and *L. gray* (Swaminathan, 2001). It is an ubiquitous gram-positive, short rod shaped and non-sporeforming bacterium (Rocourt, 1999). *L. monocytogenes* is aerobic or facultative anaerobic, catalase positive, oxidase negative (Farber and Peterkin, 1991). It can be found in soil, silage, plants and water and can be isolated from

numerous species of domestic and wild animals. The optimum growth temperature of *L. monocytogenes* is between 30 °C to 37 °C, and temperature limits for growth were reported to be from 0 °C to 45 °C (Walker *et al.*, 1990). It is able to grow in 10-12% NaCl and grows in high populations in moderate salt concentration (6.5%). This bacterium can survive for a long period in high salt concentration (Swaminathan, 2001).

L. monocytogenes is one of the major food borne pathogens. Generally they enter the host during the consumption of contaminated food. It has been the cause of numerous sporadic illnesses and associated with high mortality rates in large outbreaks (Messina *et al.*, 1988). Listeriosis is the common name given to the general group of disorders caused by the bacterium *L. monocytogenes.* The ingestion and penetration of *L. monocytogenes* in the gastrointestinal tract is one of the means by which listeriosis can occur. People with underlying illnesses or the elders with weakened immune systems are more prone to listeriosis, which is a life-threatening foodborne disease. Immuno-suppressed individuals, pregnant women, fetuses and neonates are most susceptible to *Listeria* infection (Swaminathan, 2001). Human listeriosis is characterized by high mortality rates, with clinical features that include mild influenza-like symptoms, encephalitis, meningitis, fatal bacteremia, puerperal sepsis, and flu-like illness during pregnancy (Schlech, 1996). It can also cause miscarriages, stillbirths, preterm labor by cross-placenta and utero-fetal infection (Mclauchlin, 1996). *L. monocytogenes* can grow in cold-smoked salmon, contamination occurs primarily during processing. Due to its physiological and genetic similarities to *L. monocytogenes* (Glaser *et al.*, 2001; Schmid *et al.*, 2005), *L. innocua* is used as its non-pathogenic surrogate (Li *et al.*, 2011), as it was also in the current study.

2.3 Specific fish spoilage bacteria

Besides LAB, other fish spoilage bacteria such as *P. phosphoreum*, *V. vulificus* and *P. putida* are also important to be discussed. In vacuum packaged CSS, *P. phosphoreum* present with LAB at the end of the shelf-life (Hansen *et al*., 1995). *P. phosphoreum* produce strong off-odour in fish products (as reviewed by Leroi, 2010). *Vibrio* spp. are also present in cold-smoked salmon (Joffraud *et al.*, 2006; Matamoros *et al.*, 2009) without making off-odors (Joffraud *et al.*, 2006). This bacterium can spoil in shellfish products. *Pseudomonas* spp. may dominate spoilage flora of fresh iced fish from temperate waters (Gram and Huss, 1996).

2.3.1 *Photobacterium phosphoreum*

P. phosphoreum is a gram-negative bacteria, easily isolated from intestines of various fish (Gram and Huss, 1996). This organism is widespread in the marine environment and is the principal spoilage bacterium of many different fish products made from fresh and salt water fish. *P. phosphoreum* plays a major role in the spoilage of freshwater fish (Gram and Huss, 1996). Leroi (1998) found that *P. phosphoreum* requires approximately 2.5% salt for its development (Leroi 1998), higher concentrations greatly lower their growth and no growth occurred at concentrations above 6% (as reviewed by Leroi, 2010). The optimum pH value for different *P. phosphorum* strains varies between 7.0 and 8.5 (Kuts and Ismailov, 2009). In fish products, *P. phosphoreum* has been found to produce trimethylamine (TMA) from trimethylamine oxide (TMAO) in stored fish and to contribute to histamine production (Gram and Huss, 1996). Due to the reduction of TMAO, this bacterium produces strong off-odours in fish products (as reviewed by Leroi, 2010).

2.3.2 *Vibrio vulnificus*

V. vulnificus is a gram-negative halophilic, motile, and rod-shaped bacterium present in marine environments such as estuaries, brackish ponds, or coastal areas (Drake *et al.*, 2007; Han and Ge, 2010; Ji *et al.*, 2011; Quan *et al.*, 2010). It is an opportunistic human pathogen and has been isolated from a variety of seafood, including shrimps, fish and shellfish (oysters, crabs and clams) (Gopal *et al.*, 2005; Ji *et al.*, 2011). *V. vulnificus* has the ability to cause serious and often-fatal infections including primary septicemia, gastroenteritis, and wound infections in humans (Chiang and Chuang, 2003; Ji *et al.*, 2011). People vulnerable to infection include those with chronic disease involving elevated serum iron levels, immune function abnormalities and other chronic disorders (Ji *et al.*, 2011; Oliver and Kaper, 2001). The level of *V. vulnificus* is strongly influenced by salinity and temperature of water in its natural habitats. Low salinity (0,5 – 2,5 %) and relatively high temperatures (> 20 ºC) are favorable for this organism (Motes et al., 1998). *Vibrio* spp. are suspected to be frequently present in CSS (Joffraud *et al.*, 2006; Matamoros *et al.*, 2009) but it is not responsible for any off-odors production (Joffraud *et al.*, 2006).

2.3.3 Pseudomonas putida

Pseudomonas putida is a rod-shaped, gram-negative bacterium that is found in most soil and water habitats where there is oxygen. It grows optimally at 25-30 ºC and can be easily isolated. *P. putida* is saprophytic and deemed safe bacteria; other species in the genus are opportunistic pathogens such as *Pseudomonas aeruginosa* and *Pseudomonas syringae* (Altinok *et al.*, 2006). *Pseudomonas* spp. are not recognized as spoilage bacteria for CSS but they are specific spoilers of ice stored tropical freshwater fish (Gram and Huss, 1996). Fruity, rotten, sulfhydryl odors and flavors are typical of the *Pseudomonas* spp. spoilage of iced fish. *Pseudomonas* spp. produces a number of volatile aldehydes, ketones, esters and sulphides (Gram and Huss, 1996).

Different bacteria have different characteristics such as shape, gram staining, optimum temperature, pH for growth and etc. Characteristics of LAB, Listeria and spoilage bacteria (*Photobacteria, Psuedomonas* and *Vibrio*) are mentioned in TABLE 2.1.

TABLE 2.1 Characteristics of LAB, Listeria and spoilage bacteria (*Photobacteria, Psuedomonas* and *Vibrio*)

Name	Cocci/ Rod	Gram (+/-)	Aerobic (+/-)	Motile (+/-)	Optimum Temp. (ºC)	Optimum pH	Habitat	Relevant seafood products
Listeria	Rod	+	Facultative anaerobic	+	30-37	7	Ubiquitous	LPFP, smoked fish and other seafood products.
Photobacteria	Rod	-	Facultative	+	18-25	7.0-8.5	Fish	CSS
Pseudomonas	Rods and cocci	-	+	+	25-30	--	Soil and water	Fish
Vibrio	Rod	-	Facultative	+	37	7.8	Seawater	Shellfish and fish
Lactobacillus	Rod	+	Facultative anaerobic	+	30-40	5.5-6.2	Grastrointestinal tract, oral cavity,high level of soluble carbohydrate.	CSS
Lactoccous	Cocci	+	Facultative	-	~30	~6.2	Milk, fermented milk, cheese,gratointestinal tract.	MAP products, seafood salad
Carnobacterium	Rod	+	Facultative	+	30	~7	Ubiquitous	CSS
E. faecalis	Cocci	+	Facultative anaerobic	-	45	9.6	Gastrointestinal tract, water, soil.	CSS, Cooked MAP shrimp,

Data collected from (Anonymous, 2001b; Barbes, 2008; Duwat *et al.*, 2000; Farber and Peterkin, 1991; Giraffa, 2007; Gram and Huss, 1996; Hartman *et al.*, ; Joborn *et al.*, 1999; Kuts and Ismailov, 2009; Leisner *et al.*, 2007; Leroi, 2010; Quan *et al.*, 2010; Rocourt, 1999; Schlegel, 1992a; Swaminathan, 2001; Thevenot *et al.*, 2006)

2.4 Cold smoked fish

2.4.1 Definition

The definition of cold smoked fish is vague. Gram and Huss (2000) characterized cold smoked fish as 'lightly preserved' including fish products preserved with low levels of salt (<6% NaCl) in the water phase, and the addition of preservatives such as sorbate, benzoate, NO_2 or smoke. These products have high pH (>5.0), they are often packaged under vacuum and must be stored and distributed at refrigeration temperatures (≤ 5º C). These products are usually consumed as a ready to eat product without further heat treatment (Ward, 2001).

2.4.2 Processing

Smoking is a traditional method of preserving fish. There are principally three steps of processing that contributes to the preserving effect, salting, dehydration and smoking. Salting and dehydration (which occurs during the smoking step) lowers the water activity (a_w), thereby, inhibiting the growth of bacteria and mold, which generally cannot grow when a_w is lower than 0.90. In addition, the chloride ions of salts are toxic for some microorganisms. The bacteriostatic effect of smoke is mainly due to phenols (Leroi *et al.*, 2000).

Two types of smoking processes are used in the food industry, hot smoking and cold smoking. Today the most common of fish smoking is cold smoking (Sunen *et al.*, 2003). The smoking temperature in this process is 25-30 ºC, this heat treatment is not sufficient to kill the microorganisms (Sunen *et al.*, 2003), meaning that the antimicrobial effect of such heat treatment is relatively small.

The traditional smoking method, smoke is generated from the incomplete burning of wood which leads to the production of polyaromatic hydrocarbon compounds (Vitt *et al.*, 2001). LS is formulated from condensation of wood smoke and water, and then filtered to remove materials that contains polyaromatic hydrocarbons which are known to be carcinogenic (Paranjpye *et al.*, 2004; Vitt *et al.*, 2001). Traditional smoke and LS are both known for their antimicrobial properties. Smoke compounds contain phenols (guaiacol and euhenol is responsible for taste, syringol is responsible for odor), acids, aldehydes, alcohol and hydrocarbon (Duffes, 1999). LS

also contains phenols, carbohydrates and organic acids and it has potential use as an antibacterial agent. The above mentioned compounds in smoke or LS can affect food colour, flavour and preservation. Duffes (1999) found that LS (0.2 mg phenols per 10 g total deposition) had a significant effect on *L. monocytogenes* growth, whereas wood smoke was less efficient.

2.4.3 Microbiology

LPFP does not include only cold smoked fish, but also other seafood products such as carpaccio-type marinated fish, , pickled fish, seafood in brine, peeled shrimp stored in MAP or brine (as reviewed by Leroi, 2010). CSS is also a lightly preserved fish product, which typically is vacuum-packed and stored at chilled temperatures. At the end of the shelf-life, the product typically contains three different microfloras, dominated by LAB, LAB and *Enterobacteriaceae* and *P. phosphoreum* with occasional high levels of LAB (Hansen *et al.*, 1995).

By comparing the CSS from Norway, the Faroe Islands and Chile, Hansen and Huss, 1998 found that *L. curvatus* dominated among LAB, and *L. sake*, *L. plantarum*, *Carnobacterium* spp. and *Leucosnostoc* spp. were present in smaller numbers (Leroi *et al.*, 1998). Leroi et al. (1998) found, however, that *C. maltaromaticum* dominated the LAB in Norwegian farmed salmon smoked in France. The flora of spoilage bacteria depends on the type of processing. Marine Vibrio dominated at the onset of spoilage in the normal dry-salted salmon, while a mixture of LAB and *Enterobacteriaceae* dominated the injection brined salmon (Hansen *et al.*, 1996). The majority of bacteria in cold-smoked salmon do not contribute to spoilage (Gram and Huss, 1996), but Leroi et al. (1998) presents data indicating that *P. phosphoreum* and most LAB are involved in spoilage.

L. monocytogenes is able to survive the smoke and salt steps performed during processing of cold-smoked salmon (Guyer and Jemmi, 1991), and to grow well both aerobically and anaerobically (vacuum packaged) at refrigerator temperatures.

2.5 Effects of preservatives

2.5.1 Salt

The preservation of fish by salting is one of the ancient techniques. Sodium salts such as lactate and acetate can be used to inhibit the growth of spoilage bacteria and foodborne pathogens, and thereby increase the shelf-life of refrigerated seafood. Earlier studies indicated that these organic acid salts have the ability to control microbial growth, improve sensory attributes, and extend the shelf-life (Kilinc *et al.*, 2009; Mendonca *et al.*, 1989; Sallam, 2007; Zhuang *et al.*, 1996). Sodium diacetate (SDA) is also used in foods as a flavouring and an antimicrobial agent. In 2000, the U.S. Department of Agriculture- Food Safety and Inspection Service increased the permissible level of Sodium lactate (SL) or K-L to 4.8% and SDA limited to less than 0.25% of the weight of the finished products.

Generally salting is applied as a pre-smoking treatment in order to provide flavour, to prevent discolouration and to firm the fish. Salt is added in fish in two different ways; dry salting or wet salting (brining). In dry salting, fish is covered with an amount of salt corresponding to 5-10% of the weight of the fish and the brine formed is allowed to drain away. In brining, the product is immersed in a prepared salt solution of a particular degree of salt saturation (Dillon *et al.*, 1994; Horner, 1997).

Salt is effective as a preservative because it reduces the a_w of foods. The a_w of a food is the amount of unbound water available for microbial growth and chemical reactions. The ability of salt to decrease water activity is thought to be due to the ability of sodium and chloride ions to associate with water molecules. According to Davidson et al. (2001), adding salt to foods can cause microbial cells to undergo osmotic shock, resulting in the loss of water from the cell and thereby causing cell death or retarded growth (Davidson, 2001). The mechanism of sodium lactate not only includes lowering the a_w but also other properties such as cytoplasmic acidification and specific anionic effects (Kin *et al.*, 2011).

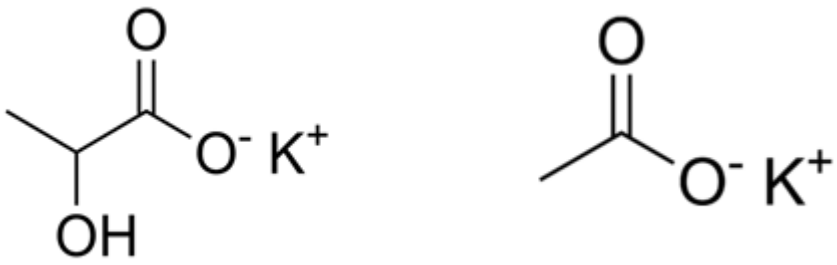


FIGURE 2.1 Structure of K-L (left) and K-A (right). PURAC solution (PURASAL Opti.From PPA PLUS), used in this study, contains 72.4% K-L and 5% K-A.

CSS with a high salt content (4.6% in water phase) stored vacuum packed at 5 and 10 ºC were sensory acceptable for at least 2-3 weeks longer than similar products with low salt (2.2%) stored at the same conditions (Hansen *et al.*, 1995). Increasing the storage temperature from 5 to 10 ºC shortened the shelf life of 1-2 weeks for samples with high salt content and 2-3 weeks for those with low (Hansen *et al.*, 1995). Results from a study by Peterson et al. (1993) indicated that either 3 or 5 % water phase NaCl was sufficient to prevent the growth of *L. monocytogenes* in either vacuum packaged or permeable film-packaged CSS, storage at 5 or 10 ºC. But when the salt concentration increased to 6%, growth was significantly inhibited by the low storage temperature. This salt concentration is sensorally too high, meaning that a combined effect of several inhibitory components is necessary to inhibit the growth of *L. monocytogenes* in cold-smoked salmon, regardless of packaging method.

A study by Leroi et al. (2000) showed that the inhibition of bacteria in cold-smoked salmon stored at 5 ºC with 5% salt (w/w) and smoke for 5 weeks, was linearly proportional to salt and phenol content (the higher the concentration, the greater the inhibition). Gram–negative bacteria are considered to be more salt sensitive than gram-positive bacteria. A study published by Leroi el at. (1998), reported that *P. phosphoreum* required approximately 2.5% salt (NaCl) for its development, but higher concentration (5% in liquid culture medium) greatly lowered their growth and no growth occurred at concentration higher than 6% (as reviewed by Leroi, 2010).

2.5.2 Liquid smoke

Liquid smoke contains antimicrobial compounds such as phenols, carbohydrates and organic acids and this food ingredients has potential use as antimicrobial agents (Vitt *et al.*, 2001). The main purpose of smoking food products with liquid smoke is that it not only acts as a colouring and flavouring agent, but also has antibacterial and antioxidative properties. The phenolic compound present in liquid smoke destroys the cytoplasmic membrane and causes leakage of cells (Davidson, 2001). Liquid smoke or smoke compounds have been tested in smoked salmon (Poysky *et al.*, 1997; Thurette *et al.*, 1998), beef franks (Messina *et al.*, 1988) and laboratory media (Niedziela *et al.*, 1998). Commercial LS (Aro-smoke P-50) has been used to control the hazard of *L. monocytogenes* in processed meat products, reported by Messina et al (1988).

2.6 Storage temperature

Storage temperature is the most important environmental parameter influencing the growth rate and type of spoilage microorganisms of highly perishable foods, such as seafood products. Storage at chilled temperatures, one of the most widely practised methods of controlling microbial growth, reduces contaminating microorganisms that cause spoilage and prolongs the shelf-life of the fish. Storage temperature below the growth optimum lead to extended generation times and lag time, and the growth rate decreases. Storage temperatures below the growth minimum result in a continued extension of the lag-time until multiplication ceases and the growth of the microorganism stops (Jackson *et al.*, 2001).

LAB tends to grow slowly at refrigerated temperatures. Under anaerobic conditions, LAB are capable of growth at 5 ºC or below, enabling successful competition with other psychrotrophic spoilage microorganism (Jackson *et al.*, 2001).

2.7 Packaging

Different forms of packaging, such as modified atmosphere packaging (MAP) and vacuum packaging (VP) are used to extend the shelf-life, and to keep the microbiological quality of fresh and processed fish as high as possible. Furthermore, packaging protects the fish product from contamination, fat oxidation, shrinkage, and colour deterioration. MAP involves replacement of air in the package with different gases such as CO_2, O_2 and N_2. VP is the method of packaging without oxygen (air) in the container.

In the presence of CO_2, the number of gram negative bacteria decreases, particularly the respiratory microorganism like *Pseudomonas* and *Shewanella*. However, *P. phosphoreum* is resistant to CO_2. MAP favours the development of LAB in fresh fish (as reviewed by Leroi, 2010). Research carried out on herring fillets showed that preservation under 100% CO_2, clearly led to predominance of *Lactobacillus* spp., while under air *Pseudomonas* were more common. However, high CO_2 levels resulted in poor sensory characteristics due to the carbonated flavour (as reviewed by Leroi, 2010). Emborg et al (2001) showed that *P. phospohreum* was the dominant bacterial species in fresh salmon MAP fillets (60% CO_2, 40% N_2). When the fillets were frozen for four weeks at -20 ºC and thawed before packaging, *P. phosphreum,* being a very cold-sensitive species, was eliminated so that *C. maltaromatium* became dominant (Emborg *et al.*, 2002).

2.8 Bacterial growth

The growth of microorganisms reproducing by binary fusion can be depicted graphically by plotting the logarithm of cell numbers or viable counts versus time. The resulting growth curve is sigmoidal in shape and has four different phases: (A) lag phase, (B) exponential (logarithmic) phase, (C) stationary phase and (D) death phase as mentioned in FIGURE 2.2.

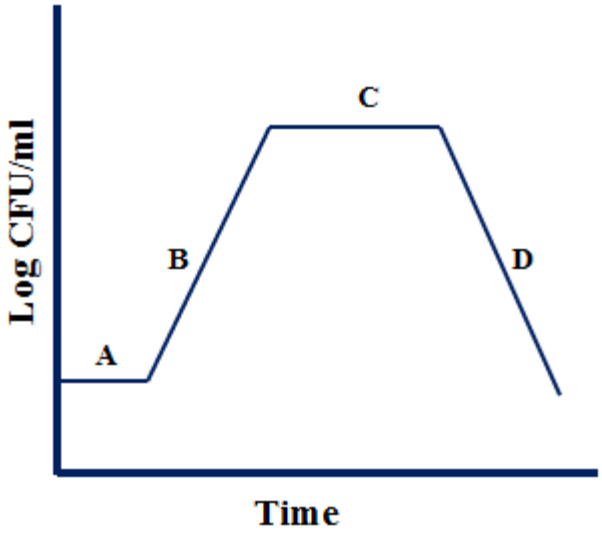


FIGURE 2.2 Bacterial growth curve, CFU/ml denotes colony forming units per ml

During the lag phase, cells adjust to their new environment by inducing enzyme synthesis and activity, initiating chromosome and plasmid replications. The length of the lag phase depends on the temperature, the inoculum size (larger inoculum usually results in shorter lag phases) and suitability of the nutrient medium (Montville and Matthews, 2001). As per Robinson concept, the lag time depends on two elements : (i) the amount of energy required of the cell to adjust to a new environment and/or repair injury due to the shift to the new environment, and (ii) the rate at which those repairs and adjustments can be made (Robinson *et al.*, 1998).

The exponential phase, or logarithmic growth phase, is a period characterized by a cell doubling. During the exponential phase, each microorganism is dividing at a constant rate. Exponential growth cannot continue indefinitely, however, because the medium is eventually depleted of nutrients and enriched with wastes that may be inhibiting bacterial growth, or even toxic.

The third phase in the growth of bacteria is a stationary phase, when the rate of bacterial growth is equal to the rate of bacterial death. The factors that cause cells to enter stationary phase are related to changes in the environment. Typically, the stationary phase is caused by high cell concentrations, low partial pressure of oxygen, and accumulation of toxic metabolic end products (Schlegel, 1992b).

The final phase of the growth cycles is the death phase. In this phase, the cells quickly lose the ability to divide even if they are placed in fresh medium. In some cases, cell lysis occurs due to release of intracellular lytic enzymes. All phases can be slowed by lowering the temperature.

2.9 Bioscreen C

The ‘Bioscreen’ is an automated turbidity reader linked to an integrated PC, which measures optical density (OD) by the changes in turbidity or the formation of turbidity in the culture medium due to the growth of microorganisms. All microorganisms increase turbidity of the broth when growing and multiplying in it. OD, measured in a Bioscreen, can be used as a measure of the concentration of bacteria in a suspension. As visible light passes through a cell suspension the light is scattered. Greater scatter indicates that more bacteria or other material is present. In the Bioscreen, OD is measured kinetically with a vertical photometer in which the light beam passes up through the bottom of the plate well, through the sample suspension to a detector.

This equipment has been used for many applications in microbiology, including basic research, studies on the effects of chemical compounds on the growth of microorganisms, determination of the lag time of individual cells, growth kinetics of bacteria and others (Johnston, 1998).

2.10 Apparent lag time

In food microbiology, the lag time is mostly defined as the time before increase of number of cells occurs, or, more accurately, as the point of intersection of the tangent at the steepest slope of the growth curve (Smelt *et al.*, 2001). In the review by Swinnen et al. (2004) the various definitions of lag time are covered. For instance, Pirt (1975) refers to the lag time of individual cells as true lag defined as the time from inoculation of one cell until the time of division. According to Smelt and Brul 2007, apparent lag is defined as the time needed for a whole population to multiply by a factor of 2. The apparent lag time is subjected to a larger variability and dependent on the previous history of the population and also on the size of the inoculum (Smelt *et al.*, 2001).

In the present thesis, apparent lag time is defined as the time taken for the OD to double. When OD doubles, the population may also double. However, I cannot present experimental evidence that doubling of OD truly corresponds to a doubling of the bacterial population. Thus, it must be stressed that this is a simplified means of estimating (apparent) lag time. OD can vary dependent on the size and shape of the cell (Løvdal *et al.,* 2008), and also on bacterial shedding of extracellular material to the medium. Additionally, the present approach may be even more sensitive to inoculum size. However, the purpose of this work was to observe quantitative difference on growth of bacteria as an effect of the preservatives applied, and not the qualitative difference in true lag. As such, the present approach is efficient in medium scale experiments. In the present study, ‘apparent lag time’ should be considered an operational term.

2.11 Analysis of Bioscreen data

The data was analyzed using a data spreadsheet (Excel), and calculating ratio OD for each bacteria in different treatment. Ratio OD was calculated by following equations:

$$\boldsymbol{X_0 = \frac{X}{n}} \qquad \text{Equation 1}$$

$$\boldsymbol{Y_0 = \frac{y}{n}} \qquad \text{Equation 2}$$

$$\boldsymbol{Z_0 = Y_0 - X_0} \qquad \text{Equation 3}$$

$$\boldsymbol{Z_2 = Z_1 - Z_0} \qquad \text{Equation 4}$$

$$\boldsymbol{Z = \frac{Z_2}{Z_3}} \qquad \text{Equation 5}$$

X = Mean value of the OD of the unamended TSBYE solution (without bacteria) in the triplicate at each time interval.

X_0 = Baseline value for TSBYE solution (without bacteria).

n= Total sample number.

Y = Mean value of the OD of the amended stock solutions (without bacteria) in the triplicate at each time interval.

Y_0 = Baseline value for TSBYE with additives (without bacteria).

Z_0 = OD value of the preservatives.

Z_1 = OD value of the stock solutions (with bacteria).

Z_2 = Correction value of each sampling (t_0……t_n).

Z_3 = Correction value of the first sampling (time zero sample).

Z = Ratio OD of each sample.

The data for growth bacteria and pH values for all bacteria in different combinations were compared using analysis of variance (ANOVA) in Minitab. Statistical significance was set at $p<0.05$.

2.12 Real-time PCR

The polymerase chain reaction (PCR) is one of the most powerful technologies in molecular biology. Since it was described (Mullis and Faloona, 1987), the PCR technique has undergone significant improvements in methodology, and has revolutionized molecular biology from the conventional thoughts. It is a basic technique that can amplify a small amount of template DNA or reverse transcribed RNA, also called complementary DNA (cDNA), into large quantities in a few hours. However, conventional end-point PCR has a drawback. It lacks reliable quantification and does not easily yield reproducible results. This limitation was resolved twenty years ago, with the invention of real-time PCR by Higuchi et al. (1992) . A novel "real time" quantitative PCR (qPCR) method with greater specificity was also invented by Heid et al. (1996). The method detects the increasing amount of template while the amplification is still progressing. Real-time qPCR has been used in a growing number of research applications including gene expression quantification.

qPCR uses two basic quantification methods which are increasingly used and suitable for different applications: Absolute quantification and relative quantification. Absolute quantification is used to determine the absolute/exact quantity of a genomic DNA or RNA template within an unknown sample by using a standard curve that is prepared form a dilution series of control template of known concentration. Relative quantification is used to measure the relative concentration of template (or target gene) in unknown samples normalized to a stably expressed reference gene, and compared relatively to a calibrator sample (for example time zero, or untreated sample).

Real-time PCR uses several different fluorescence detection technologies to detect PCR products. One of them is SYBR Green which is used as a dye for the quantification of double stranded DNA (dsDNA) PCR products. This fluorescent dye must be added in the reaction mixture which contains template cDNA (or genomic DNA), gene specific primers (forward and reverse), and buffer. After annealing of the primers, a few dye molecules binds to the double stranded DNA, resulting in a significant increase of molecules to emit light upon excitation. With each cycle, more and more dye molecules bind with newly synthesized DNA. If the reaction is monitored continuously, an increase of fluorescence can be viewed by using a computer.

During the initial cycles of real-time PCR, the signal level can be denoted by the base line of the reaction. Generally there is little change in fluorescence signal within cycles of 3 to 15. The background or the noise of the reaction can be linked with the low-level signal of the baseline. To eliminate the background in the early cycles, it is necessary to take into consideration enough cycles when determining the baseline in the real-time PCR. However, the cycles in which the amplification signal begins to rise above the background, should be avoided. The baseline value should be set in a way that the threshold cycle (Ct) can be determined accurately.

The threshold of the real-time PCR reaction is the level of signal that reflects a statistically significant increase over the calculated baseline signal. It is set to distinguish relevant amplification signal from the background. Usually, real-time PCR instrument software automatically sets the threshold above 10 times the standard deviation of the fluorescence value of the baseline. However, the positioning of the threshold can be set at any point in the exponential phase of PCR amplification. The threshold cycle is the cycle (Ct) number at which the fluorescence signal of the reaction crosses the threshold. The Ct is used to calculate the initial cDNA copy number, because the Ct value is inversely related to the amount of starting template. The Rn value is the ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye. The delta Rn value, or normalized reporter value, is the Rn value of an experimental reaction minus the Rn value of the baseline signal generated by the instrument.

After finishing a sufficient number of cycles (30 to 50), melting curve analysis can be performed to verify primer specificity when applying SybrGreen chemistry. Generally, a melting curve shows a single amplified product for a gene, but it is also possible to have more amplified product from primer-dimer and genomic DNA, resulting in multiple peaks. Therefore, melting curve analysis can confirm the specificity of a quantitative reverse transcription PCR reaction. The melting curve can be found when dsDNA starts to melt by heating. As the temperature is raised, double stranded DNA becomes a single stranded DNA and the dye (SYBR Green based) dissociates from the DNA. Agarose gel electrophoresis can be performed (prior to dissociation) to estimate the size of the amplified product (after amplification with gene-specific primers). .

2.13 Agarose gel-electrophoresis

Gel electrophoresis is one of the most widespread methods of studying nucleic acids. Introduction of electrophoresis methods has revolutionized the research on molecular genetics. Electrophoresis is the procedure by which charged molecules are allowed to migrate in an electric field, the rate of migration being determined by the size of the molecules and their electric charge. In gel electrophoresis, the nucleic acid is suspended in a gel, usually made of polyacrylamide or agarose. The gel is a complex network of fibrils, and the pore size of the gel can be controlled by the way in which the gel is prepared. The nucleic acid molecules migrate through the pores of the gel at rates dependent on their molecular weight and molecular shape. Small molecular or compact molecules migrate more rapidly than large or loose molecules. After a defined period of time of migration (up to a few hours), the locations of the cDNA molecules in the gel are assessed by making the cDNA molecules fluorescent and observing the gel with ultra violet radiation (Madigan and Martinko, 1997).

2.14 Molecular microbiology

Real-time quantitative PCR (RT-qPCR) has been used as a powerful tool for gene expression analysis in bacteria (Costantini *et al.*, 2011; Reiter *et al.*, 2011; Zhao *et al.*, 2011). For accurate RT-qPCR analysis, it is necessary to correct non-specific experimental variations such as difference in starting material and quality between samples. These variations can be controlled by normalizing gene expression data using one or more appropriate reference genes (Løvdal and Lillo, 2009; Vandesompele *et al.*, 2002b; Zhao *et al.*, 2011). The selection of suitable references for the normalization of gene expression is a prerequisite for reliable results in quantitative RT-PCR (Vandesompele *et al.*, 2002b). The ideal reference should be unaffected by experimental treatment and should be expressed at a constant level among different cells in an organism. Vandesompele et al. (2002) recommended using multiple reference genes to normalize gene expression analysis because no unique gene that is stably expressed under all experimental conditions has yet been reported.

In this study, eight candidate reference genes of *L. curvatus*, namely the *tuf* gene (elongation factor Tu, involved in protein synthesis), *6PGD* (6-phosphogluconate dehydrogenase), *rpoB* (RNA polymerase beta subunit), recA (recombinase A), *pheS* (phenylalanyl-tRNA synthesis), *GAP* (glyceraldehyde-3-phosphate), *RpL11* (ribosomal protein L11), and *RpS7* (ribosomal

protein S7) were evaluated for their potential as reference genes under conditions of variable temperature growth conditions. One genes of interest; *hsp60* (also known as *groEL,* 60-kDa heat shock protein) *L. curvatus* was included in the analysis. The main goal of this experiment was to find stably expressed reference genes in different temperatures as an experimental condition.

3 Material and Methods

Two different methods are mentioned in this chapter. The first method is for the growth experiments conducted with the Bioscreen, and the second is for the real time PCR gene expression experiments.

3.1 Bioscreen experiment

3.1.1 Bacterial cultures

The fish spoilage bacteria (*P. phosphoreum* CCUG 16288, *P. putida* ATCC 49128 , and *V. vulnificus* ATCC 27562 *),* two stains of *Listeria innocua*; *L. innocua* ATCC 33090 and *L. innocua* ATCC 51742 (as a *L. monocytogenes* non–pathogenic substitute) and LAB (*L. curvatus* CCUG 30669, *L. lactis subsp. lactis* CCUG 32211, *C. maltaromaticum* CCUG 30142, *C. inhibens* CCUG 31728, *E. faecalis* ATCC 29212) were used in Bioscreen experiments. Initially they were grown in rich medium at two different temperatures; 30 ºC and 20 ºC according to their temperature optima.

Bacterial strains where obtained from Oxoid (Cambridge, UK) and the Culture Collection at the University of Gothenburg (CCUG). Culti-loops (Oxoid) and freeze dried (CCUG) bacterial strains were revived according to the manufacturers. The bacteria (*L. innocua* ATCC 33090, *L. innocua* ATCC 51742, *P. phosphoreum, P. putida, V. vulnificus, L. lactis* and *E. faecalis*) were streaked out onto TSAYE (tryptic soy agar (Oxoid, Basingstoke , UK) with 0.6 % yeast extract (Merck , Darmstadt , Germany)) Petri dishes with a plastic sterile loop and incubated at 30 ºC for 24 hours, whereas *P. phosphoreum, C. maltaromaticum* and *C. inhibens* were grown at 25 ºC . A different medium, De Man, Rogosa and Sharpe (MRS), was used to grow *L. curvatus* at 30 ºC for 48 hours.

The bacteria were taken from single colonies of the TSAYE agar plate with a sterile plastic loop and transferred to the Microbank tubes (Pro-lab Diagnostics, Canada). Microbank tubes were shaken 5 times, so that bacteria would attach to the rings and then the bacteria where stored at -70 °C on the rings until further use.

Before the experiment, the rings from frozen storage were transferred to TSBYE (tryptic soy broth (Oxoid, Basingstoke, UK) with 0.6% yeast extract). The frozen beads for each bacteria were inoculated in a 100 ml Erlenmeyer flask containing 50 ml TSBYE and incubated at first for 24 hours (growth condition for *L . innocua* ATCC 33090, *L. innocua* ATCC 51742, *P .putida, V. vulnificus* : 30 ºC, 150 revolution per minute; rpm and for *P. phosphoreum,*: 20 ºC, 150 rpm. The well grown bacteria were transferred into new TSBYE medium three times sequentially after every 24 hours. Sterile technique was maintained to avoid contamination during transferring. The frozen beads of *L. curvatus* were transferred to 50 ml MRS broth in a 100 ml Erlenmeyer flask and incubated at 30 ºC, 150 rpm for 2 days. After two days, about 1 ml old culture was transferred to 50 ml TSBYE in a 100 ml Erlenmeyer flask and placed in a shaking incubator at 20 ºC with 150 rpm for 3 days. Then the well grown bacteria were transferred into new TSBYE medium three times sequentially after every 72 hours to adapt to temperature and medium. *L. curvatus* took much longer time to grow in TSBYE than MRS broth which contained polysorbate (Tween 80), acetate, magnesium and manganese, known to act as special growth factors for *Lactobacillus* spp. (Leroy and Vuyst, 2001).

Another four LAB (*L. lactis*, *C. maltaromaticum*, *C. inhibens*, *E. faecalis*) were grown in TSBYE medium over night to let the bacteria grow well (growth condition for *L. lactis* and *E. faecalis*: 30 ºC, 150 rpm, *C. maltaromaticum* and *C. inhibens* : 20 ºC, 150 rpm). All bacteria were transferred several times in TSBYE, with decreasing temperature, until they grew well at the target temperature of 20 °C.

3.1.2 Preparation of stock solution of TSBYE with additives:

Stock solutions were prepared with PURAC solution [K-L and K-A] and LS in TSBYE as shown in TABLE 3.1. Preparation of stock solutions is outlined in Appendix 9.2 and chemical properties of liquid smoke and PURAC solution in Appendix 9.2.9 and 9.2.10.

TABLE 3.1 Experimental design for control, PURAC solution [K-L (%) + K-A (%)], and liquid smoke (%)

Treatment	K-L (%)	K-A (%)	Liquid smoke (%)
1(Control)*	0	0	0
2	0	0	0,07
3	0	0	0,14
4	3	0,21	0
5	3	0,21	0,07
6	3	0,21	0,14
7	6	0,42	0
8	6	0,42	0,07
9	6	0,42	0,14

*treatment 1 is the control where only TSBYE is added. PURAC solution, liquid smoke, and combined PURAC and liquid smoke solution, were diluted in TSBYE medium in other treatments.

3.1.3 Addition of bacterial culture into TSBYE and different stocks

Prior to Bioscreen experiments, cultures of all bacteria was left to grow for 20 hours, except for *L. curvatus* which was grown for 48 hours. The bacteria was then diluted in TSBYE medium amended with PURAC and/or liquid smoke to a concentration of approximately 10^3 CFU/ml. One tube of each treatment (TABLE 2.1) remained without bacteria to serve as a negative control. From each tube, inoculum of 250 µl was added to microtiter plate wells in triplicate. The microtiter plates were placed in the Bioscreen C where they were incubated at a constant temperature of 20 ºC. Before each measurement, the plates were shaken for 10 seconds at medium intensity (20 move instruction). Bioscreen C monitored the growth bacteria by reading optical density (OD) at a wavelength of 600 nm ($OD_{600)}$ at regular time intervals (every 10 minutes). The total duration of the Bioscreen C experiment was six days.

3.1.4 Enumeration of bacteria in culture

To calculate the number of cells in the cultures, bacterial cultures were plated on TSAYE (two stains of *L. innocua*, and one strain each of *P. phosphoreum, P.putida, V. vulnificus, L. curvatus* , *L. lactis* , *C. maltaromaticum*, *C. inhibens* and *E. faecalis),* blood agar (*L. lactis* and *E. faecalis*) and MRS (*L. curvatus*) using EDDY JET (IUL Instruments, Barcelona, Spain). Each Eppendorf tube was shaken by Vortex (IK-A-Labortechnik, Staufen, Germany) and then placed manually under the nozzle of the EDDY JET. The TSAYE dishes of *P. putida* were overlaid with soft agar (melted TSAYE) to get countable colonies. All of the agar plates were incubated at 30 ºC (Two stains of *L. innocua* , *P. putida, V. vulnificus, L. curvatus* , *L. lactis* and *E. faecalis*) and 25 ºC (*P. phosphoreum, C. maltaromaticum* and *C. inhibens)* for two days. To check for additional growth, they were left for two more days in the incubator at the above mentioned temperature, and bacterial colonies were counted by the Stuart-Colony Counter SC6. To calculate the weighted mean of each bacteria, Equation 6 was used where $a_1 \ldots a_n$ are the mean values of CFU/ml in the different dilutions, and $c_1 \ldots . c_n$ are the respective dilutions factors.

$$\boldsymbol{w_{mean}} = \frac{(\boldsymbol{a_1}\times\boldsymbol{c_1})+(\boldsymbol{a_2}\times\boldsymbol{c_2})+\cdots\cdots(\boldsymbol{a_n}\times\boldsymbol{c_n})}{(\boldsymbol{c_1}+\boldsymbol{c_2}+\cdots\cdots\boldsymbol{c_n})} \qquad \text{Equation 6}$$

3.1.5 pH measurement

Four sets of microtiter plates (for time 0, after 20 hours, after 44 hours, after 68 hours and after 164 hours) were filled with bacterial cultures same as filled for Bioscreen C plate. One set of microtiter plates (at time 0) was stored at -80 ºC at the beginning of the experiment and the remaining sets were placed into an incubator at 20 ºC with 150 rpm. One set of plates were taken out from the incubator after 24 hours and stored at -80 ºC. The remaining two sets of plates were taken out after 48 and 68 hours respectively and stored at -80 ºC. The final plates were taken out directly from the Bioscreen C at the end of the experiment and stored at -80 ºC in the freezer until measurement. All samples were incubated at 20 ºC with 150 rpm for six days until the Bioscreen was stopped. The temperature was recorded every day by a thermometer which was placed inside the incubator. Before pH measurement, the micrometer pH (Jenco model 60 portable digital pH meter –USA) was calibrated according to the instruction of the manufacturer.

3.2 Gene expression analysis with real time PCR

The protocol of RNA isolation and cDNA synthesis was modified in gene expression analysis. In this modified method, Promega product for DNase treatment and Roche kits were used instead of Qiagen product. DNase treatment in RNA isolation and in cDNA synthesis procedures are the important parts in these methods. Furthermore, RNA protect reagent had to be added to bacterial culture to stabilize RNA before the bacterial cells are lysed. Modified protocols of RNA isolation, cDNA synthesis and Standard real-time PCR have been described in details in Section 3.2.1, 3.2.2 and 3.2.3. Section 3.2.4 describes how the modified method was evolved through optimization.

3.2.1 RNA isolation

In gene expression analysis, it is very important to prepare high quality RNA because RNA can be contaminated by genomic DNA. To reduce contaminating genomic DNA from RNA, proper RNA isolation and cDNA synthesis procedures need to follow. RNA isolation was carried out with the RNeasy Mini Kit (Qiagen, Germany). Exactly 500 µl diluted *L. curvatus* culture was transferred to a 2 ml eppendorf tube and mixed with 1000 µl RNA protect bacterial reagent. The mixture was mixed immediately by vortexing for 5 s and then it was incubated at room temperature for 5 minutes and later centrifuged for 10 minutes at 5000×*g*. The supernatant was discarded and residual supernatant was removed by gently dabbing the inverted tube onto a paper towel. A mixture of 200 µl TE buffer (30 mM Tris-Cl, 1 mM EDTA) containing lysosome (15 mg/ml) and 20 µl Qiagen proteinase K was prepared and then added to the pellet which was mixed by vortexing for 10 s thereafter, the mixture was incubated on a shaker-incubator at room temperature for 10 minutes. Seven hundred µl RNeasy lysis buffer; RLT (with 10 µl β-mercaptoethanol per ml) was added and vortexed vigorously. Then, 96-100% ethanol (500 µl) was added and it was mixed immediately by pipetting. The half of the mixture (700 µl) was transferred to an RNeasy spin column and centrifuged for 15 s at 8000×g. The flow through was discarded after centrifugation and reinserted in the collection tube. This step was repeated twice in order to collect all bacterial lysate. Bacterial lysate was washed by 350 µl buffer RW1 and centrifuged for 15 s at 8000×g and the flow though discarded.

DNase 1 (Qiagen, Germany) was prepared according to the manufactures instruction. Ten ul DNase 1 was added to 70 µl Buffer RDD, and added directly onto the RNeasy spin column membrane before incubation at room temperature for 15 min. gDNA was removed from the mini spin column by buffer RW1 (350 µl) by centrifuging for 15 s at ≥ 8000×g . The flow through was discarded and the mini spin columns placed in new tubes.

RPE buffer (500 µl) was added two times to the same RNeasy spin column in two separate steps and centrifuged at 8000 g for 15 seconds and 2 minutes, respectively, prior to discarding the flow through from the collection tube. RNA was eluted into a new 1.5 ml collection tube by adding 30 µl RNase-free water directly to the spin column and centrifuging at ≥ 8000×g for 1 minute. This step was repeated twice to elute all RNA.

Nano-Drop spectrophotometer was used to determine RNA concentration and purity. Three µl eluted RNA was used to determine the concentration by measuring A_{260} absorbance for each sample. Purity was assessed by calculating the A_{260}/A_{280} ratio. A_{260}/A_{280} ratio, values between 1.8 and 2.0 were used for gene expression study. Nuclease-free water was used as blanks. All RNA samples were diluted to 10 ng μl^{-1} in nuclease–free water and subjected to cDNA synthesis.

3.2.2 cDNA synthesis

cDNA synthesis was performed using the Quantitect reverse transcription kit (Qiagen, Germany) and consisted of three different steps which are described below.

In the first step, 1× gDNA wipeout buffer (2 µl) was added to 12 µl RNA (10 ng/ul), as shown in TABLE 3.2, and incubated at 42 ºC for 2 minutes.

TABLE 3.2 Genomic DNA elimination reaction components

Component	Volume/reaction
gDNA Wipeout buffer, 7×	2 µl
RNA (10 ng μl^{-1})	12 µl
Total volume	14 µl

In the second step, 2× Reverse Transcription master mix was prepared using the kit components and supplemented with 14 µl of template RNA, as shown in TABLE 3.3. A reverse transcriptase

control (RT control) was prepared with all components except that water was used as substitute for Quantiscript reverse transcriptase. The reason of using RT control was to confirm that RNA was totally free of genomic DNA.

TABLE 3.3 Reverse transcription master mix preparation

Component	Volume/ reaction
Reverse-transcription master mix	
Quantiscript Reverse transcriptase	1 µl
Quantiscript RT buffer, 5x	4 µl
RT primer MIX	1 µl
Template RNA	
Entire genomic DNA elimination reaction	14 µl
Total volume	20µl

Note: The kit components were thawed on ice, and the RT master mix prepared on ice.

In the third step, reverse transcription was performed in a thermal cycle. The thermal cycle settings were; primer annealing at 25 ºC for 5 minutes (step 1), then reverse transcription at 42 ºC for 15 minutes (step 2), and finally denaturation of the enzyme at 85 ºC for 3 minutes to stop the reaction (step 3). The final cDNA products were stored at -20 ºC until further use.

3.2.3 Standard real time PCR

Before using the real time PCR machine, a StepOnePlus (Applied Biosystems), it was calibrated according to the manual supplied by the manufacturer. Ninety six-well plates were used in real-time PCR experiments. The real-time PCR reaction per well was as shown in

TABLE **3.4**. 2×PCR Precision master mix containing ROX and SYBR green l (PrimerDesign, Southampton, UK), water and gene-specific primers were mixed to a master mix before adding cDNA. Instead of cDNA, water was added to master mix as negative control. Twenty µl of PCR reaction components were transferred to the wells in triplicate. The plate was centrifuged at 20 ºC for 3 minutes at 1500 rpm. Centrifugation was performed to get all the contents at the bottom of the wells.

TABLE 3.4 Real time PCR reaction mix

Component	Volume/ Reaction (µl)
2× PCR master mix	10
Forward primer (10µM)	0.4
Reverse primer (10µM)	0.4
Nuclease –free water	8.2
cDNA (10ng/µl)	1
Total volume/well	20

The thermo cycling profile of the PCR machine was set-up as shown in TABLE 3.5. Reactions were run for 40 cycles and after that, the amplified PCR products were routinely subjected to melting curve analysis (65-95 ºC) to verify specificity of the amplification. The gel electrophoresis was only performed initially to confirm correct cDNA band size with each primer pair.

TABLE 3.5 Thermal cycle profile

Temperature (ºC)	Time (min:sec)
50	2:00
95	10:00
95	00:15

3.2.4 Initial optimization steps

To develop a RNA isolation and cDNA synthesis method avoiding genomic DNA contamination and high quality RNA and subsequent cDNA, some steps in the original procedures supplied by the manufacturers were modified, as described below.

1) RNA protect agent was added to bacterial samples in order to stabilize and to protect RNA from degradation before RNA was isolated from the cells.
2) Generally most of the genomic DNA can be removed effectively from RNA by filtering through RNeasy silica-membranes. Thus, further DNase treatment (in addition to the

DNA wipe-out buffer) is often not required for isolating RNA. However, I observed significant amplification in the RT controls, with similar Ct values in both RT controls and cDNA, indicating genomic DNA contamination in the samples (see Result; FIGURE 4.13). To remove DNA completely, DNase treatment also on the filter was carried out.

3) After adding RNA protect reagent to culture samples, a pellet was formed by centrifugation and later mixed with Qiagen proteinase K, RNeasy lysis buffer and ethanol. The total volume of the mixture was 1470 µl, and from this, 1400 µl was loaded onto RNeasy spin column, in two steps of 700 ul.
4) Promega RQ1 RNase-free DNase (instead of Qiagen product) was used during some of the initial optimization of the RNA isolation method. The mixture of DNase 1 was prepared according to TABLE 3.6

TABLE 3.6 DNase 1 mix preparation

Contents	Volume
RQ1 RNase free DNase	10 µl
RQ1 RNase free DNase 10× reaction buffer	10 µl
Nuclease free water	80 µl

Note: DNase 1 form promega product

3.3 Gene expression experiment at different temperature

After modified protocol of RNA isolation and cDNA synthesis, gene expression experiment was performed in different temperatures. Frozen beads containing of *L. curvatus* CCUG 30668 was grown in MRS broth at 30 ºC, 150 rpm for 2 days. The MRS medium was kept at 5 ºC, 25 ºC and 40 ºC overnight. The bacterial culture was diluted 10 times and 0.5 ml of diluted bacterial culture was transferred to a 2 ml eppendorf tube and added 1 ml RNA protect reagent. This procedure was carried out in duplicate in 2 eppendorf tubes (t_{01}, t_{02}) at time zero (0 hour). Four ml of bacterial culture was transferred to 15 ml falcon tube and centrifuging at 4000 rpm for 15 min and this step was done for 3 falcon tubes at respectively 5 ºC, 25 ºC and 40 ºC. The supernatant was discarded and the pellet was re-suspended in MRS medium with respective temperature of 5 ºC, 25 ºC and 40 ºC. The sample was transferred to 50 ml falcon tube and filled up to 40 ml with

MRS medium which was kept at 5 ºC, 25 ºC and 40 ºC. Three samples were incubated at respective temperatures. The temperature was monitored at start and each sampling. Exactly 0.5 ml sample from each treatment was transferred to two different 2 ml eppendorf tubes sequentially after 1 hour, 2 hour, 4 hour, 8 hour and 24 hour and added 1 ml RNA protect reagent and later on RNA isolation (3.2.1), cDNA synthesis (3.2.2) protocol and real-time PCR (3.2.3) were performed.

3.4 Primer design and efficiency

Eight candidate reference genes (*Tuf, 6PGD, RpoB, RecA, GAP, RpL11, RpS7* and *PheS*) and a gene of interest (*HSP60*) were considered for gene expression analysis in *L. curvatus*. The primers were designed by my supervisor, Trond Løvdal, using Primer 3 (Version 0.4.0 http://frodo.wi.mit.edu) based on the available DNA sequences of *L. curvatus* (Genbank accession numbers; TABLE 3.7) and the secondary structure was checked in MFOLD (http://frontend.bioinfo.rpi.edu/applications/mfold/egi-bin/dna-form1.cgi) according to D'haene et al., (2010).

A standard curve was constructed to calculate the gene-specific PCR efficiencies from a 10-fold series dilution of cDNA template for each primer pair. The correlation coefficients (R^2) and slope values can be obtained from the standard curve, and the efficiency (E) of PCR was calculated according to the equation $E = 10^{-1/slope} - 1$ (TABLE 3.7). The dilution series was made of pooled cDNA samples from *L. curvatus* cDNA, diluted in steps of ten fold over five dilution points. Real-time PCR was performed in triplicate as described above, except 2 ul template was used instead of one.

TABLE 3.7 Primer sequences of eight reference genes and gene of interest (HSP60)

Name	Accession number	Forward primer (primer sequence 5`-3`)	Reverse primer (primer sequence 5`-3`)	Primer efficiency (%)	R^2	Amplicon length (bp)
RpoB	AF515647	ACATGGACGTTTCGCCTAAG	AAGGCACGGTTTGAATCATC	102.7	0.990	85
PheS	AM087758	AATGGAAGGCCAAGTGATTG	GTAACTTGGCCGGAAACGTA	90.4	0.991	118
HSP60	AY424345	AAGTGGCTGCTGTTTCATCA	TGCATCCCTTCAACAACG	115.9	0.946	142
6PGD	AJ582212	CTTCTTTGGCGATACAATTCG	CTAATGCGCCTAATTCACCA	95.1	0.962	98
RecA	AJ621633	ACTGCCCTCGGGGTTAAT	GCGACTGAATCGACAACTAAAA	84	0.970	128
Tuf	AJ418928	ACGTGACACAGACAAACCATTC	TACGACCTGAAGCAACAGTACC	91.8	0.999	83
GAP	AGBU01000043 *	TTGAATTGACCATGAGCTGTATC	TCGGACGTATTGGTCGTTTA	81.3	0.981	142
RpL11	AGBU01000037 †	AATCCCATGCTTCTTGCAGTA	TGGCGAACCAAACACTAAGA	75.9	0.999	144
RpS7	AGBU01000012 ‡	CCGCTTAATGATTGATGGTAAA	GTACTGGCATGATGTTGTTCATAG	98.5	0.995	134

* GAP locus tag = CRL705_1529 corresponding to nucleotides 12052-13068 on the *L. curvatus* strain CRL705 whole genome shotgun sequence (wgs) acc. no AGBU01000043 (Hebert *et al.*, 2012).

† RpL11 locus tag = CRL705_1418 corresponding to nucleotides 6756-7181 on the *L. curvatus* strain CRL705 wgs acc. no AGBU01000037.

‡ RpS7 locus tag = CRL705_732 corresponding to nucleotides 15652-16122 on the *L. curvatus* strain CRL705 wgs acc. no AGBU01000012.

3.5 Agarose gel electrophoresis

To prepare the gel, 1 g of agarose (Seakem LE agarose, RocK-Land, ME, USA) and 50 ml TAE buffer (Tris-acetate-EDTA buffer) were melted in a microwave oven in a 100 ml bottle and cooled to about 60 ºC. Then 2 µl ethidium bromides (10 mg/ml) was mixed with the melted gel and poured into a gel holder. After solidification, the gel was run in a horizontal electrophoresis chamber (Scie-plas, UK) with TAE buffer at 70 V for 1 hour.

Five microliters of each PCR product and 1 µl of loading buffer (6× blue juice loading buffer) were subjected to electrophoresis on an agarose gel containing agarose at a concentration of 2% (wt/vol) in TAE buffer. DNA molecular weight marker XIII (Roche) was used as a size standard (Appendix 9.3). It was loaded into the first well, followed by loading of the samples. This ladder consists of fifteen DNA fragments ranging in size from 50 to 1000 base pairs.

The gel was exposed to UV light to obtain a photograph for documentation of the results. The photograph of the gel was analysed by genesnap software (Syngene).

4 Results

4.1 Introduction

To prevent seafood products from LAB, *L. innocua* and fish spoilage bacteria, it is important to use appropriate volumes of preservatives besides correct storage temperature to increase the shelf life. This chapter show the results of the effect of high and low levels of preservatives (salt and liquid smoke) and in medium (TSBYE) on growth of bacteria at 20 ºC. The effect of different salt like NaCl, K-A, K-A+ K-L and NaCl + K-A with different volume on growth of *V. vulnificus* are also presented in this chapter. The Tables show the total time to reach different levels of ratio OD and also the time to reach from one level to the next level of ratio OD, i.e. the doubling in absorbance value read at 600 nm.

4.2 Bacterial growth as affected by preservatives (PURAC solution and liquid smoke)

4.2.1 Lactic acid bacteria

Five species of LAB; *C. maltaromaticum, C. inhibens, E. faecalis, L. lactis* subsp. *lactis*, and *L. curvatus* were treated with eight different preservative formulations (TABLE 3.1) to study the inhibitory effect on their growth, and to compare with growth in the unamended control medium, TSBYE, assumed to represent near-optimal growth at the respective temperature. Except *L. curvatus*, all of the LAB species showed similar inhibition effect on their growth in the preservatives. In contrast, in TSBYE solution, all these aforementioned bacteria grew very well.

4.2.1.1 *C. maltaromaticum, C. inhibens, E. faecalis and L. lactis*

Apparent lag time of *C. maltaromaticum* was 2524 minutes (42 hours) for 6% PURAC solution and 1508 minutes (25 hours) for 3% PURAC solution which indicates that inhibitory effect increases with increase of concentration of salt. In presence of different concentration of LS (0.07% or 0.14%) in 6% PURAC did not have significant ($p<0.001$) inhibitory effect on the growth of *C. maltaromaticum* (TABLE 4.1). Detailed results of the effect of preservatives on *C. maltaromaticum* are presented in TABLE 4.1.

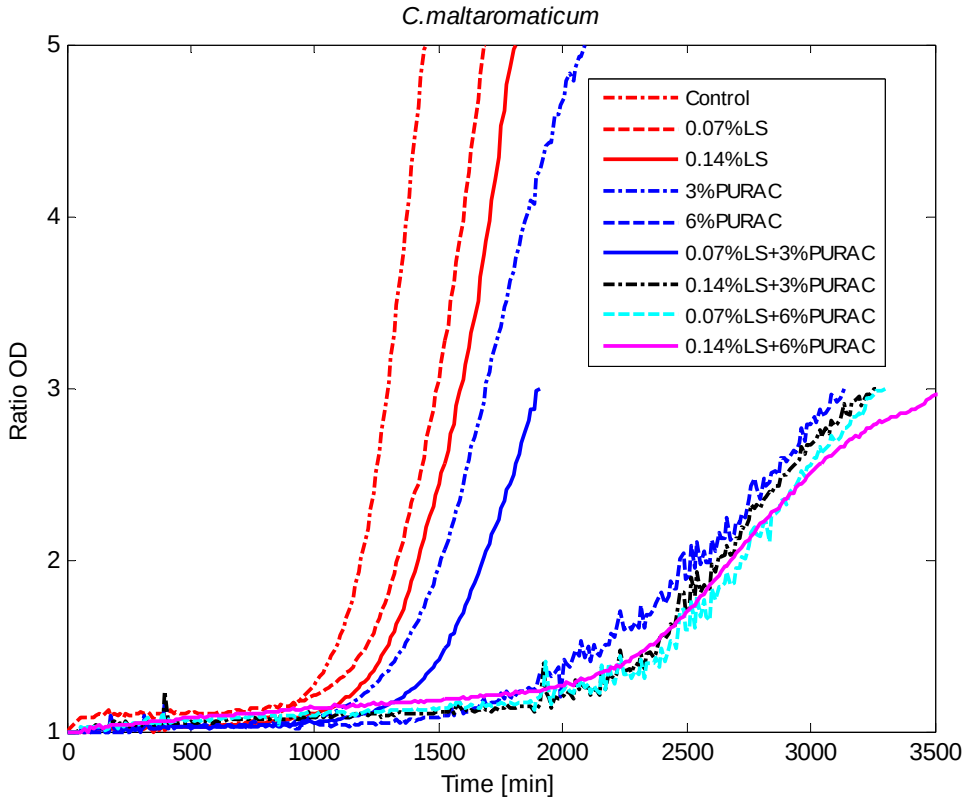


FIGURE 4.1 Ratio OD of *C. maltaromaticum* as a function of time in different treatments at 20 ºC.

TABLE 4.1 Time to reach ratio from 2 to 6 and delta time for *C. maltaromaticum* in different solutions

			Ratio OD		
	2(Apparent lag time)	**3**	**4**	**5**	**6**
Time (min)					
Control	1172 ± 6.9[a]	1284 ± 12.0[a]	1360 ± 14.0[a]	1428 ± 12.0[a]	1496 ± 18.0[a]
0.07%LS	1340 ± 25[b]	1492 ± 30.2[b]	1604 ± 36.7[b]	1684 ± 42.0[b]	1760 ± 48.0[b]
0.14%LS	1416 ± 0.0[b]	1584 ± 12.0[bc]	1708 ± 6.9[c]	1801 ± 14.0[c]	1904 ± 18.0[c]
3% PURAC	1508 ± 6.9[c]	1696 ± 6.9[c]	1856 ± 14.0[d]	2096 ± 55.4[d]	--
0.07%LS-3% PURAC	1676 ± 6.9[d]	1900 ± 6.9[d]	2076 ± 0.0[e]	2308 ± 28.0[e]	--
0.14%LS-3% PURAC	2612.7 ±62.4[e]	3256 ± 138[e]	--	--	--
6% PURAC	2524 ± 122.6[f]	3128 ± 124.9[e]	--	--	--
0.07%LS-6% PURAC	2724 ± 33.9[g]	3264 ± 67.9[e]	--	--	--
0.14%LS-6% PURAC	2676 ± 43.3[g]	3528 ± 83.1[f]	--	--	--
P-value	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***

Delta time(min)	**2-3**	**3-4**	**4-5**	**5-6**
Control	112	76	68	68
0.07%LS	152	112	80	76
0.14%LS	168	124	93	102
3% PURAC	188	160	240	--
0.07%LS-3% PURAC	224	176	232	--
0.14%LS-3% PURAC	643	--	--	--
6% PURAC	604	--	--	--
0.07%LS-6% PURAC	540	--	--	--
0.14%LS-6% PURAC	852	--	--	--

All values of Time (min) are mean± standard deviations of three parallels.
Same letters in a column indicate no significant differences and different letters indicates significant difference ($p<0,001$).
LS-Liquid smoke, 3% PURAC- 3% K-L + 0.21% K-A, 6% PURAC- 6%K-L+ 0.42%K-A.

Inhibition effect of combined solution of LS and PURAC of different concentration on growth of *C.inhibens* and *E.faecalis,* found similar. For both bacteria, different concentration of LS with low concentration PURAC (3%) played a significant role to increase the inhibition effect. However, with high concentration PURAC (6%), different concentration of LS made a little change in apparent lag time. In a scenario of when LS or PURAC worked as a sole preservative, PURAC was found to be capable of increase the inhibition effect significantly ($p<0.001$) with increasing concentration (see FIGURE 4.2, FIGURE 4.3, TABLE 4.2 and TABLE 4.3). As expected, control medium was the least where the bacteria did not experience growth inhibition compared to other treatments.

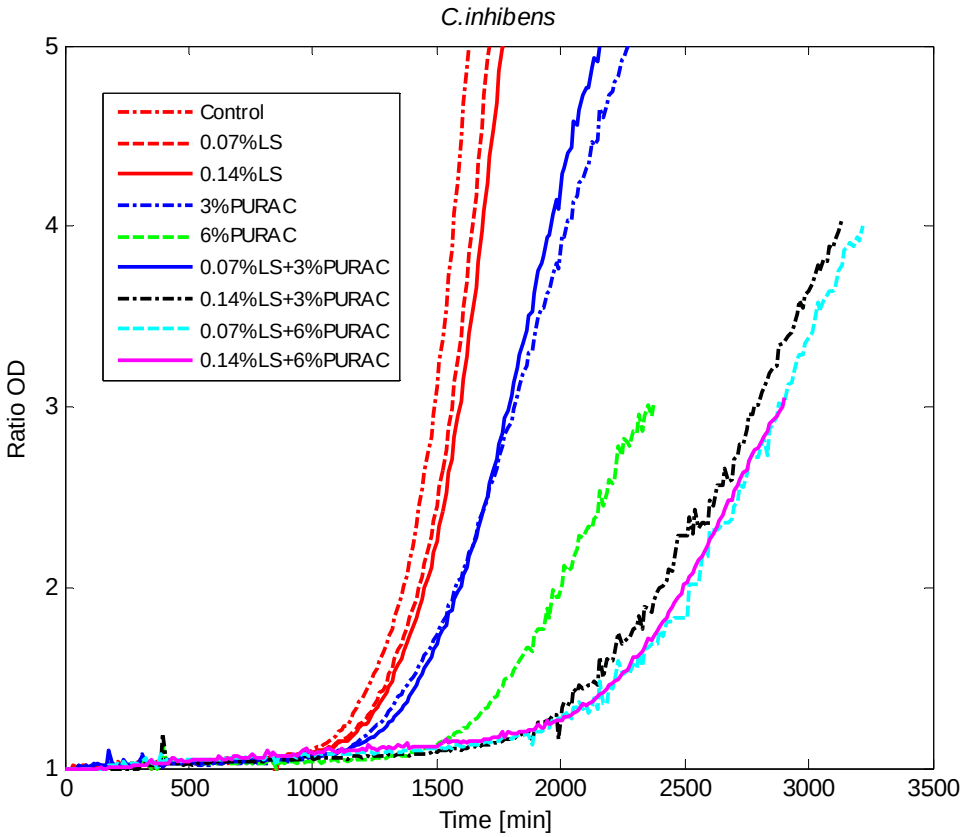


FIGURE 4.2 Ratio OD of *C. inhibens* as a function of time (min) in different treatments at 20 ºC.

TABLE 4.2 Time to reach ratio from 2 to 6 and delta time for *C. inhibens* in different solutions

	Ratio OD				
	2(Apparent lag time)	**3**	**4**	**5**	**6**
Time (min)					
Control	1368± 0.0[a]	1492±6,9[a]	1568±6,9[a]	1628±6.9[a]	1692±0.0[a]
0.07%LS	1428±12.0[b]	1560±12.0[b]	1644±12.0[b]	1708±13.9[b]	--
0.14%LS	1452±12.0[b]	1596±12.0[b]	1684±6.9[b]	1768±6.9[c]	1836±12.0[b]
3% PURAC	1576±18,3[c]	1812±24.0[c]	2020±18.3[c]	2272±6.9[d]	2548±56.7[c]
0.07%LS-3% PURAC	1588±13.9[c]	1788±12.0[c]	1936±27.2[d]	2148±12.0[e]	2408±25.0[d]
0.14%LS-3% PURAC	2396±18.3[d]	2792±38.6[d]	3124±42.1[e]	--	--
6% PURAC	1956±52.3[e]	2344±45.4[e]	--	--	--
0.07%LS-6% PURAC	2480±13.9[f]	2872±6.9[f]	3216±24.0[f]	3624±43.3[f]	--
0.14%LS-6% PURAC	2488±13.9[f]	2884±18.3[f]	3416±102.1[g]	--	--
P-value	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***

Delta time(min)	**2-3**	**3-4**	**4-5**	**5-6**
Control	124	76	60	64
0.07%LS	132	84	64	--
0.14%LS	144	88	84	68
3% PURAC	236	208	252	276
0.07%LS-3% PURAC	200	148	211	260
0.14%LS-3% PURAC	396	332	--	--
6% PURAC	388	--	--	--
0.07%LS-6% PURAC	392	344	408	--
0.14%LS-6% PURAC	396	532	60	--

All values of Time (min) are mean± standard deviations of three parallels.
Same letters in a column indicate no significant differences and different letters indicates significant difference ($p<0,001$).
LS-Liquid smoke, 3% PURAC- 3% K-L + 0.21% K-A, 6% PURAC- 6%K-L+ 0.42%K-A

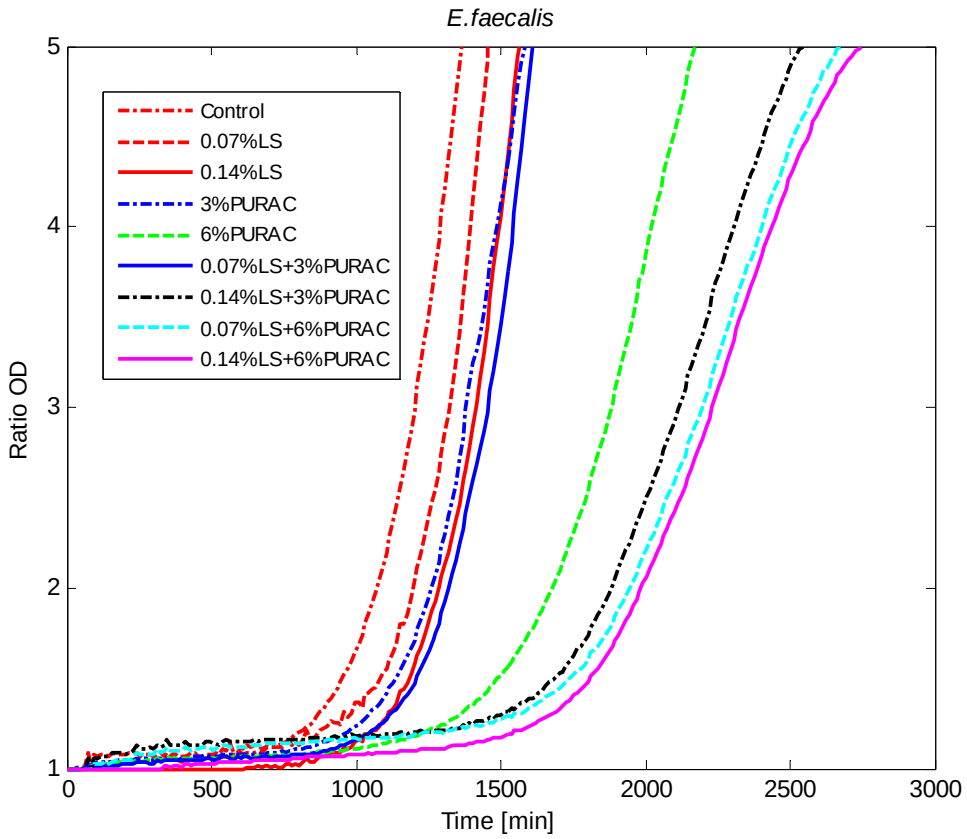


FIGURE 4.3 Ratio OD of *E. faecalis* as a function of time (min) in different treatments at 20 ºC.

TABLE 4.3 Time to reach ratio from 2 to 6 and delta time for *E. faecalis* in different solutions

	Ratio OD				
	2(Apparent lag time)	**3**	**4**	**5**	**6**
Time (min)					
Control	1072 ± 6.6[a]	1200 ± 12.0[a]	1291± 18.0[a]	1363± 18.0[a]	1423 ± 21.8[a]
0.07%LS	1197 ± 13.1[b]	1324± 6.6[b]	1396± 6.6[b]	1456± 6.6[b]	1504± 6.6[a]
0.14%LS	1281 ± 5.4[c]	1416 ± 8.5[c]	1497± 5.4[c]	1564± 6.6[c]	1648 ± 6.6[a]
3% PURAC	1262 ± 35.4[c]	1387± 44.6[c]	1485± 43.0[c]	1581± 37.0[c]	1672± 52.0[a]
0.07%LS-3% PURAC	1320 ± 8.5[d]	1454.± 10.0[d]	1545± 13.0[d]	1608 ± 14.7[c]	1675 ± 13.7[a]
0.14%LS-3% PURAC	1879 ±26.3[e]	2114± 19.7[e]	2304 ± 14.7[e]	2534 ± 17.8[d]	--
6% PURAC	1680 ± 42.4[f]	1881.± 45.2[f]	2035± 65.0[f]	2196 ± 114[e]	2385± 170[b]
0.07%LS-6% PURAC	1944 ± 14.7[g]	2193± 13.1[g]	2402 ± 21.5[g]	2678± 49.0[f]	--
0.14%LS-6% PURAC	1982 ± 10.0[g]	2232 ± 8.5[g]	2443 ± 13.7[h]	2764 ± 40.0[g]	--
P-value	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***

Delta time(min)	**2-3**	**3-4**	**4-5**	**5-6**
Control	127	91	72	60
0.07%LS	127	72	60	48
0.14%LS	134	81	67	84
3% PURAC	124	98	96	91
0.07%LS-3% PURAC	134	91	62	67
0.14%LS-3% PURAC	235	189	230	--
6% PURAC	201	153	160	189
0.07%LS-6% PURAC	249	208	276	--
0.14%LS-6% PURAC	249	211	321	--

All values of Time (min) are mean± standard deviations of five parallels.
Same letters in a column indicate no significant differences and different letters indicates significant difference ($p<0,001$).
LS-Liquid smoke, 3% PURAC- 3% K-L + 0.21% K-A, 6% PURAC- 6%K-L+ 0.42%K-A

L. lactic was also inhibited in the same manner as *C. inhibens* and *E. faecalis,* but surprisingly, increase of LS solution in 6% PURAC rather decreased the inhibition effect (see FIGURE 4.4). The maximum apparent lag time (app. 50 hours) was observed in the case of when *L. lactic* was treated in high concentration LS (0.14%)+ 3% PURAC while the minimum apparent lag time (app. 16 hours) was observed when treated on control medium (TABLE 4.4).

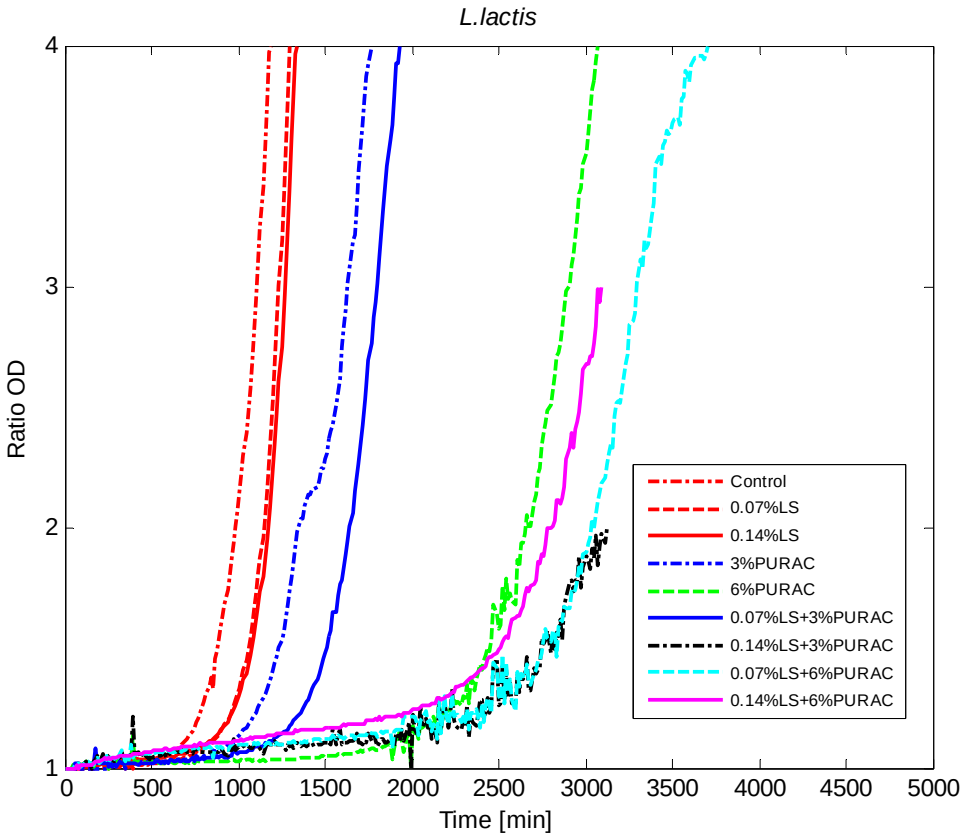


FIGURE 4.4 Ratio OD of *L. lactis* as a function of time (min) in different treatments at 20 ºC.

TABLE 4.4 Time to reach ratio from 2 to 4 and delta time for *L. lactis* in different solutions

	Ratio OD		
	2(Apparent lag time)	**3**	**4**
Time (min)			
Control	984 ± 0.0[a]	1104 ± 0.0[a]	1176±0.0[a]
0.07%LS	1148 ± 30.0[b]	1236 ± 32[b]	1296 ± 32[b]
0.14%LS	1176 ± 24.0[b]	1272 ± 24[b]	1336 ± 30[b]
3% PURAC	1344 ± 20.8[c]	1632 ± 12[c]	1756 ± 30[c]
0.07%LS-3% PURAC	1644 ± 0.0[d]	1800 ± 17[d]	1914 ±8.5[d]
0.14%LS-3% PURAC	3120± 0.0[e]	--	--
6% PURAC	2660 ± 45.4[f]	2904 ± 42[e]	3072 ± 52[e]
0.07%LS-6% PURAC	3028 ± 42.1[g]	3296 ± 66[f]	3628 ± 78[f]
0.14%LS-6% PURAC	2808 ± 119[h]	3090 ± 144[g]	--
P-value	***<0.001***	***<0.001***	***<0.001***

Delta time(min)	**2-3**	**3-4**
Control	120	72
0.07%LS	88	60
0.14%LS	96	64
3% PURAC	288	124
0.07%LS-3% PURAC	156	114
0.14%LS-3% PURAC	--	--
6% PURAC	244	168
0.07%LS-6% PURAC	268	332
0.14%LS-6% PURAC	282	--

All values of Time (min) are mean± standard deviations of three parallels.
Same letters in a column indicate no significant differences and different letters indicates significant difference ($p<0,001$).
LS-Liquid smoke, 3% PURAC- 3% K-L + 0.21% K-A, 6% PURAC- 6%K-L+ 0.42%K-A

4.2.1.2 L. curvatus

As mentioned before, *L. curvatus* did not show the similar effect of preservatives on growth rate like the other LAB species, and it took very long time to grow in the preservatives. Surprisingly, *L. curvatus* grew faster in LS solution and in mixture of LS solution and lower concentration 3% PURAC solution, compared to the control TSBYE medium. To quantify the effect of preservatives on *L. curvatus*, the experiment was run for six days and results up to the 5th day (FIGURE 4.5 and TABLE 4.5) were analyzed.

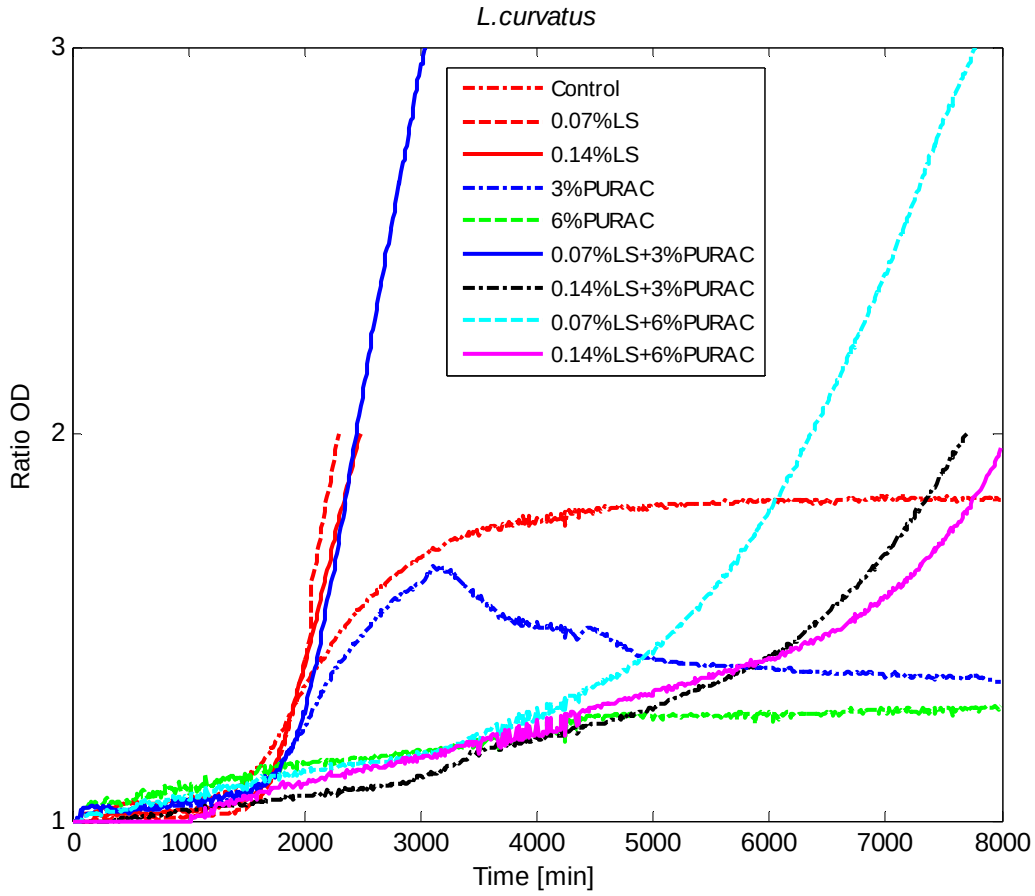


FIGURE 4.5 Ratio OD of *L. curvatus* as a function of time (min) in different treatments at 20 ºC

TABLE 4.5 Time to reach ratio from 2 to 3 and delta time for *L. curvatus* in different solutions.

	Ratio OD	
	2(Apparent lag time)	**3**
Time (min)		
0.07%LS	2292 ± 0.0[a]	--
0.07%LS-3% PURAC	2443 ± 34.6[a]	3036 ± 44.9[a]
0.14%LS-3% PURAC	7706 ± 246.6[b]	--
0.07%LS-6% PURAC	6386 ± 200.3[c]	7749 ± 265.0[b]
P-value	***<0.001***	***<0.001***

Delta time(min)	**2-3**
0.07%LS	--
0.07%LS-3% PURAC	592
0.14%LS-3% PURAC	--
0.07%LS-6 % PURAC	1363

All values of Time (min) are mean± standard deviations of five parallels.
Same letters in a column indicate no significant differences and different letters indicates significant difference ($p<0,001$).
LS-Liquid smoke, 3% PURAC- 3% K-L + 0.21% K-A, 6% PURAC- 6%K-L+ 0.42%K-A

TABLE 4.5 shows that *L. curvatus* declined to grow after approximately 2000 minutes (~33 hours) in 0.14% LS, thus, apparent lag time could not be calculated for this particular treatment. However, in the mixture with 6 % PURAC and 0.07% LS, the apparent lag time of *L. curvatus* was calculated to 6386 minutes (106 hours), whereas in 0.07% LS, the apparent lag time was 2292 minutes (38 hours).

4.2.2 *Listeria innocua*

Two different strains of *L. innocua; L. innocua* ATCC 33090 and *L. innocua* ATCC 51742 were studied in this experiment. When both strains were treated alone in salt solution (PURAC) and in LS, it was observed that the salt solution has higher inhibition effect than LS**,** and the effect increased accordingly with concentration of salt and LS. When these bacteria were treated in presence of LS in 3% PURAC, inhibition differed significantly with the increment of LS concentration. However, in 6% PURAC, presence of LS of different concentration did not significantly affect the growth inhibition (FIGURE 4.6 and FIGURE 4.7). All sort of treatment of LS and PURAC, either as combined or sole preservative, inhibited the growth of these bacteria more than in control medium. Apparent lag time and delta time is presented TABLE 4.6 and TABLE 4.7.

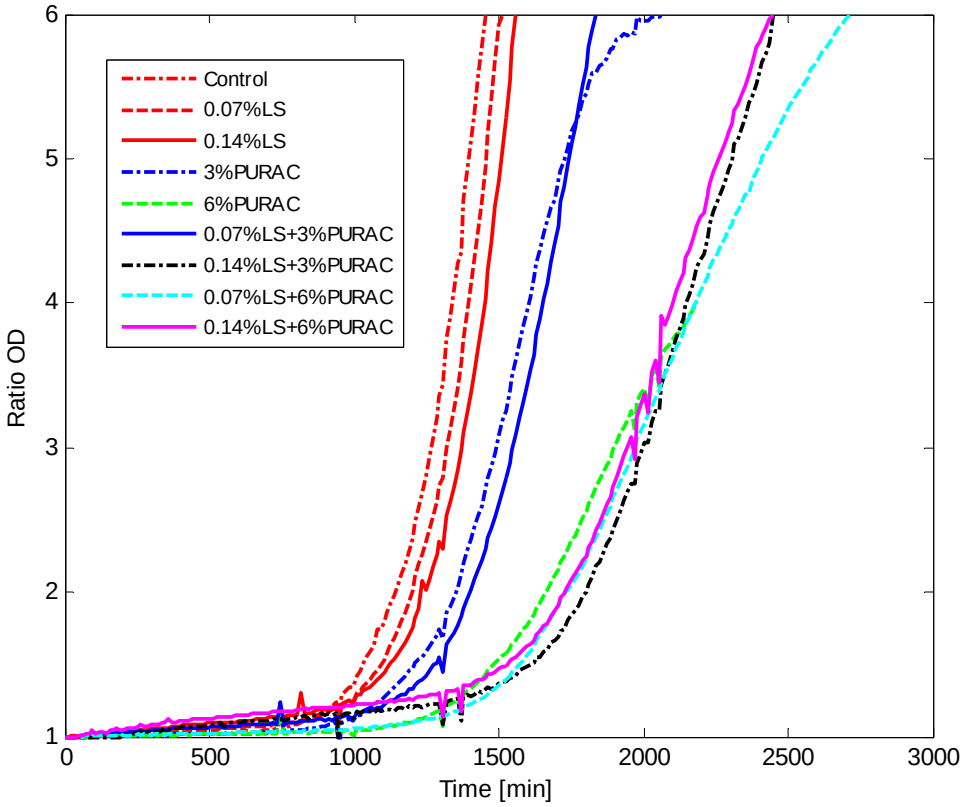


FIGURE 4.6 Ratio OD of *L. innocua* ATCC 33090 in different treatments at 20 ºC.

TABLE 4.6.Time to reach ratio from 2 to 6 and delta time for *L. inncua* ATCC 33090 in different solutions.

	Ratio OD				
	2(Apparent lag time)	**3**	**4**	**5**	**6**
Time (min)					
Control	1140 ± 12.0[a]	1268 ± 6.9[a]	1340 ± 6.9[a]	1396 ± 6.9[a]	1448 ± 6.9[a]
0.07%LS	1208 ± 6.93[b]	1336 ± 6.9[b]	1412 ± 6.9[b]	1468 ± 6.9[b]	1512 ± 0[b]
0.14%LS	1244 ± 6.93[c]	1384 ± 6.9[c]	1464 ± 0[c]	1524 ± 0[c]	1572 ± 0[c]
3% PURAC	1364 ± 13.7[d]	1504 ± 6.9[d]	1616 ± 13.9[d]	1744 ± 6.9[d]	2000 ± 28[d]
0.07%LS-3% PURAC	1416 ± 12.0[e]	1560 ± 12.0[e]	1668 ± 12[e]	1756 ± 18.3[e]	1848 ± 24[e]
0.14%LS-3% PURAC	1804 ± 6.9[f]	2008 ± 6.9[f]	2164 ± 6.9[f]	2320 ± 6.9[f]	2460 ± 0[f]
6% PURAC	1668 ± 0.0[g]	1912 ± 6.9[g]	2196 ± 12[g]	--	--
0.07%LS-6% PURAC	1740 ± 0.0[h]	1980 ± 0[h]	2196 ± 0[h]	2424 ± 12[g]	2728 ± 30.2[g]
0.14%LS-6% PURAC	1744 ± 13.9[h]	1960 ± 13.9[h]	2108 ± 27.7[i]	2272 ± 13.9[h]	2448 ± 20.8[f]
P-value	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***

Delta time(min)	**2-3**	**3-4**	**4-5**	**5-6**
Control	128	72	56	52
0.07%LS	128	76	56	44
0.14%LS	140	80	60	48
3% PURAC	140	112	128	256
0.07%LS-3% PURAC	144	108	88	92
0.14%LS-3% PURAC	204	156	156	140
6% PURAC	244	284	--	--
0.07%LS-6% PURAC	240	216	228	304
0.14%LS-6% PURAC	216	148	164	176

All values of Time (min) are mean ± standard deviations of three parallels.
Same letters in a column indicate no significant differences and different letters indicates significant difference ($p<0,05$).
LS-Liquid smoke, 3% PURAC- 3% K-L + 0.21% K-A, 6% PURAC- 6%K-L+ 0.42%K-A

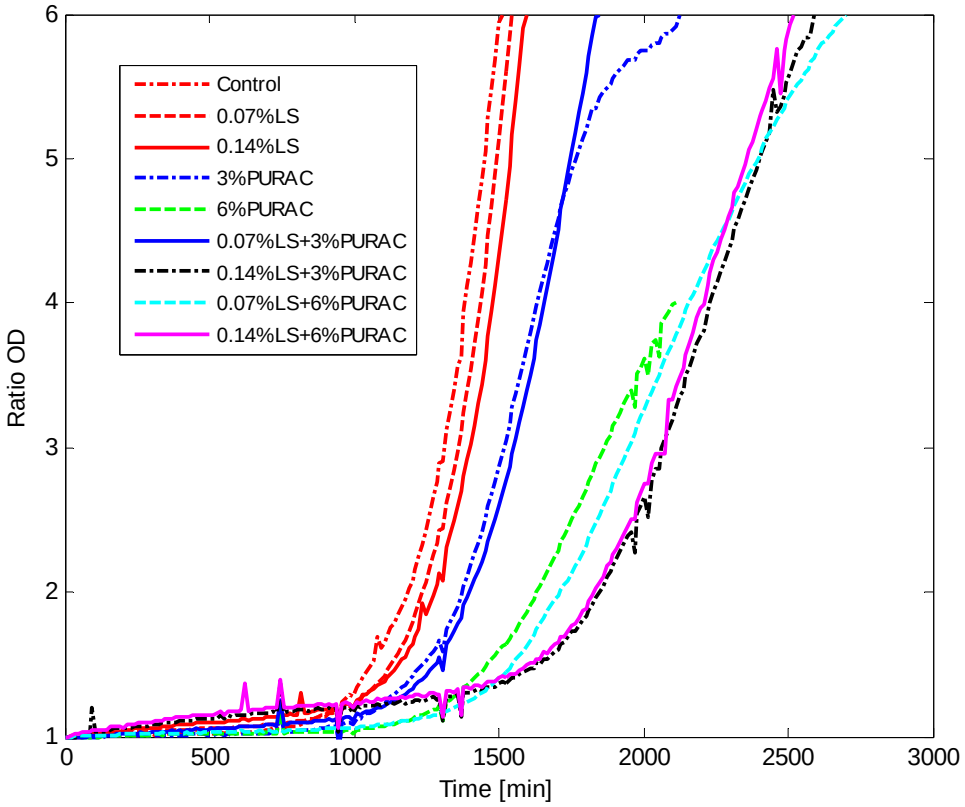


FIGURE 4.7 Ratio OD of *L. innocua* ATCC 51742 as a function of time (min) in different treatments at 20 ºC.

TABLE 4.7 Time to reach ratio from 2 to 6 and delta time for *L. inncua* ATCC 51742 in different solutions.

	Ratio OD				
	2(Apparent lag time)	**3**	**4**	**5**	**6**
Time (min)					
Control	1188 ± 0.0[a]	1308 ± 0.0[a]	1384 ± 6.9[a]	1452 ± 0.0[a]	1500 ± 0.0[a]
0.07%LS	1236 ± 0.0[b]	1356 ± 0.0[b]	1428 ± 0.0[b]	1472 ± 27.7[a]	1532 ± 6.9[a]
0.14%LS	1272 ± 0.0[c]	1404 ± 0.0[c]	1476 ± 0.0[c]	1536 ± 0.0[b]	1584 ± 0.0[b]
3% PURAC	1368 ± 0.0[d]	1516 ± 6.9[d]	1632 ± 12.0[d]	1760 ± 6.9[c]	2188 ± 6.9[c]
0.07%LS-3% PURAC	1396 ± 6.9[e]	1556 ± 13.9[e]	1660 ± 6.9[e]	1748 ± 13.9[c]	1840 ± 6.9[d]
0.14%LS-3% PURAC	1844 ± 18.3[f]	2060 ± 18.3[f]	2228 ± 18.3[f]	2400 ± 36.0[d]	2596 ± 54.1[e]
6% PURAC	1628 ± 6.9[g]	1856 ± 6.9[g]	2104 ± 18.3[g]	--	--
0.07%LS-6% PURAC	1704 ± 0.0[h]	1944 ± 0.0[h]	2148 ± 0.0[h]	2388 ± 0.0[d]	2696 ± 6.9[f]
0.14%LS-6% PURAC	1828 ± 6.9[i]	2040 ± 0.0[i]	2204 ± 13.9[i]	2356 ± 6.9[e]	2520 ± 12.0[g]
P-value	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***

Delta time(min)	**2-3**	**3-4**	**4-5**	**5-6**
Control	120	76	68	48
0.07%LS	120	72	44	60
0.14%LS	132	72	60	48
3% PURAC	148	116	128	428
0.07%LS-3% PURAC	160	104	88	92
0.14%LS-3% PURAC	120	168	172	196
6% PURAC	228	248	--	--
0.07%LS-6% PURAC	240	204	240	308
0.14%LS-6% PURAC	212	164	152	164

All values of Time (min) are mean± standard deviations of three parallels.
Same letters in a column indicate no significant differences and different letters indicates significant difference ($p<0,001$).
LS-Liquid smoke, 3% PURAC- 3% K-L + 0.21% K-A, 6% PURAC- 6%K-L+ 0.42%K-A

4.2.3 Specific fish spoilage bacteria

4.2.3.1 Photobacterium phosphoreum

P. phosphoreum grew faster in 3% PURAC solution than in 0.07% LS solution. Inhibitory effects on growth were more profound in higher concentration of LS and combination of LS and PURAC solution. The apparent lag time of *P. phosphoreum* was around 1100 minutes (18 hours), 1580 minutes (26 hours) and 4254 minutes (71 hours) in 3% PURAC solution, combined mixture of 0.07%LS and 3% PURAC and in combination of 0.14% LS and 3% PURAC, respectively. The apparent lag time was significantly longer in the presence of LS solution in salt solution, compared to when bacteria was treated in salt solution alone. Also, upon increasing the amount of LS solution, apparent lag time increased accordingly, which is also true for PURAC solution.

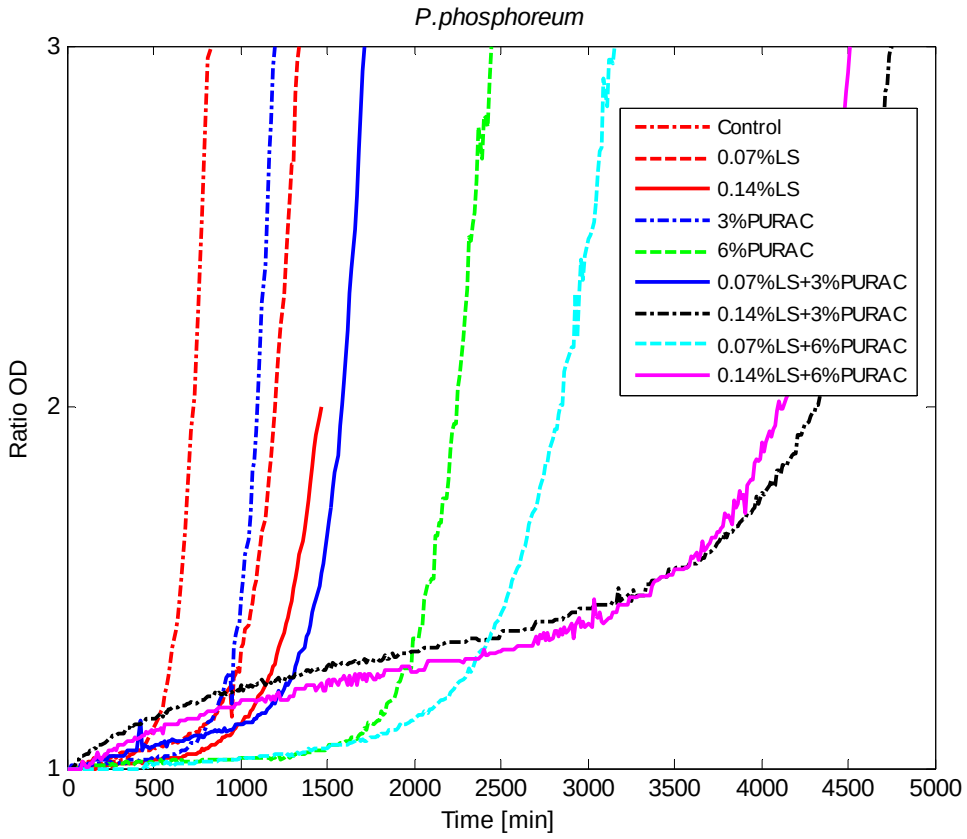


FIGURE 4.8 Ratio OD of *P. phosphoreum* as a function of time (min) in different treatments at 20 ºC.

TABLE 4.8 Time to reach ratio from 2 to 4 and delta time for *P. phosphoreum* in different solutions.

	Ratio OD		
	2(Apparent lag time)	**3**	**4**
Time (min)			
Control	732 ± 0.0[a]	816 ± 0.0[a]	956 ± 6.9[a]
0.07%LS	1200 ± 0.0[b]	1336 ± 6.9[b]	--
0.14%LS	--	--	--
3% PURAC	1100 ± 6.9[c]	1196 ± 25.0[c]	1352 ± 30.2[b]
0.07%LS-3% PURAC	1580 ± 13.9[d]	1712 ± 13.9[d]	1804 ± 18.3[c]
0.14%LS-3% PURAC	4254 ± 8.5[e]	4734 ± 59.4[e]	4950 ± 110.0[d]
6% PURAC	2224 ± 6.9[f]	2460 ± 55.0[f]	--
0.07%LS-6% PURAC	2852 ± 123.0[g]	3160 ± 153.0[g]	--
0.14%LS-6% PURAC	4104 ± 55.0[h]	4512 ± 43.3[h]	4676 ± 42.1[a]
P-value	***<0.001***	***<0.001***	***<0.001***

Delta time(min)	**2-3**	**3-4**
Control	84	140
0.07%LS	136	--
0.14%LS	--	--
3% PURAC	96	156
0.07%LS-3% PURAC	132	92
0.14%LS-3% PURAC	480	216
6% PURAC	236	--
0.07%LS-6% PURAC	308	--
0.14%LS-6% PURAC	408	164

All values of Time (min) are mean± standard deviations of three parallels.
Same letters in a column indicate no significant differences and different letters indicates significant difference ($p<0,001$).
LS-Liquid smoke, 3% PURAC- 3% K-L + 0.21% K-A, 6% PURAC- 6%K-L+ 0.42%K-A

4.2.3.2 Pseudomonas putida

Different concentrations of LS did not have profound effect on the inhibition of *P. putida* growth compared to when it was treated in control medium. It was found that higher concentration of salt have inhibition effect on growth of this particular bacterium either with or without the presence of LS. However, high concentration of LS only increased inhibition effect when it worked with low concentration salt solution. On the contrary, with high concentration salt solution, LS did not make any significant difference on growth of this bacterium (FIGURE 4.9). Detailed results are presented in TABLE 4.9

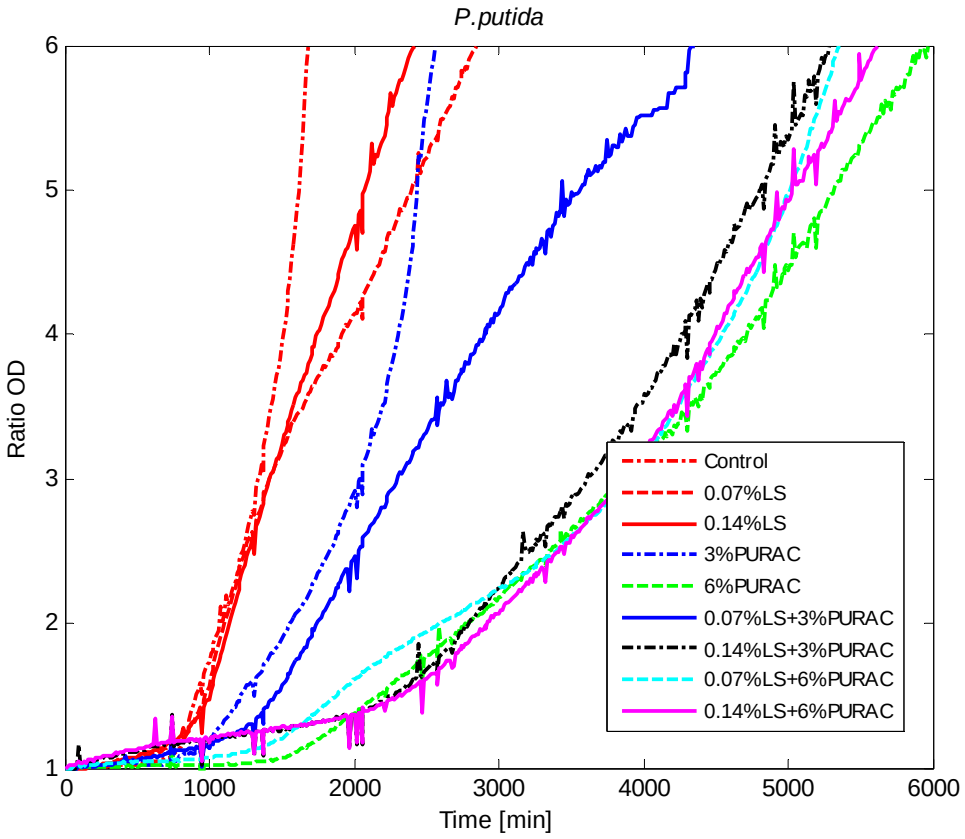


FIGURE 4.9 Ratio OD of *P. putida* as a function of time (min) in different treatments at 20 ºC

TABLE 4.9 Time to reach ratio from 2 to 6 and delta time for *P. putida* in different solutions.

	Ratio OD				
	2(Apparent lag time)	**3**	**4**	**5**	**6**
Time (min)					
Control	1076 ± 13.9[a]	1336 ± 6.9[a]	1512 ±0.0[a]	1620 ± 12.0[a]	1676 ± 7.0[a]
0.07%LS	1104 ± 0.0[a]	1408 ± 25.0[a]	1904±109[b]	2380 ± 96.0[b]	2824±115.0[b]
0.14%LS	1128 ± 0.0[a]	1416 ± 0.0[a]	1732 ± 14.0[b]	2076 ± 12.0[c]	2404 ± 54.0[c]
3% PURAC	1540 ± 36.7[b]	2020 ± 25.0[b]	2308 ± 28.0[c]	2456 ± 60.0[d]	2528 ± 78.0[c]
0.07%LS-3% PURAC	1744 ± 30.2[c]	2304 ± 62.0[c]	2896 ± 92.0[d]	3548±145.0[e]	4416±150.0[d]
0.14%LS-3% PURAC	2776 ± 34.6[d]	3588 ± 12.0[d]	4248 ± 12.0[e]	4780±66.1[f]	5252±105.0[e]
6% PURAC	2592 ± 0.0[e]	3824 ±198.0[e]	4684±216.0[f]	5296±218.0[g]	5884±225.0[f]
0.07%LS-6% PURAC	2512 ± 81.7[f]	3848 ± 90.0[e]	4532±114.0[f]	5004±115.0[h]	5348±121.0[g]
0.14%LS-6% PURAC	2880 ± 31.7[g]	3832 ± 60.0[e]	4484±117.0[f]	4988±157.0[h]	5596±275.0[g]
P-value	***<0.001***	***<0.001***	***<0.001***	***<0.001***	***<0.001***

Delta time(min)	**2-3**	**3-4**	**4-5**	**5-6**
Control	260	176	108	56
0.07%LS	304	496	476	444
0.14%LS	288	316	344	328
3% PURAC	480	288	148	72
0.07%LS-3% PURAC	560	592	652	868
0.14%LS-3% PURAC	812	660	532	472
6% PURAC	1232	860	612	588
0.07%LS-6% PURAC	1336	684	472	344
0.14%LS-6% PURAC	952	652	504	608

All values of Time (min) are mean± standard deviations of three parallels.
Same letters in a column indicate no significant differences and different letters indicates significant difference ($p<0,001$).
LS-Liquid smoke, 3% PURAC- 3% K-L + 0.21% K-A, 6% PURAC- 6%K-L+ 0.42%K-A

4.2.3.3 Vibrio vulnificus

V. vulnificus was highly inhibited when grown in 6% PURAC solution, almost no growth was found in this solution. However, in 3% PURAC solution, *V. vulnificus* had moderate growth where the apparent lag time was longer compared to control. The growth was also inhibited in liquid smoke (0.07% and 0.14%). FIGURE 4.10 shows that combined mixture of LS and PURAC solution had higher inhibition effect than in only LS solution.

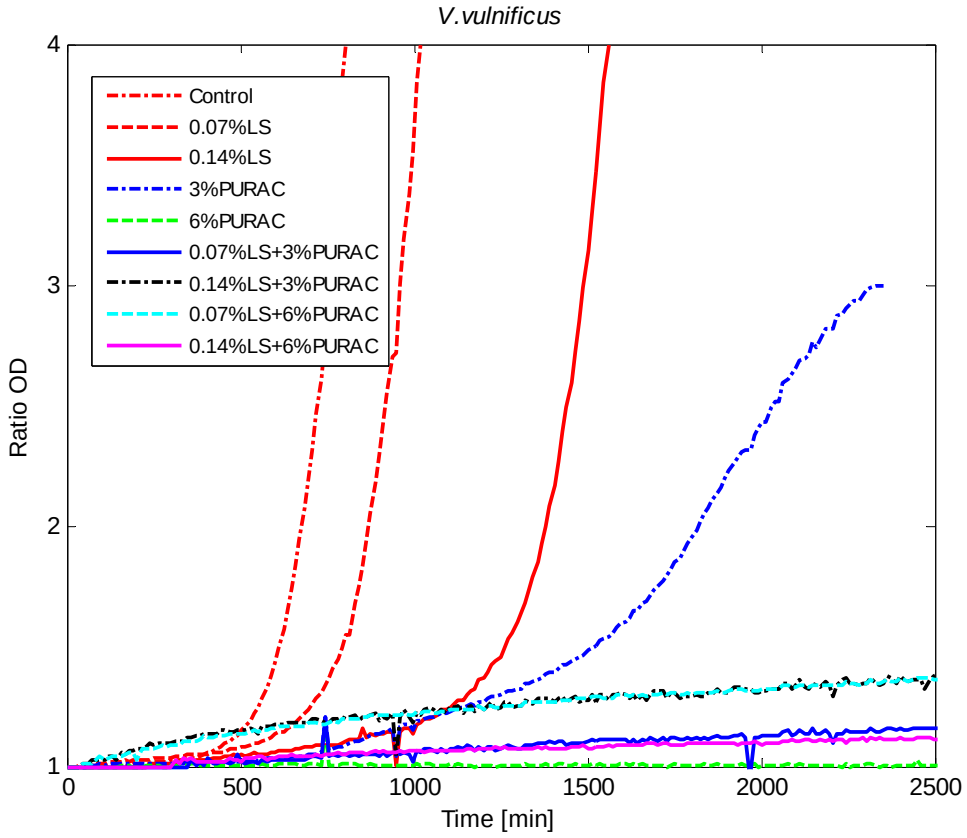


FIGURE 4.10 Ratio OD of *V. vulnificus* as a function of time in different treatments at 20 ºC

According to Quan et al. (2010), *V. vulnificus* are naturally found in sea water (3%-4% salt) so they can be able to grow in salt water. Supporting this phenomenon, one may presume that *V. vulnificus* are also able to grow in PURAC, even though it is not a sea born salt. But as shown (FIGURE 4.10), *V. vulnificus* grew poorly in PURAC. It must, however, be noted that the chemistries of PURAC and sea water are highly diverse. Therefore, to further investigate the

growth of *V. vulnificus* in alternative salts, another experiment was performed with salts such as NaCl, K-A, and PURAC solution supplemented with both K-L and K-A at 20 ºC (FIGURE 4.11

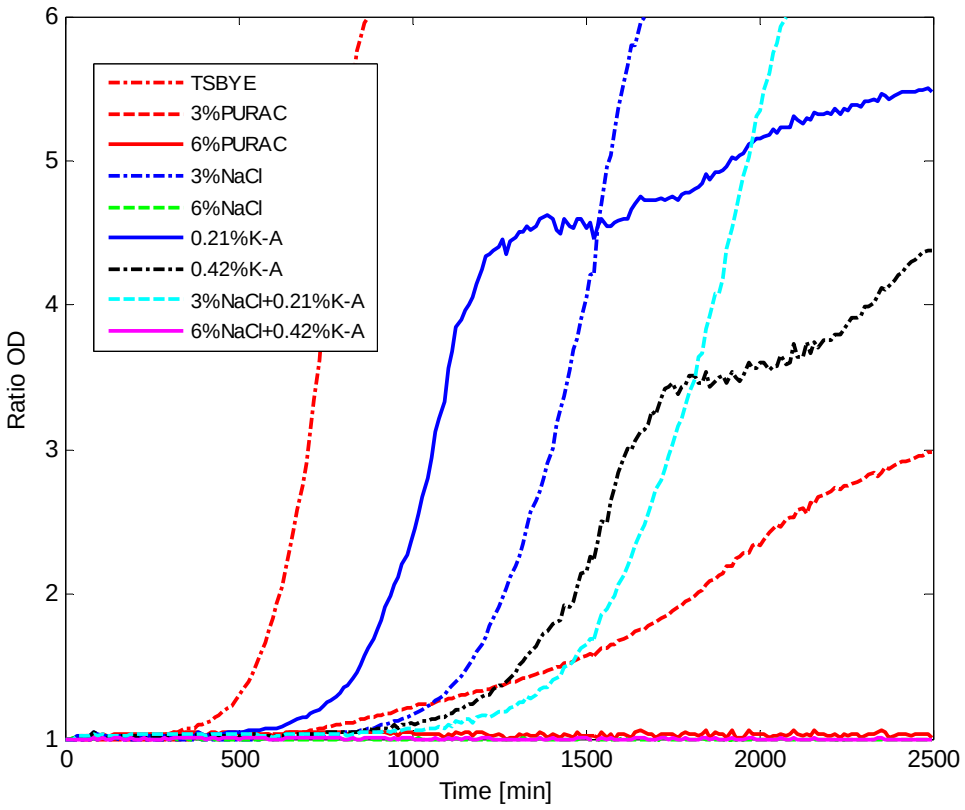


FIGURE 4.11 Ratio OD of *V.vulnificus* as a function of time in different salt treatments at 20 ºC.

The growth was inhibited mostly in 3% PURAC solution (3%K-L + 0.21%K-A), it was also inhibited in 3% NaCl + 0.21% K-A, but to a lesser extent (FIGURE 4.11). When they were treated in control medium, they grew well but inhibited significantly when treated with NaCl (FIGURE 4.11). Addition of 0.21% K-A in 3% NaCl, inhibited the growth of *V. vulnificus* more compared to in 3% NaCl alone. In these two treatments, the difference in inhibition effect was found due to presence of potassium acetate. When *V. vulnificus* was inoculated in 0.21% K-A, growth was inhibited more compared to control and the inhibition was enhanced with an increment of K-A. The presence of 0.42% K-A with 6% NaCl, had highly inhibitory effect on this bacterium, and no growth occurred in this situation (FIGURE 4.11). *V.vulnificus* did not

grow in the solutions with the highest level of PURAC (6%), 0.21% K-A + 6% PURAC, 0.42% K-A + 6% PURAC and 3% NaCl + 0.42% K-A (FIGURE 4.11).

TABLE 4.10 Time to reach ratio from 2 to 4 and delta time for *V. vulnificus* in different solutions.

		Ratio OD	
	2(Apparent lag time)	**3**	**4**
Time (min)			
Control	676 ± 6.9[a]	756 ± 0.0[a]	804±0.0[a]
0.07%LS	864 ± 0.0[c]	960 ± 0.0[c]	1020±0.0[c]
0.14%LS	1368 ± 0.0[d]	1488 ± 0.0[d]	1560±0.0[d]
3%PURAC	1808 ± 6.9[b]	2286 ±21.6[b]	3444±0.0[b]
P-value	***<0.001***	***<0.001***	***<0.001***

Delta time(min)	**2-3**	**3-4**
Control	80	48
0.07%LS	96	60
0.14%LS	120	72
3%PURAC	478	1158

All values of Time (min) are mean± standard deviations of five parallels.
Same letters in a column indicate no significant differences and different letters indicates significant difference ($p<0,001$).
LS-Liquid smoke, 3% PURAC- 3% K-L + 0.21% K-A, 6% PURAC- 6%K-L+ 0.42%K-A

4.3 pH

4.3.1 pH in treatment solutions and stability during storage

Addition of LS (0.07 and 0.14 %) and PURAC premix (3 and 6 %) to the growth medium (TSBYE) affected the pH of the solution. The pH of the TSBYE solution itself (Control) was 7.1±0.07. Addition of 0.14 % LS caused a slight increase in the pH (7.2±0.05), while addition of 0.07 % LS to the medium did not affect the pH (7.1±0.08). So it can be expected that there will be no major pH-effect on the bacteria exposed for the solutions supplemented with LS as compared to the control (TSBYE).

Addition of 3 % PURAC premix to the growth medium increased the pH from 7.1±0.07 to 7.6±0.02. A similar increase was also observed in the solutions supplemented with 3 % PURAC in combination with either 0.07 % (7.6±0.03) or 0.14 % (7.6±0.04) LS. The average pH 0.5 increase may contribute with a significant effect on the bacteria exposed to these solutions. However, the observed increase is a direct consequence of using PURAC premix as an additive and the single effect of pH on the bacteria will not in the following be separated and quantified from the total effect that addition of PURAC premix contributes with on the bacteria.

The highest addition level of PURAC premix (6 %) further increased the pH, as compared to adding 3 % premix. The pH of the solutions supplemented with 6 % PURAC premix, 0.07 % LS + 6 % PURAC premix and 0.14% LS + 6 % PURAC premix was 7.8±0.05, 7.7±0.03 and 7.8±0.18, respectively. As described above, only the total effect of adding PURAC premix will be investigated in the following due to the changed pH is considered as a secondary effect, and a consequence of adding premix to the solution.

The pH range of the treatment solutions at the start of the experiment is thus from 7.1 to 7.8, which is regarded as neutral to slightly alkaline but still within the range of what can be observed in stream water. Hence, the pH environment in this experiment can be assumed to be relevant to what can be found in several food products and at production plants.

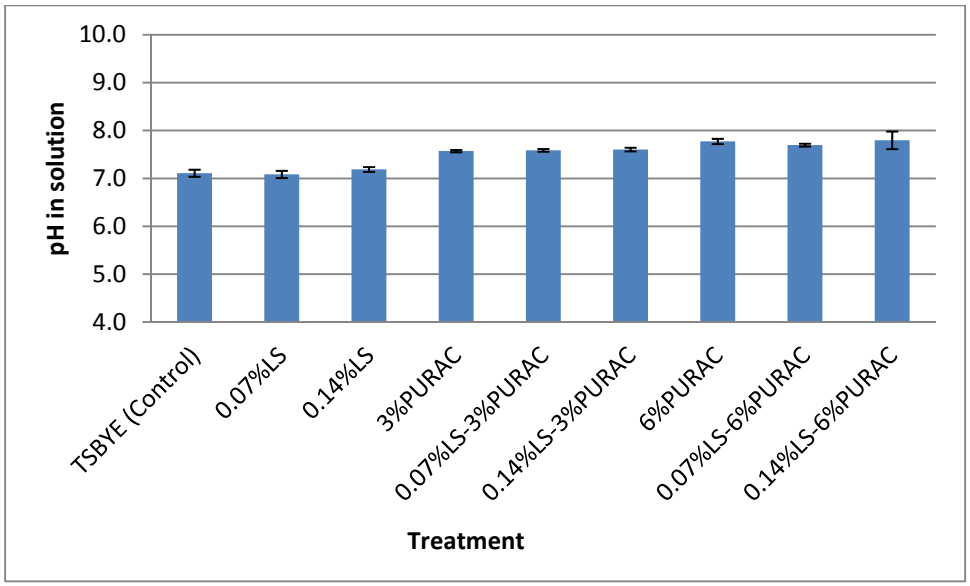


FIGURE 4.12 pH value in different treatments (without bacteria)

The pH stability/development in the "blank" treatment solutions during storage (0-4320 min) was investigated (data not shown). Only minor fluctuations (0.1) in the pH as a function of time were observed. Due to this it can be assumed that any changes in pH during storage solely are caused by bacterial activity and not chemical reactions/changes of the additives themselves.

4.4 Real-time PCR

4.4.1 RNA isolation and cDNA synthesis

It is well known that the quality of RNA is critical for successful gene expression analysis. RNA samples may get contaminated with genomic DNA. To remove contaminated genomic DNA from RNA, two DNase treatments were used; one during the RNA isolation (on the filter), and another included in the cDNA synthesis procedure (gDNA wipe-out reagent). In initial experiments, DNase treatment was performed only in the cDNA synthesis procedure (gDNA wipeout reagent) as proposed by the manufactures, leading to positive RTcontrols, indicating the presence of genomic DNA in the samples (FIGURE 4.13).

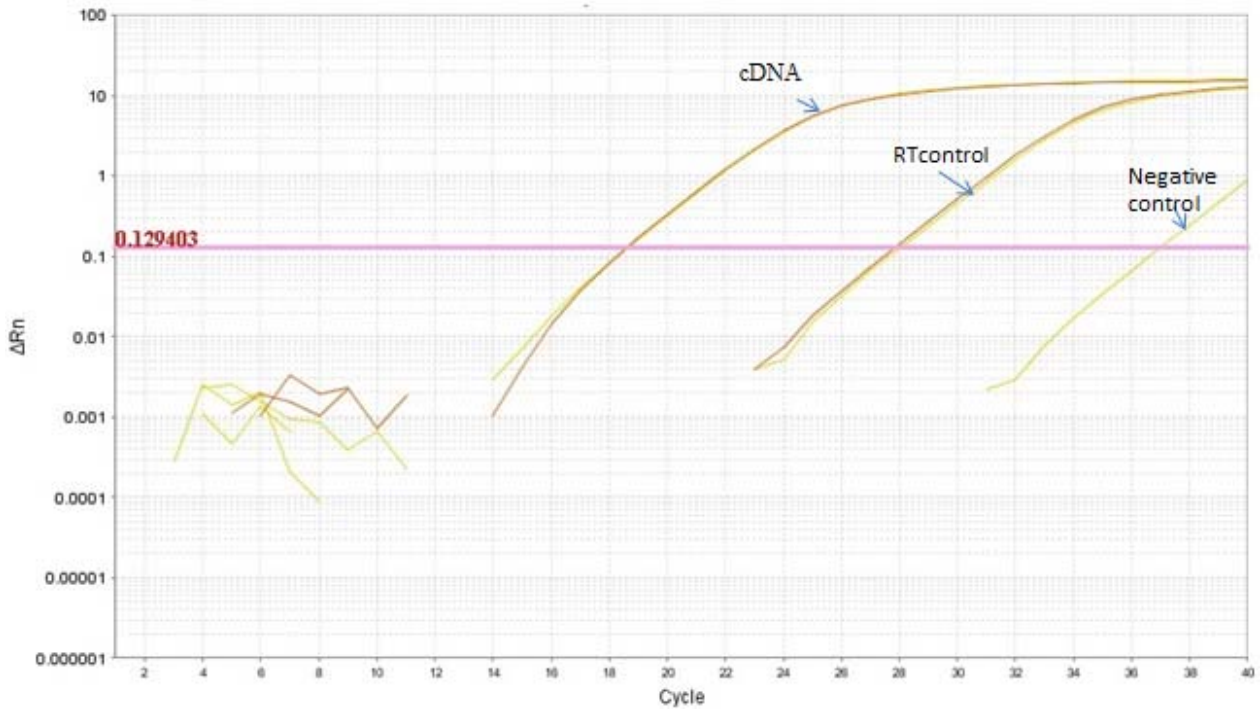


FIGURE 4.13 Amplification curve of cDNA, RTconrol and negative sample treated with one DNase treatment.

For optimization, DNase treatment was included also in the RNA isolation procedure. By the inclusion of an additional DNase treatment, genomic DNA was nearly eliminated from the samples (see FIGURE 4.14). The Ct value of RT control and cDNA was 36, compared to 24 for the reference sample, respectively, indicating only minor traces of genomic DNA in the samples. Thus, the optimized procedure was deemed satisfactory for further analysis.

When Qiagen product was used for DNase treatment in RNA isolation and cDNA synthesis, the Ct of the RTcontrol and reference cDNA was 33 and 19, respectively (FIGURE 4.15). The difference in Ct value of cDNA and RT control, shown in FIGURE 4.13 and FIGURE 4.14, clearly demonstrated the importance of two DNase treatments.

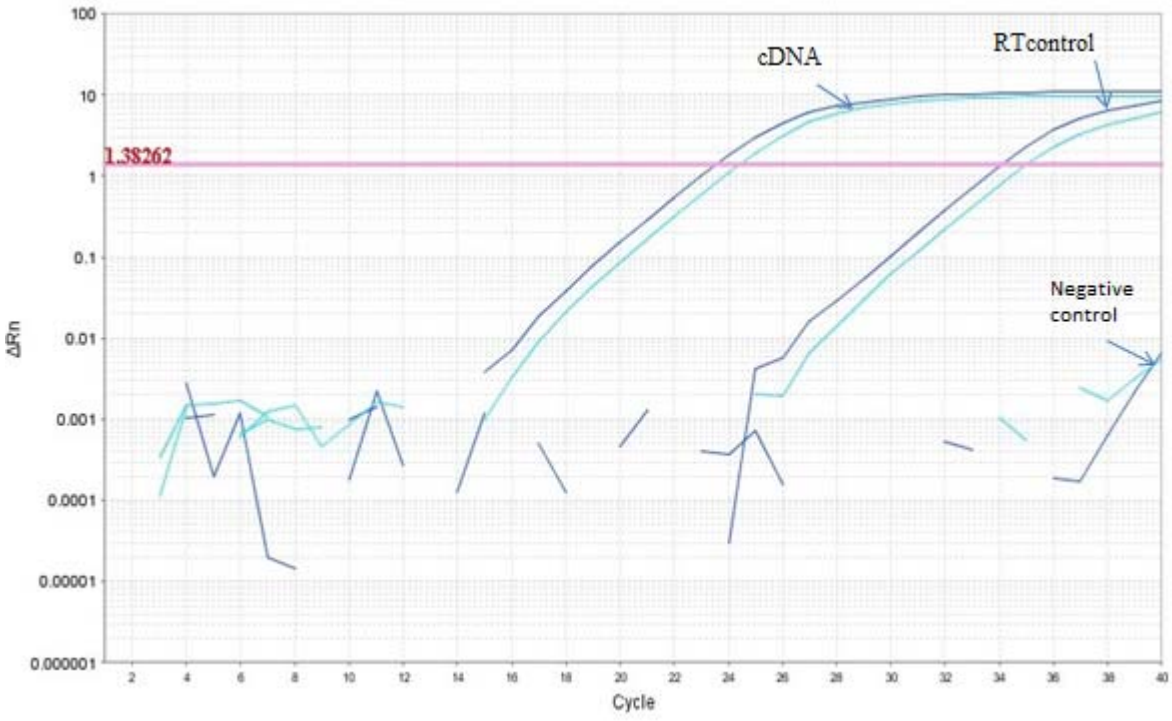


FIGURE 4.14 Amplification curve of cDNA, RTconrol and negative control with two DNase treatments. Promega products for DNase treatment was used in RNA isolation.

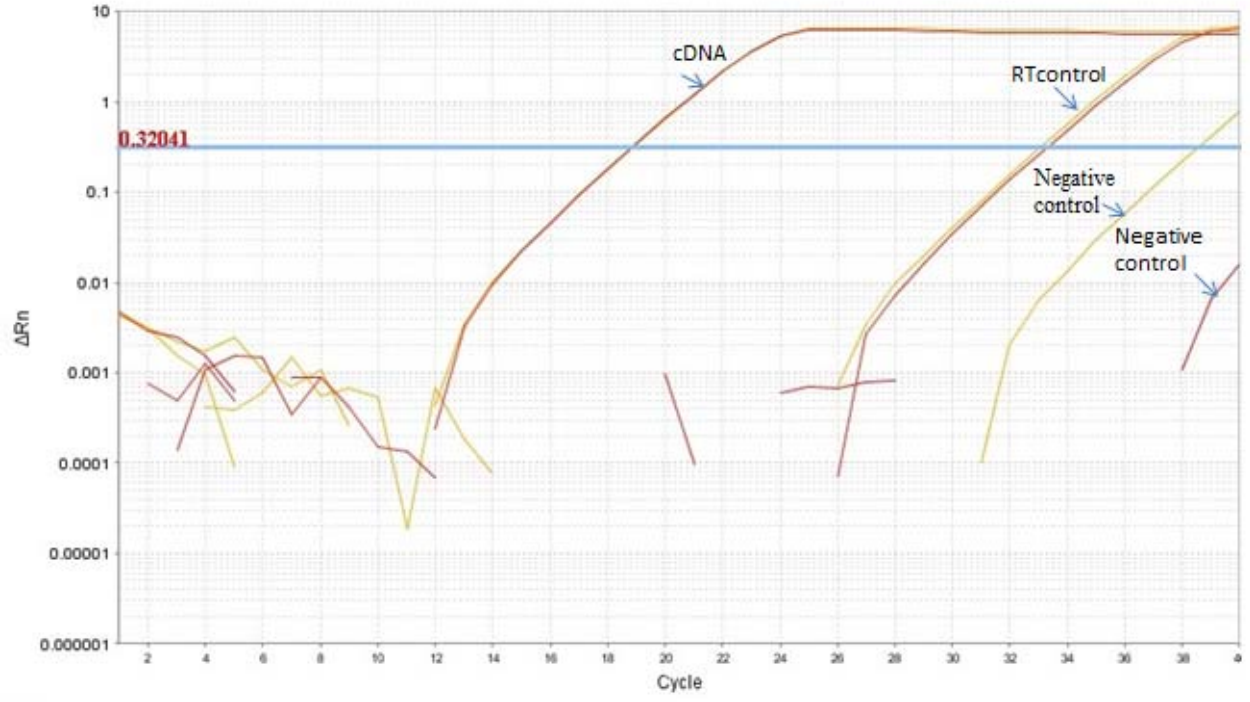
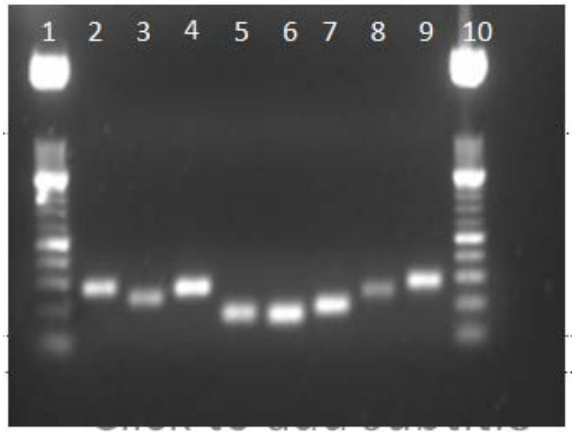


FIGURE 4.15 Amplification curve of cDNA, RTconrol and negative sample with two DNase treatments. Qiagen products for DNase treatment was used in RNA isolation.

4.4.2 Confirmation of amplicon length by agarose gel electrophoresis

The agrose gel electrophoresis was used to check the amplicon size of amplified PCR products. After finishing thermal cycling reactions, the undiluted amplified PCR products in eight reference genes were used to check the band size of amplified cDNA with specific primer sequences. DNA molecular weight marker XIII (Roche) was used as a standard (see Appendix 9.3).

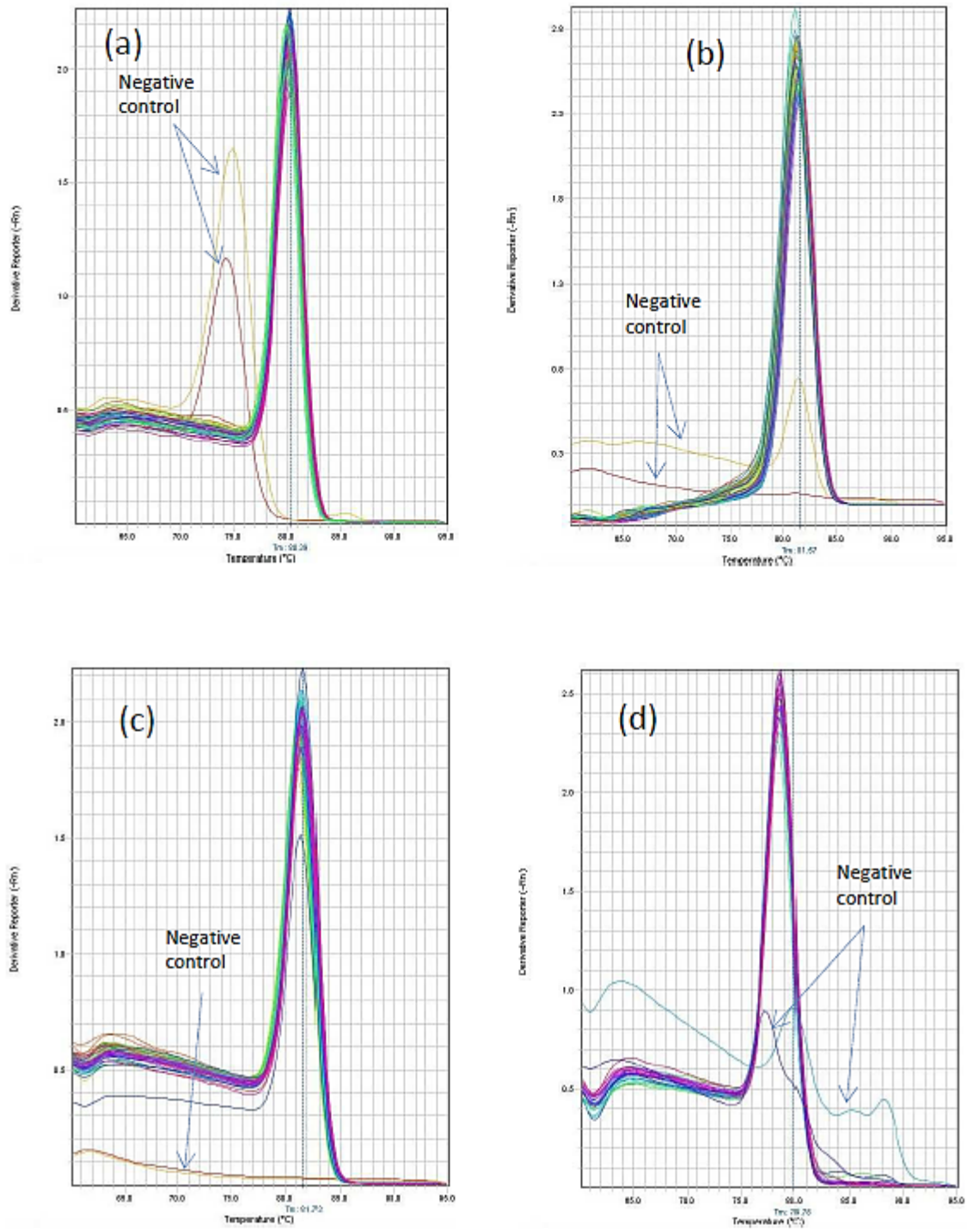


1) Ladder
2) GAP
3) PheS
4) RpS7
5) RpoB
6) Tuf
7) 6PGD
8) RecA
9) RpL11
10) Ladder

FIGURE 4.16 Amplified PCR product of eight genes.

4.4.3 Melting curve analysis

The melting curve analysis showed that all amplicons produced single-peak melting curves at the expected temperature, implying specific amplification. FIGURE 4.17 shows single-peak melting curve in each visible picture and also negative control which did not have any peaks, meaning that no amplification performed. FIGURE 4.18 shows amplification curve and melting curve of HSP60, indicates absence of contaminating products in negative control which did not have amplification curve and no peak in melting curve.


(a)
Negative control
(b)
Negative control
(c)
Negative control
(d)
Negative control

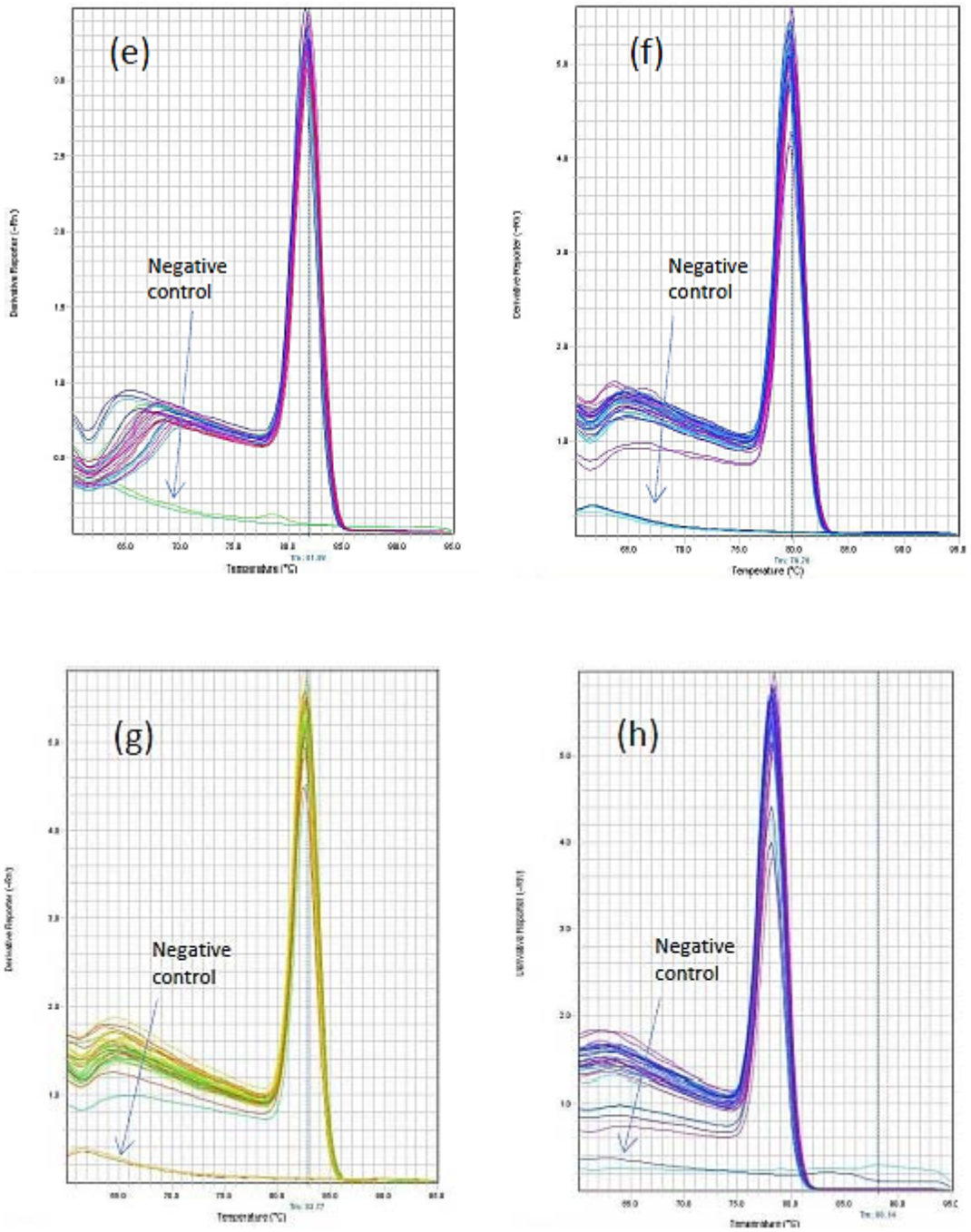


FIGURE 4.17 Melting curves of *L.curvatus* of eight reference genes: a) *6PGD* b)*Tuf* c) *RpoB* d) *Phes* e) *RecA* f) *GAP* g) *RpL11* h) *RpS7*

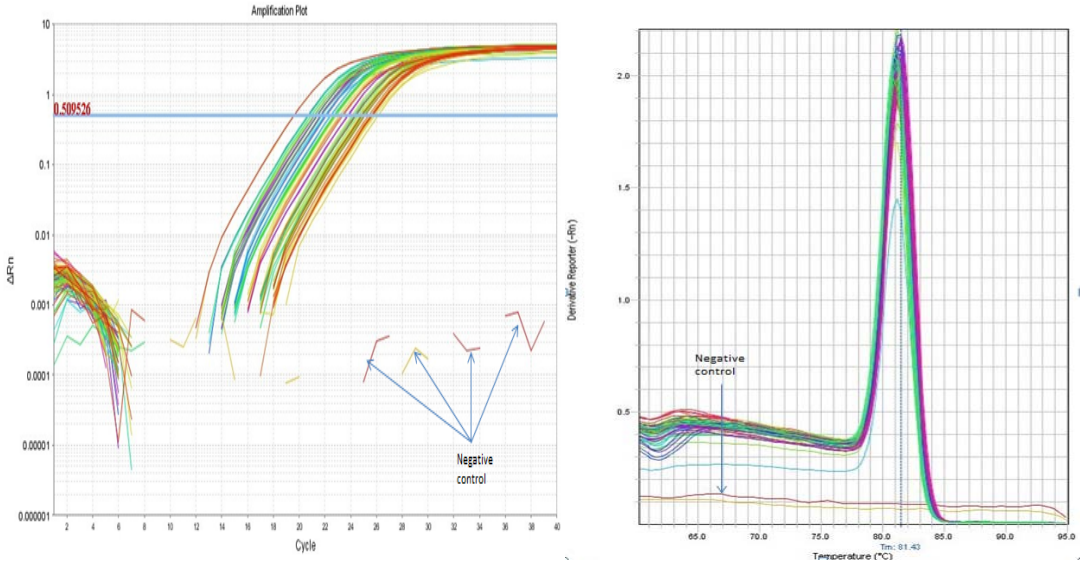


FIGURE 4.18 Amplification curve and melting curve of *HSP60*.

4.4.4 Standard curve and PCR efficiency

To check PCR efficiency of each primer pair, ten-fold dilution series of cDNA were prepared. Nine genes as listed in TABLE 3.7 were tested. After real-time PCR, the Ct value was plotted against the dilution factor. The efficiency plots for each of the candidate genes were found to have linear correlation (R^2 ≥0.946). The efficiencies of all genes were ranged from 75% to 115.9%. The 100% efficiency means that the template doubles after each cycle during exponential amplification. On the other hand, the presence of PCR inhibitors in one or more reagents can produce lower, and the presence of primer-dimers and/or secondary structures, higher efficiency (D'haene *et al.*, 2010).

4.5 Reference gene selection in *L. curvatus* subjected to temperature stress

To evaluate the stability of expression of candidate reference genes, the two algorithms geNormPlus and NormFinder were used. geNormPlus calculates the average gene stability coefficient '*M*' by determining the average pair wise variation between a particular candidate reference gene and all the candidate reference genes. Genes with lower values of '*M*' correspond to the most stable genes and hence are the most appropriate ones for normalization. In addition, the pairwise variation (*V*), is implemented as a proxy for the number of genes to include when

normalization is performed using multiple reference genes. Vandesompele et al., (2002) proposed 0.15 as a cutoff value for *V*, below which the inclusion of an additional reference genes is not required. In this study, the most stable candidate reference genes were; *tuf, 6PGD, recA, RpL11, GAP* and *PheS* as their *M* values were low (< 0.5) at the given experimental conditions (FIGURE 4.19). The *M* values for *tuf* and *6PGD* were less than 0.325 (see FIGURE 4.19) and the *V* value was 0.110 (see FIGURE 4.20). This low *V* value (<0.15) indicates that the use of two reference genes, *tuf* and *6PGD,* is sufficient for reliable normalization during temperature-related experiments.

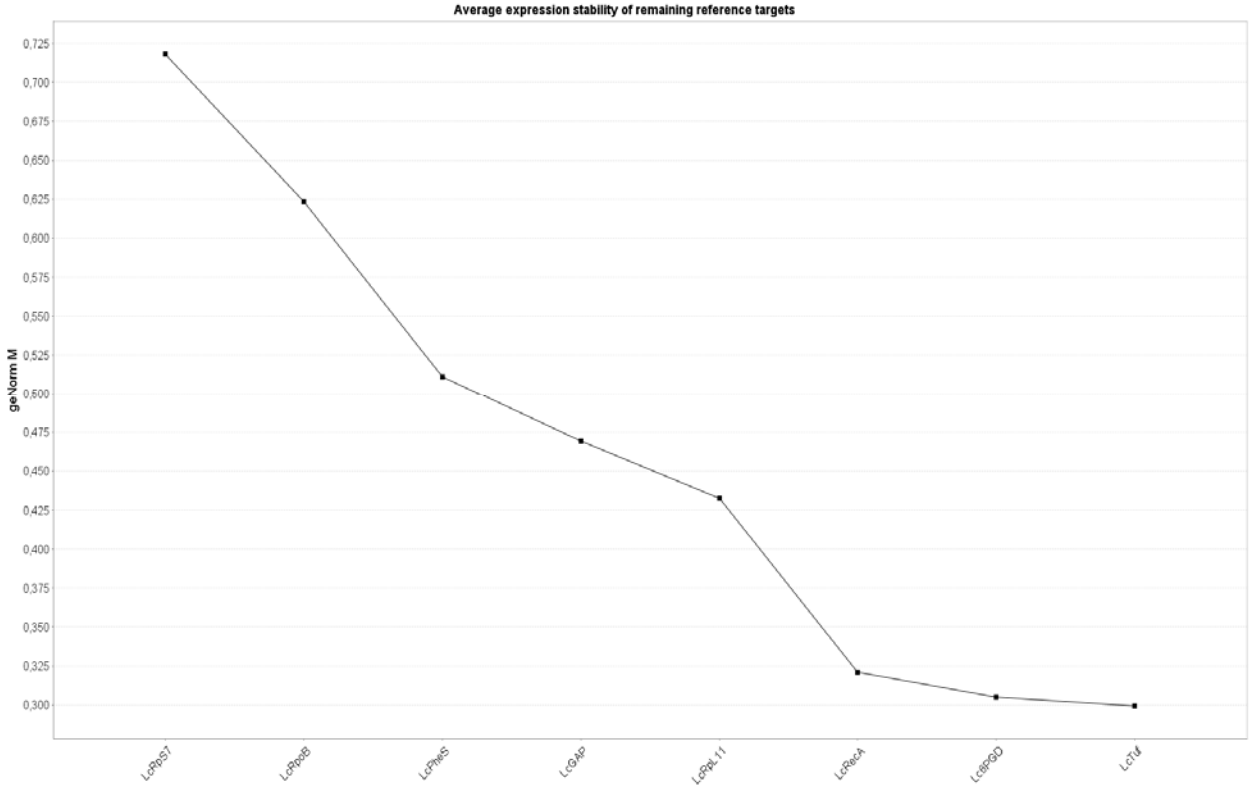


FIGURE 4.19 Average expression stability values (*M*) of candidate reference genes in different temperatures by geNorm analysis.

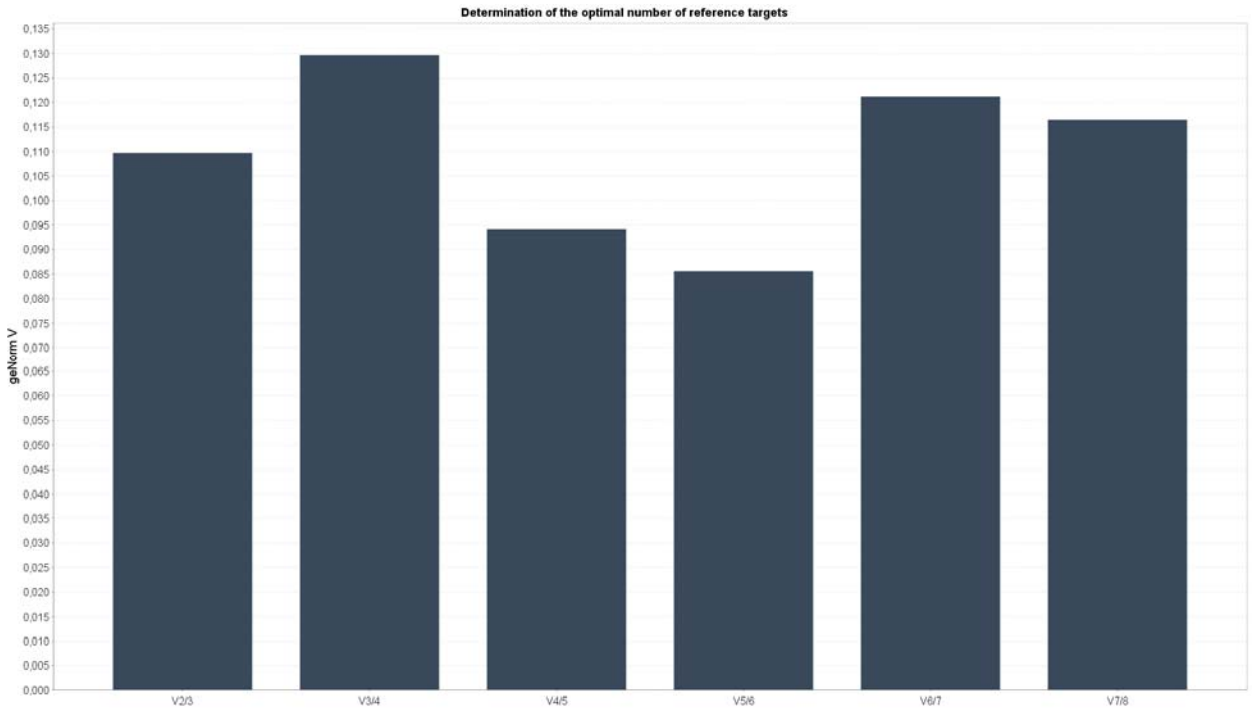


FIGURE 4.20 Determination of number of reference genes for normalization by geNorm analysis.

The NormFinder program is a model-based variance estimated approach, applied to evaluate the expression stability of candidate reference genes for all samples, and it automatically calculates the stability value for all candidate reference genes tested on a sample set containing minimum amount of samples for reliability, organized in any given number of groups (Andersen *et al.*, 2004). The lower average expression stability values indicate the most stably expressed genes. The lower values were found for the same candidate reference genes (see FIGURE 4.21) which were found by the geNormPlus program. However, both programs have shown the most stable genes and least stable genes. These most two stable reference genes (*tuf* and *6PGD*) were used for the normalization of expression level of target genes (*hsp60*) by using geometric mean of *tuf* and *6PGD* as normalization factor.

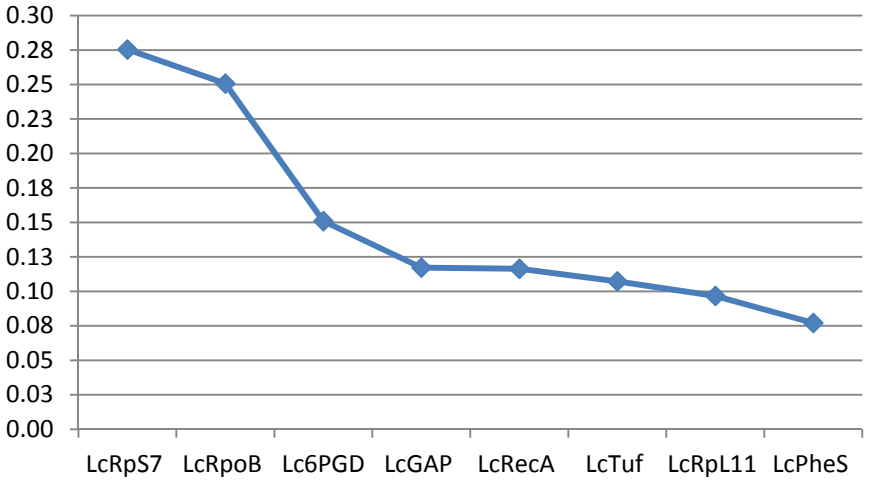


FIGURE 4.21 Average expression stability values (*M*) of candidate reference genes in different temperatures by NormFinder analysis.

FIGURE 4.22 shows the results of expression of *L. curvatus hsp60* under different temperature (5 ºC, 25 ºC and 40 ºC). All samples were calibrated with one (average value for all samples). The gene of interest (*hsp60*) of *L. curvatus* was normalized using geometric mean of *tuf* and *6PGD*. In two different temperatures (5 ºC and 25 ºC) with different time intervals (after 1, 2, 4, 8 and 24 hours), the expression of *hsp60* did not differ significantly (FIGURE 4.22). High expression of *hsp60* was only found when bacterial cell was inoculated at higher temperature 40 ºC. The expression increased 250 fold at 40 ºC after 8 hours.

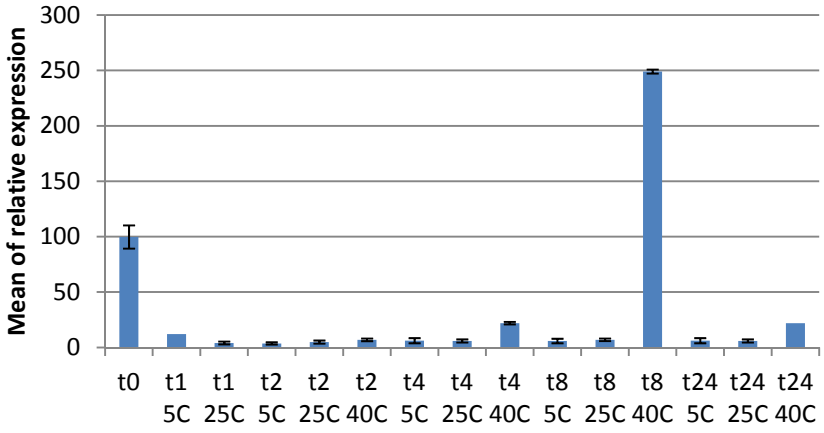


FIGURE 4.22 Expression of gene of interest (*hsp60*) under different temperature as an experimental condition. Mean of two replicates with Standard error.

5 Discussion

5.1 Effect of preservatives on growth of bacteria

In this study bacterial growth was measured indirectly by using Bioscreen. The main reason of this indirect growth measurement of LAB, specific fish spoilage bacteria, and *L. innocua* was to find the effects of additives in different concentrations. Viable count is a traditional, sensitive method for estimating the microbial growth curve, but it is time-consuming and labor-intensive (Dalgaard *et al.*, 1994; McClure *et al.*, 1993). Therefore, Bioscreen was chosen to find the effects of preservatives on growth of bacteria by measuring optical density (absorbance).

As mentioned in Chapter 3, the presence of high concentration PURAC (6%) profoundly affected the growth of species of LAB at 20 ºC. The only exception was *L. curvatus*, their growth was stable for 5 days within the vicinity of ratio OD of less than 1.5 So, the apparent lag time could not be calculated by the current approach. Low concentration PURAC solution (3%) also inhibited growth of *L. curvatus* but not as effective as high concentration PURAC (6%). Interestingly, *L. curvatus* grew faster in LS solution (0.07% or 0.14%) compared to in TSBYE. Since the pH value was the same (7.1) for both TSBYE and 0.14% LS+TSBYE solution, it may be assumed that *L. curvatus* grew well because of the presence of LS solution.

L. curvatus growth was influenced by LS solution, and it was also able to grow in the presence of 0.07% LS + 6% PURAC solution, but not when 6% PURAC was the sole preservative. The apparent lag time for 6% PURAC (106 hours) was three-fold longer compared to 0.07% LS alone. The effect of preservatives on growth of *L. curvatus* observed in this study can be compared with what Marceau *et al.*, (2003) found. They experimented with *L. sakei*, which are genetically closely related (Hebert et al., 2012) to *L. curvatus*. Growth of *L. sakei* was completely inhibited at 4 ºC with 9% NaCl, but it was able to survive for a prolonged period of 24 to 25 weeks at this condition (Marceu et al., 2003). It is quite noticeable that alike *L. sakei, L. curvatus* are also capable of surviving in higher concentration of salt with a relatively slow growth for a comparatively prolonged period of time and varying temperature.

Stekelenburg and Kant-Muermans, (2001) found that the growth of *L. curvatus* in cooked cured ham products was inhibited in the presence of Na-lactate (Purasal, PURAC Biochem.), the lag

time extended from 41 to 89 hours as the concentration of Na-lactate increased from 2.5% to 3.3%. In this present study, the apparent lag time increased form 41 hours to 106 hours when the concentration of PURAC (with 0.07% LS) increased form 3% to 6%. The only difference between the preservatives in these two studies is that, in the present study PURAC (contained K-A and K-L) was mixed with 0.07% LS while Stekelenburg and Kant-Muermans (2001) used 3.3% Na- lactate alone. This indicates that LS may have some effect on shortening the apparent lag time in this experiment.

Since *L. curvatus* was well adapted to TSBYE, it was assumed that they would be able to grow after transferring to different treatments (TABLE 2.1). However, when *L. curvatus* was treated in TSBYE as a control, 6% PURAC, 6% PURAC+0.07% LS, 6% PURAC+0.14% LS and 3% PURAC+0.14% LS for five days (FIGURE 4.5), surprisingly no doubling in optical density was observed. It has been observed that *L. curvatus* need longer time to grow in TSBYE compare to in MRS broth. It would be interesting to run the same experiment with MRS broth and compare the results with TSBYE. It is known that the compounds of MRS broth such as polysorbate (tween 80), acetate, magnesium and manganese help to grow *Lactobacillus* spp. (Leroy and Vuyst, 2001). This present experiment on *L. curvatus* was also carried out before by Løvdal and Birkeland (unpublished data) and their results are in good agreement with the present study.

Combined effects of PURAC and LS on the other LAB (*C. maltaromaticum, C. inhibens, L. lactis* and *E. faecalis*) was found notable; their growth was delayed significantly ($p<0.001$) in presence of LS in PURAC compared to in higher concentration of PURAC solution alone. LS as a sole preservative had minor effect on growth inhibition but when LS combined with PURAC solution, the inhibition effect was enhanced. High concentration of PURAC solution acted as a main inhibitory preservative. However, all these above mentioned bacteria grew well in control medium compared to in different treatments.

Kim and Austin (2008) found that the optimum conditions for growth of *C. maltaromaticum* were between 2% and 3% of salinity at 25 ºC. They also observed that this bacterium was able to grow at high pH (e.g. pH 9) and at 15% (w/v) of salinity. From the present study, it was observed that *C. maltaromaticum* was able to grow at high concentration of salt (6%) solution to a lesser extent. From both the present study and study done by Kim and Austin (2008), it can be comprehended that *C. maltaromaticum* are able to grow in high concentration of salt. In vacuum-

packed cold-smoked or sugar-salted ('gravad') halibut, rainbow trout, salmon and tuna with 3-7% NaCl in water phase, pH value of 5.8-6.5, high concentrations of *C. maltaromaticum* and *C. divergens* are particularly common (Leisner *et al.*, 2007). Laursen (2005) isolated this particular LAB species from vacuum packaged cold smoked salmon stored at 5 ºC having pH value of 6.2, and 3.6 to 5.7% NaCl. From the references discussed above, and the present study, it may be assumed that *C. maltaromaticum* and *C. inhibens* can grow in the presence of high salt concentrations.

L. innocua were found capable of having higher growth in both low (0.07%) and high (0.14%) concentration LS compared to in PURAC alone. Growth was inhibited slightly in LS solution compared to the control medium. Guilbaud et al. (2008) found that the growth of *L. monocytogenes* started to decrease slightly after 20 hours in presence of 15 μg ml^{-1} in brain heart infusion medium and decreased rapidly when phenol content increased to 30 μg ml^{-1} after 8 hours. From their experiment, one may say that *L. monocytogenes* growth inhibition increase with increment of phenol concentration. In this present study, it was observed that *L. innocua* took approximately 18 hours to start growing in presence of LS (0.07% LS; 14 μg ml^{-1} phenol or 0.14% LS; 28 μg ml^{-1} phenol).

When high concentrations of PURAC was used, it had significant inhibition effect ($p<0.001$) on the growth of *L. innocua* (FIGURE 4.6) delayed apparent lag time compared to high concentration of LS solution. Hwang (2009) found that the growth probability of *L. monocytogenes* was affected more profoundly by salt and storage temperature than by phenol. He also mentioned that the growth of *L. monocytogenes* in tryptic soy broth (TSB) decreased when the salt and/or phenol concentration increased, and at lower storage temperature. *L. monocytogenes* are able to grow at 10-12% NaCl (Swaminathan, 2001). Low temperature and high concentration of salt or phenol can be used to decrease their growth.

In an experiment on *L. monocytogenes*, Vogel (2006) found that the growth was prevented for up to 42 days when 2.1% K-L and 0.12% Sodium Diacetate (SDA) (PURASAL opti.From PD 4) was brine injected in cold smoked salmon, under vacuum packaged storage at 10 ºC. Treatments with lower concentration of K-L (1.9%) and SDA (0.09%) resulted in an extended lag phase and slowed the growth of *L. monocytogenes*. Since these concentrations of salt solution were able to

prevent their growth up to 42 days at 10 ºC, it can be said that high salt solution and low temperature might have profound inhibition effect on growth of this particular bacteria.

Effects of preservatives on *P. phosphoreum* were found different from other examined bacteria, growth was inhibited significantly ($p<0.001$) in 3% PURAC solution when compared to control medium but compared to LS, the growth was significantly enhanced ($p<0.001$). Combined solution of high concentration PURAC and LS restrained their growth. They grew fast in presence of 3% PURAC but in 6% PURAC solution, slow growth was observed (FIGURE 4.8). Leroi (1998) found that *P. phosphoreum* was able to grow in presence of 2.5% salt medium but the growth was prevented completely when treated in more than 6% salt (as reviewed by Leroi 2010).

As gram-negative bacteria, *P. putida* and *V. vulnificus* did not reveal the same characteristic as *P. phosphoreum,* they did not grow well in 3% PURAC solution but in LS solution and in control medium. Sallam et al. (2007) found that the population of *Pseudomonas* spp. in sliced salmon was significantly reduced ($p<0.05$) when treated with 2.5% aqueous solutions of sodium lactate/sodium acetate/sodium citrate. In control sample, it took 9 days to approach a population of 7 $\log_{10}$ CFU/g whereas in samples treated with sodium salts, they never reached the same number during 15 days of storage at 1 ºC. The growth of *V. vulnificus* was prevented completely in 6% PURAC (FIGURE 4.10), extended apparent lag time was observed when treated in 3% PURAC solution, which was quite surprising since it is defined as a halophilic (salt-requiring) bacterium (Daniels, 2011; Ji *et al.*, 2011; Oliver and Kaper, 2001). Being halophilic bacteria, one may expect their growth to be enhanced in 3% PURAC solution.

To verify the results, the above mentioned experiment was replicated twice with the same results (data not shown). Since the results were rather unexpected and interesting, especially as compared to the other bacteria not regarded as halophilic, another experiment was carried out where NaCl, lactate, acetate was used as preservatives. The results revealed that PURAC has better inhibition effect on *V. vulnificus*, than sodium salt within the same concentration range.

These halophilic bacteria grow best in warm water (temperature >22º C) (Daniels, 2011), especially in summer time (Ji *et al*., 2011; Daniels, 2011; Motes *et al.,* 1998). Motes et al., (1998) reported that low salinity (0.5 – 2.5 %) and relatively high temperature (> 20 ºC) are favorable

growth conditions for this organism. Kelly (1985) found that the optimal temperature and salinity for growth of *V. vulnificus* were 37 ºC and <2% NaCl respectively. He also found that the growth rate of *V. vulnificus* was optimal in media (brain heart infusion broth) containing 0.5% to 2.0% NaCl, growth was retarded in media containing 4.0 to 6.5% NaCl, and no growth was noted in the presence of 8.5 or 10.5% NaCl.

In the present study, it was found that *V.vulnificus* had moderate growth in low concentration of PURAC and no growth was observed in high concentration of PURAC. Kaspart (1993) found that the number of *V. vulnificus* increased during 6 days of incubation in the temperature range from 13 to 22 ºC. Temperature outside of this range reduced the survival time of *V. vulnificus* in sterile 1.0 % salinity (Kaspar and Tamplin, 1993).

From the above findings on *V. vulnificus*, one may suggest that the growth of this halophilic bacterium enhances in presence of high temperature and low salinity, but high salinity prevents its growth. However, in this present study, it was found that this bacterial growth was inhibited in 3% PURAC and prevented in 6% PURAC solution. In addition, the inhibition effect on growth was also observed in the presence of 0.21% K-A and compared with control. When K-A was added in NaCl, the inhibition effect was enhanced compared to NaCl alone. Since PURAC solution contains both K-L and K-A, the growth was inhibited more compared to in presence of K-A alone. K-A influenced inhibition effect on growth of this bacterium.

Zhuang and others (1996) concluded that catfish fillets treated with 2% sodium acetate had the potential to extend the shelf-life of refrigerated catfish fillets by 3 days while 2% sodium lactate had little effect on microbial growth when compared to untreated catfish fillets. Serdengecti et al., (2006) suggested that preservatives which contain combinations of sodium salts are more effective than preservative with individual sodium salt. Kin et al., (2011) found that combination of K-L and K-A enhanced sensory quality and extended the shelf-life of refrigerated catfish fillets.

From the above discussion, it is clear that high concentrations of PURAC, used either combined with LS (0.07% or 0.14%) or alone, may have high inhibition effect on growth of LAB, spoilage bacteria, and *L. innocua* at 20 ºC. Additionally, the composition of PURAC solution (K-A + K-L) also played a big role on the growth inhibition of bacteria. At low temperature, it can be assumed

that the same effect can be obtained with high concentration of PURAC to increase the shelf-life of fish products.

5.2 Evaluation of candidate reference genes for normalization of quantitative real-time PCR data

Before the gene expression studies, appropriate protocols for RNA isolation and cDNA synthesis, was established. The necessity of evaluation and potential optimization of such methods is that to ensure pure RNA of high quality, and eliminating contaminating genomic DNA. Two DNase treatments were imperative to sufficiently reduce genomic DNA from RNA, as found in this study (FIGURE 4.13 and 4.14), and also reported elsewhere (Takle *et al.*, 2007; Vandesompele *et al.*, 2002a).

Among the eight candidate reference genes, six genes (*Tuf, 6PGD, RecA, RpL11, GAP* and *PheS*) were found to be stably expressed under the given experimental conditions. This was verified by two algorithms (geNorm and NormFinder). In geNorm program, the most stable reference genes ranked form *Tuf, 6PGD, RecA, RpL11, GAP a*nd *PheS* whereas in NormFinder program, the most stable reference genes ranked from *PheS, RpL11, tuf, recA* and *6PGD*. geNorm program determines the most stable candidate reference genes and the optimal number of genes which are required for normalization of RT-PCR data (Vandesompele *et al.*, 2002b). NormFinder can determine only the most stable candidate reference genes (Andersen *et al.*, 2004).

Both programs determined the same stable reference genes, the only difference was in the ranking of the most stable reference genes. In geNorm program, the two most stable reference genes were *Tuf* and *6PGD* and in NormFinder, the two most stable reference genes were *PheS* and *RpL1*. Anderson et al., (2004) mentioned that geNorm may be insensitive to coregulated reference genes. Coregulated reference genes should be avoided when using multiple references genes because they may bias the results. When *Tuf* was removed from the analysis, there was no difference observed in the ranking of the rest of stable reference genes. This indicates that there was no coregulation occurring.

Tuf and *6PGD* were sufficient for normalization of gene expression analysis because of their low *V* values (<0.15), found from geNorm analysis. *Tuf* and *6PGD* encoding protein synthesis and 6-

phosphogluconate dehydrogenase respectively, were used for normalization of gene expression data. *Hsp60* gene in *L. curvatus* was expressed significantly when *L. curvatus* was inoculated at high temperature (40 ºC) after 8 hours (mention as a t8 40 ºC in FIGURE 4.22).

6 Conclusion

The effects of different concentration of preservatives on the growth of selected fish spoilage bacteria, LAB and *L. innocua* were investigated at 20 ºC based on optical density data. The most inhibition effect on growth of bacteria was found in presence of high concentration of PURAC (6%), used either combined with liquid smoke (0.07% or 0.14%) or alone. The minor inhibition effect on growth of bacteria was found in presence of liquid smoke alone (0.07% or 0.14%). The only exception was *L. curvatus*, which grew quite well in presence of LS compared to control medium. This bacterium was also able to grow in the presence of 0.07% LS + 6% PURAC, but not when 6% PURAC was the sole preservative. The growth of another fish spoilage bacterium, *V. vulnificus* was prevented in higher concentration PURAC solution (6%), and significantly inhibited in presence of lower concentration of PURAC solution (3%). When *V. vulnificus* was treated in NaCl, K-A and PURAC, it was observed that PURAC (contain K-L and K-A) had better inhibition effect than sodium salt within the same concentration range.

Appropriate protocols for RNA isolation and cDNA synthesis were established. Two DNase treatments were imperative to sufficiently reduce genomic DNA from RNA, as found in this study. Eight candidate reference genes and one gene of interest were used in gene expression analysis. Among eight reference genes, six candidate reference genes were stably expressed under experimental condition (different temperature). Two reference genes were sufficient for normalization of gene expression analysis found form geNorm anlysis. The expression of *hsp60* was induced significantly when inoculated at 40 ºC.

7 Perspective for future work

For the future work, it would be interesting to investigate the effect of preservatives (salt and liquid smoke) on growth of LAB, *L. innocua* and selected fish spoilage bacteria at refrigerated temperature. The growth of aforementioned bacteria can be monitored in co-culture by conventional plating at two different temperatures (moderate and refrigerator) to estimate the putative inhibitory effect (Matamoros et al., 2009) of specific LAB on the growth of other seafood related bacteria. Gene expression analysis can be performed to investigate effect of salt and pH for bacteria.

8 References

Altinok, I., Kayis, S. and Capkin, E. (2006). *Pseudomonas putida* infection in rainbow trout. *Aquaculture* **261**, 850-855.

Andersen, C. L., Jensen, J. L. and Orntoft, T. F. (2004). Normalization of real-time Quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, Applied to bladder and colon cancer data sets. *Cancer Research* **64**, 5245-5250.

Anonymous (2001a). Special supplement- processing parameters needed to control pathogens in cold-smoked fish-preface. *Journal of Food Science* **66**, 1059-1066.

Anonymous (2001b).*Vibrio vulnificus*: Prepared for the Ministry of Health by ESR Ltd.

Austin, B. and Austin, D. A. (1992). The sanitary quality of waters associated with fresh water aquaculture *Microbiol Aliment Nutr* **10**, 303-311.

Barbes, C. (2008). *Biology of effector organisms for probiotic and replacement therapy.* Washington DC.: ASM Press.

Chiang, S. R. and Chuang, Y. C. (2003). *Vibrio vulnificus* infection: clinical manifestations, pathogenesis, and antimicrobial therapy. *Journal of Microbiology Immunology and Infection* **36(2)**, 81-88.

Costantini, A., Vaudano, E., Rantsiou, K., Cocolin, L. and Moruno, E. G. (2011). Quantitative expression analysis of *mleP* gene and two genes involved in the ABC transport system in *Oenococcus oeni* during rehydration. *Applied MicrobiolBiotechnol* **91**, 1601-1609.

D'haene, B., Vandesompele, J. and Hellemans, J. (2010). Accurate and objective copy number profiling using real-time quantitive PCR. *Method* **50**, 262-270.

Dalgaard, P., Ross, T., Kamperman, L., Neumeyer, K. and McMeekin, T. A. (1994). Estimation of bacterial growth rates from turbidimetric and viable count data. *International Journal of Food Microbiology* **23**, 391-404.

Daniels, N. A. (2011). *Vibrio vulnificus* oysters: Pearls and Perils. *Clinical Infectious Diseases* **52 (6)**, 788-792.

Davidson, M. P. (2001). Chemical preservatives and natural antimicrobial compounds. In: Doyle, M.P., Beuchat, L.R. and Montville, T.J. (eds). Food Microbiology: Fundamentals and Frontiers, 2nd ed, ASM Press, Washington, pp. 593-627.

Dillon, R., Patel, T. R. and Martin, A. M. (1994). *Microbiological control for fish smoking operations.* London: Chapman and Hall.

Drake, S. L., Depaola, A. and Jaykus, L. A. (2007). An overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. *Comprehensive Reviews in Food Science and Food Safety* **6(4)**, 120-144.

Duffes, F. (1999). Improving the control of *Listeria monocytogenes* in CSS. *Trends in Food Science and Technology* **10**, 211-216.

Duwat, P., Hammer, K., Bolotin, A. and Gruss, A. (2000). *Genetics of lactococci*. Washington DC.: American Society for Microbiology.

Emborg, J., Laursen, B. G., Rathjen, T. and Dalgaard, P. (2002). Microbial spoilage and formation of biogenic amines in fresh and thawed modified atmosphere-packed salmon (*Salmon salar*) at 2 degree C. *Journal of Applied Microbiology* **92**, 790-799.

Farber, J. M. and Peterkin, P. I. (1991). *Listeria monocytogenes*, a food-borne pathogen. . *Microbiological-Review* **55 (3)**, 476-511.

Giraffa, G. (2007). *Enteroccoci and Dairy products.*: John Wiley and Sonc. Inc.

Glaser, P., Frangeu, l. L., Buchriwser, C., Rusniok, C., Ahmend, A. and Cossart, P. (2001). Comparative genomics of *Listeria* species. *Science* **294**, 849-852.

Gopal, S., Otta, S. K., Kumar, S., Karunasagar, I. and Nishibuchi, M. (2005). The occurrence of Vibrio species in tropical shrimp culture environments; implications for food safety. *International Journal of Food Microbiology* **102(2)**, 151-159.

Gram, L. and Huss, H. H. (1996). Microbiological spoilage of fish and fish products. *International Journal of Food Microbiology* **33**, 121-137.

Guilbaud, M., Chafsey, I., Pilet, M. F., Prevost, H., Hebraud, M. and Dousset, X. (2008). Response of *Listeria monocytogenes* to liquid smoke. *Journal of Applied Bacteriology* **1744-1753**.

Guyer, S. and Jemmi, T. (1991). Behavior of *Listeria monocytogenes* during fabrication and storage of experimentally contaminated smoked salmon. *Applied and Environmental Microbiology* **57**, 1523-1527.

Han, F. and Ge, B. (2010). Quantitive detection of *Vibrio Vulnificus* in raw oysters by real-time loop-mediated isotermal amplification. *International Journal of Food Microbiology* **142**, 60-66.

Hansen, L. T., Gill, T. and Huss, H. H. (1995). Effects of salt and storage temperature on chemical, microbiological and sensory changes in cold-smoked salmon. *Food Research International* **28 (2)**, 123-130.

Hansen, L. T., Gill, T., Røntved, D. S. and Huss, H. H. (1996). Importance of autolysis and microbiological activity on quality of cold-smoked salmon. *Food Research International* **29 (2)**, 181-188.

Hansen, L. T. and Huss, H. H. (1998). Comparison of the microflora isolated from spoilage cold-smoked salmon from three smokehouses. *Food Research International* **31**, 703-711.

Hartman, P. A., Deibel, R. H. and Sieverding, L. M. Enterococci. In *Compendium of methods for the micrological examination of foods*, pp. 82-87.

Hebert, E. M., Saavedra, L., Taranto, M. P. and other authors (2012). Genome sequence of the bacteriocin-producing *Lactobacillus curvatus* strain CRL705. *Journal of Bacteriology* **194(2)**, 538-539.

Heid, C. A., Stevens, J., Livak, K. J. and William, P. M. (1996). Real time quantitative PCR. *Genome Research* **6(10)**, 989-994.

Higuchi, R., Dollinger, G., Walsh, P. S. and Griffith, R. (1992). Simultaneous amplification and detection of specific DNA-sequences. *Bio-Technology* **10(4)**, 413-417.

Horner, W. F. A. (1997). *Preservation fish by curing (drying, salting and smoking)*. London: Blackie Academic and Professional.

Huss, H. H., Embarek, P. K.and Jeppessen, V. F. (1995). Control of biological hazards in CSS production. *Food Control* **6(6)**, 335-340.

Hwang, A. C. (2009). The probability of growth of *Listeria monocytogenes* in cooked salmon and tryptic soy broth as affected by salt, smoke compound, and storage temperature. *Food Microbiology* **26**, 253-258.

Hwang, A. C., Sheen, S. and Juneja, K. V. (2009). Effect of salt, smoke compound and temperature on the survival of *Listeria monocytogenes* in salmon during simulated smoking processes. *Journal of Food Science* **74 (9)**.

Jackson, C. T., Marshall, L. D., Acuff, R. G. and Dickson, S. J. (2001). Meat, poultry and seafood. In: Doyle, M.P., Beuchat, L.R. and Montville, T.J. (eds). Food Microbiology: Fundamentals and Frontiers, 2nd ed, ASM Press, Washington, pp. 91-126

Jemmi, T. (1993). *Listeria monocytogenes* in smoked fish: an overview. *Archiv fur lebensmittelhygiene* **44**, 1-24.

Ji, H., Chen, Y., Guo, Y., Liu, X., Wen, J. and Liu, H. (2011). Occurrence and characteristics of *Vibrio vulnificus* in retail marine shrimp in China. *Food Control* **22**, 1935-1940.

Joborn, A., Dorsch, M., Olsson, J. C., Westerdahl, A. and Kjelleberg, S. (1999). *Carnobacterium inhibens* sp. nov., isolated from the intestine of Altantic salmon (*Salmo salar*). *International Journal of Systematic Bacteriology* **49**, 1891-1898.

Joffraud, J. J., Cardinal, M., Cornet, J., Chasles, J. S., Leon, S., Gigout, F. and Leroi, F. (2006). Effect of bacterial interactions on the spoilage of cold-smoked salmon. *International Journal of Food Microbiology* **112**, 51-61.

Johnston, D. M. (1998). A simple and rapid test for quality control of liquid media, using the bioscreen microbiological growth analyser. *Journal of Microbiological methods* **32**, 37-43.

Jørgensen, L. V. and Huss, H. H. (1998). Prevalence and growth of *Listeria monocytogenes* in naturally contaminated seafood. *International Journal of Food Microbiology* **42**, 127-131.

Jeppesen, V. T., Huss, H.H. (1993). Characteristic and antagonistic activity of lactic acid bacteria isolated from chilled fish products. *International Journal of Food Microbiology* **18**, 305-320.

Kaspar, C. W. and Tamplin, M. L. (1993). Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. *Applied and Environmental Microbiology* **59(8)**, 2425-2429.

Kelly, M. T. (1982). Effect of temperature salinity on *Vibrio* (*Beneckea*) *vulnificus* occurrence in a Gulf Coast Environment. *Applied and Environmental Microbiology* **44(4)**, 820-824.

Kilinc, B., Cakli, S., Dincer, T. and Tolasa. S. (2009). Microbiological, chemical, sensory, color and textural changes of rainbow trout fillets treated with sodium acetate, sodium lactate, sodium citrate and stored at 4 degree C. *Journal of Aquatic food product technology* **18**, 3-17.

Kim, D. H. and Austin, B. (2008). Characterization of probiotic carnobacteria isolated from rainbow trout (*Oncorhynchus mykiss*) intestine. *Letters in Applied Microbiology* **47**, 141-147.

Kin, S., Wes Schilling, M., Smith, S. B., Silva, L. J., Kim, T., Pham, J. A. and Campano, G. S. (2011). Potassium Acetate and Potassium Lactate enhance the microbiological and physical properties of marinated catfish fillets. . *Journal of Food Science* **76(4)**, 242-250.

Knøchel, S. (1983). Fermented fish products in Scandinavia. Kor. *Journal of Applied Microbiological Bioengineering* **11**, 347-351.

Kuts, V. V. and Ismailov, A. D. (2009). Physiological and Emission characteristics of the luminescent bacterium *Photobacterium phosphoreum* from the white sea. *Microbiology* **78(5)**, 554-558.

Larsen, A. T. and Asche, F. (2011). Contracts in the Salmon Aquaculture Industry: An analysis of Norwegian salmon exports. *Marine Resource Economics* **26**, 141-150.

Leisner, J. J., Millan, J. C., Huss, H. H. and Larsen, C. M. (1994). Production of histamine and tyramine by Lactic acid bacteria isolated from vacuum-packaged sugar-salted fish. *Journal of Applied Bacteriology* **76**, 417-423.

Leisner, J. J., Laursen, B. G., Prevost, H., Drider, D. and Dalgaad, P. (2007). *Carnobacterium*: positive and negative effects in the environment and in foods. *FEMS Microbiol Review* **31**, 592-613.

Leroi, F., Joffraud, J. J., Chevalier, F. and Cardinal, M. (1998). Study of the microbial ecology of cold-smoked salmon during storage at 8 degree C. *International Journal of Food Microbiology* **39**, 111-121.

Leroi, F., Joffraud, J. J. and Chevalier, F. (2000). Effect of salt and smoke on the microbiological quality of cold-smoked salmon during storage at 5 degree as estimated by the factorial design method. *Journal of food protection* **63 (4)**, 502-508.

Leroi, F. (2010). Occurrence and role of lactic acid bacteria in seafood products. *Food microbiology* **27**, 698-709.

Leroy, F. and Vuyst, L. D. (2001). Growth of the bacteriocin-producing *Lactobacillius sakei* strain CTC 494 in MRS Broth is strongly reduced due to nutrient exhaustion: a nutrient depletion model for the growth of lactic acid bacteria. *Applied and Environmental Microbiology* **67(10)**, 4407-4413.

Li, M., Pradhan, A., Cooney, L., Mauromoustakos, A., Crandall, P., Slavik, M. and Li, Y. (2011). A predictive model for the inactivation of *Listeria innocua* in cooked poultry products during postpackage pasturization. *Journal of Food Protection* **74**, 1261-1267.

Lyhs, U. and Bjorkroth, J. K. (2008). *Lactobacillius sakei* / *curvatus* is the prevailing lactic acid bacterium group in spoiled maatjes herring. *Food Microbiology* **25**, 529-533.

Lyhs U., Lahtinen J., Ahomaa M.F., Trees H.E., Elfing K. and Korkeala H. (2001). Microbiological quality and shelf-life of vacuum-packaged gravad rainbow trout stored at 3 and 8 degree C. *International Journal of Food Microbiology* **70**, 221-230.

Løvdal, T. and Lillo, C. (2009). Reference gene selection for quantitive real-time PCR normalization in tomato subjected to nitrogen, cold and light stress. *Analytical Biochemistry* **387**, 238-242.

Løvdal, T., Skjoldal, E.F., Heldal, M., Norland, S. and Thingstad, T.F. (2008). Changes in Morphology and Elemental Composition of *Vibrio splendidius* along a gradient from Carbon-limited to Phosphate-limited Growth. *Microbial Ecology* **55**, 152-161.

Madigan, T. M. and Martinko, J. M. (1997). *Biology of Microorganism*. USA: Prentice Hall International.

Marceau, A., Zagorec, M. and Champomier-Verges, M. C. (2003). Positive effects of growth at suboptimal temperature and high salt concentration on long-term survival of *L. sakei*. *Research in Microbiology* **154**, 37-42.

Matamoros, S., Pilet, M. F., Gigout, F., Prevost, H. and Leroi, F. (2009). Selection and evaluation of seafood-borne psychrotrophic lactic acid bacteria as inhibitors of pathogenic and spoilage bacteria. *Food Microbiology* **26**, 638-644.

McClure, P. J., Cole, M. B., Davies, K. W. and Anderson, W. A. (1993). The use of automated turbidimetric data for the construction of kinetic models. *Journal of Industrial Microbiology* **12**, 277-285.

Mclauchlin, J. (1996). The relationship between *Listeria* and listeriosis. *Food Control* **7**, 187-193.

Mendonca, A. F., Molins, R. A., Kraft, A. A. and Walker, H. W. (1989). Effects of potassium sorbate, sodium acetate, phosphates and sodium chloride alone or in combination on shelf life of vacuum packaged pork chops. *Journal of Food Science* **54**, 302-306.

Messina, M. C., Ahmed, H. A., Marchelo, J. A., Gerba, C. P. and Paquette, M. W. (1988). Effect of liquid smoke on *L. monocytogenes*. *Journal of Applied Microbiology* **51**, 629-631.

Montville, T. J. and Matthews, K. R. (2001). *Principles which influence microbial growth, survival, and death in foods*, 2nd edn. Washington,D.C.: ASM.

Motes, M.L., Depaola, A., Cook, D.W., Veazey, J.E., Hunsucker, J.C., Garthright, W.E., et al. (1998). Influence of water temperature and salinity on *Vibrio vulnificus* in Northern Gulf and Altantic Coast oysters (*Crassostrea virginica*). *Applied and Environmental Microbiology* **64(4)**, 1459-1465

Muller, C. P., Dalgaad, P., Huss, H. H. and Gram, L. (1998). Evaluation of the role of *Carnobacteria pisicola* in spoilage of vacuum and modified atmosphere packed cold-smoked salmon stored at 5 degree C. *International Journal of Food Microbiology* **39**, 155-166.

Mullis, B. K. and Faloona, F. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol* **155**, 335-350.

Niedziela, J. C., Macrae, M., Ogden, I. D.and Nesvadba, P. (1998). Control of *Listeria monocytogenes* in salmon; antimicrobial effect of salting, smoking and specific smoke compounds. *Lebensm-WissU-Technol* **31**, 155-161.

Oliver, J. D. and Kaper, J. B. (2001). Vibrio species. In: Doyle, M.P., Beuchat, L.R. and Montville,T.J. (eds). Food Microbiology: Fundamentals and Frontiers, 2nd ed, ASM Press, pp. 263-300.

Paranjpye, R. N., Perterson, M. E., Poysky, F. T., Pelroy, G. A. and Eklund, M. W. (2004). Control of bacterial pathogens by liquid smoke and sodium lactate during processing of cold-smoking and dried salmon strips. *Journal of Aquatic food product technology* **13(4)**.

Peterson, M. E., Pelroy, A. G., Paranjpye, R. N., Poysky, F. T., Almond, J. S. and Eklund, W. M. (1993). Parameters for control of *Listeria monocytogenes* in smoked fishery products-sodium chloride and packaging method. *Journal of Food Protection* **56**, 938-943.

Pirt, S. J. (1975). *Principles of Microbe and Cell Cultivation.* London: Blackwell Scientific.

Poysky, F. T., Paranjpye, R. N., Peterson, M. E., Pelroy, A. G., Guttman, A. E. and Eklund, M. E. (1997). Inactivation of *Listeria monocytogenes* on hot- smoked salmon by interaction of heat and smoke or liquid smoke. *Journal of Food Protection* **60**, 649-654.

Quan, Y., Choi, K. D., Chung, D. and Shin I.S. (2010). Evaluation of bactericidal activity of weakly acidic electrolyzed water (WAEW) against *Vibrio vulnificus* and *Vibrio parahaelyticus*. *International Journal of Food Microbiology* **136**, 255-260.

Reiter, L., Kolstø, A. B. and Piehler, A. P. (2011). Reference genes for quantitative, reverse-transcription PCR in *Bacillus cereus* group strain throughout the bacteria life cycle. *Journal of Microbiological methods* **86**, 210-217.

Robinson, T. P., Ocio, M. J., Kaloti, A. and Mackey, B. M. (1998). The effect of the growth environment on the lag phase of *Listeria monocytogenes*. *International Journal of Food Microbiology* **44**, 83-92.

Rocourt, J. (1999). *The Genus Listeria and Listeria monocytogenes: Phylohenetic Position, Taxonomy, and Identification.* In: Ryser, E.T., and Marth, E.H. (eds). *Listeria*, Listeriosis and Food Safety, 2nd edn. Marcel Dekker, New York, USA, pp.1-20.

Rørvik, L. M., Yndestad, M. and Skjerve, E. (1991). Growth of *Listeria monocytogenes* in vacuum-packed, smoked salmon, during storage at 4 degree C. *International Journal of Food Microbiology* **14**, 111-117.

Ringø, E. and Gatesoupe, F.J. (1998). Lactic acid bacteria in fish: a review. *Aquaculture* **160**, 177-203.

Ringø, E., Bendiksen, H.R., Wesmajervi, M.S., Olsen, R.E., Jansen, P.A. and Mikkelsen, H. (2000). Lactic acid bacteria associated with the digestive tract of Altantic salmon (*Salmon salar L.*). *J. Appl. Microbiol.* **89**, 317-322.

Gonzalez, C.J., Lopez-Diaz, T.M., Gaecia-Lopez, M.L., Prieto, M. and Otero, A. (1999). Bacterial microflora of Wild brown trout (*Salmo trutta*), Wild pike (*Esox hucius*) and aquaculture rainbow trout (*Oncorhynchus mykiss*). *Journal of Food Protection* **62**, 1270-1277

Gonzalez, C.J., Encinas, J.P., Gacia-Lopez, M.L. and Otero, A. (2000). Characterisation and identified of lactic acid bacteria from freshwater fishes. *Food Microbiology* **17**, 383-391.

Sallam, I. K. (2007). Antimicrobial and antioxidant effects of sodium acetate, sodium lactate, and sodium citrate in refrigerated sliced salmon. *Food control* **18**, 566-575.

Schlech, F. W. (1996). Overview of listeriosis. *Food Control* **7**, 181-186.

Schlegel, G. H. (1992a). *The grouping of prokaryotes*. In: General Microbiology, 7th ed, The University of Cambridge.UK, pp. 89-135

Schlegel, G. H. (1992b). *Growth of microorganisms*, In: General Microbiology, 7th ed, The University of Cambridge.UK, pp.193-231

Schlegel, H. G. (1992c). *Special fermentations*. In: General Microbiology, 7th ed, The University of Cambridge.UK, pp. 290-331.

Schmid, M. W., Ng, E. Y. W., Lampidis, R., Emmerth, M., Walcher, M., Kreft, J., Goebel, W., Wagner, M. and Schleifer, K. H. (2005). Evolutionary history of the genus Listeria and its virulence genes. *Systematic and Applied Microbiology* **28**, 1-18.

Smelt, J. P. P. M., Otten, G. D. and Bos, A. P. (2001). Modelling the effect of sublethal injury on the distribution of the lag times of the individual cells of *Lactobacillus plantareum. International Journal of Food Microbiology* **73**, 207-212.

Smelt, J.P.P.M. and Brul, S. (2007). Modelling lag-time in predictive microbiology with special reference to lag phase of bacterial spores. In: Brul, S., Van Gerwen, S. and Zwietering, M. (eds). Modelling microorganisms in food, CRC press, Cambridge, England, pp. 67-81.

Starliper, C. E., Stotts, E. B. and Brown, J. (1992). Isolation of *Carnobacterium Pisicola* and an unidentified gram-positive bacillus from sexually mature and post-spawning rainbow-trout *Oncorhynchus mykiss. Diseases of aquatic organisms* **13**, 181-187.

Stekelenburg, F. K. and Kant-Muermans, M. L. T. (2001). Effects of sodium lactate and other additives in a cooked ham products on sensory quality and development of a strain of *L. curvatus* and *L. monocytogenes*. *International Journal of Food Microbiology* **66**, 197-203.

Sunen, E., Aristimuno, C. and Galian, F. B. (2003). Activity of smoke wood condensated against Aeromonas hydrophila ans *L.monocytogenes* in vacuum-packed ,cold -smoked rainbow trout stored at 4 °C. *Food reseasch international* **36**, 11-116.

Swaminathan, B. (2001). *Listeria monocytogenes.* In: Doyle, M.P., Beuchat, L.R. and Montville,T.J. (eds). Food Microbiology: Fundamentals and Frontiers, 2nd ed, ASM Press, Washington, pp. 383-409.

Takle, G. W., Toth, I. K. and Brurberg, M. B. (2007). Evaluation of reference genes for real-time RT-PCR expression studies in the plant pathogen *Pectobacterium atrosepticum*. *BMC Plant Biology* **7:50**.

Thevenot, D., Dernburg, A. and Vernozy-Rozand, C. (2006). An updated review of Listeria monocytogenes in the pork meat industry and its products. *Journal of Applied Microbiology* **101**, 7-17.

Thurette, J., Membre, J. M., Ching, L. H., Tailliez, R. and Catteau, M. (1998). Behavior of Listeria spp. in smoked fish products affected by liquid smoke, NaCl concentartion and temperature. *Journal of Food Protection* **61(11)**, 1475-1479.

Vandesompele, J., Paepe, A. D. and Speleman, F. (2002a). Elimination of primer-dimer artifacts and genomic coamplification using a two- step SYBR Green I real-time RT-PCR. *Analytical Biochemistry* **303**, 95-98.

Vandesompele, J., Preter, D. K., Pattyn, F., Poppe, B., Roy, V. N., Paepe, D. A. and Speleman, F. (2002b). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3(7)**, research 00341.00341-00034.00311.

Vitt, M. S., Himelbloom, H. B. and Crapo, A. C. (2001). Inhibition of *L. innocua* and *L. monocytogenes* in a laboratory medium and cold-smoked salmon containing liquid smoke. *Journal of Food Safety* **21**, 111-125.

Walker, S. J., Archer, P. and Banks, J. G. (1990). Growth of *Listeria monocytogenes* at refrigeration temperatures. *Journal of Applied Bacteriology* **68**, 157-162.

Ward, D. R. (2001). Description of the stiutation. *Journal of Food Science* **66**, 1067-1071.

Zhao, W., Li, Y., Gao, P., Sun, Z., Sun, T. and Zhang, H. (2011). Validation of reference genes for real-time quantitative PCR studies in gene expression levels of *Lactobacillius casei* Zhang. *Journal of International Biotechnology* **38**, 1279-1286.

Zhuang, R. Y., Huang, Y. W. and Beuchat, L. R. (1996). Quality changes during refrigerated storage of packaged shrimp and catfish fillets treated with sodium acetate, sodium lactate or propylgallate. *Journal of Food Science* **61**, 241-261.

9 Appendix

9.1 Media

9.1.1 Tryptic Soy Agar with 0.6 % Yeast Extract (TSAYE)

- 20.0 g Tryptic Soy Agar [Oxoid CMO131]
- 3.0 g yeast [Merck 1.03353.0500]
- 500 ml Elix water
- Mix with a magnetic stirrer
- Autoclave at 121 °C for 15 min.
- Pour in 10 cm Petri dishes.
- Store at 4 °C.

9.1.2 Tryptic Soy Broth with 0.6 % Yeast Extract (TSBYE)

- 15g Tryptic Soy Broth [Oxoid CMO129]
- 3g yeast [Merck 1.03353.0500]
- 500 ml distilled water
- Mix with a magnetic stirrer
- Autoclave at 121 °C for 15 min.
- Store at 4 °C.

9.1.3 De Man, Rogosa and Sharpe medium (MRS)

- 51 g MRS Broth (69966)
- 1 litre distilled water
- 1 ml Tween 80 (Sigma P8074)
- Dissolve 51 g MRS in 1 litre distilled water
- Boil to dissolve the medium completely.
- Autoclave at 121 ºC for 15 minutes.
- Store at 4 °C.

9.1.4 TE-buffer (30 mM Tris-Cl, 1 mM EDTA) containing 15 mg/ml lysosome.

- 1.35 ml Tris
- 0.5 ml Ethylenediaminetetraacetic acid
- 70 ml distilled water
- 1,35 ml Tris into 45 ml distilled water.
- Adjust pH to 8.0.
- Add 0.5 ml EDTA.
- Add distilled water to 50 ml.
- Filter
- Add lysosome.

50x TAE (Tris-acetate-EDTA) buffer composition

50x TAE buffer	g/ l
Tris-Base	242 g
Acetic acid , pH 8.3	57.1 ml
EDTA	100 ml 0.5 M (pH 8.0)
H_2O	1000 ml

9.2 Stock solutions of K-L and liquid smoke in TSBYE

9.2.1 Preparation of 6% K-L

- 41.7 g lactate solution
- 458.3 ml TSBYE
- Total volume-500 ml

9.2.2 Preparation of 3 %NaL

- 20.9 g lactate solution
- 479.1 ml TSBYE

- Total volume-500 ml
- Fill into 500 ml line of the flask by TSBYE.
- Store at 4 °C.

9.2.2.1 0.07% Liquid smoke

- 0.35 g liquid smoke (Aro-smoke P-50)
- 499.65 ml TSBYE
- Total volume-500 ml

9.2.2.2 0.14% Liquid smoke

- 0.70 g liquid smoke
- 499.30 ml TSBYE
- Total volume-500 ml

- Fill with TSBYE until 500 ml line of the flask.
- Store at 4 °C.

9.2.2.3 0.07% liquid smoke-3 % K-L

- 0.35 g liquid smoke
- 20.9 g Lactate solution
- TSBYE fill up to 500 ml.

9.2.2.4 0.14% liquid smoke -3 % K-L

- 0.70 g liquid smoke
- 20.9 g Lactate solution
- TSBYE until fill up 500 ml scale of the flask.

9.2.2.5 0.07% liquid smoke- 6 % K-L

- 0.35 g liquid smoke
- 41.7 g Lactate solution
- TSBYE until fill up 500 ml scale of the flask.

9.2.2.6 0.14% liquid smoke -6 % K-L

- 0.70 g liquid smoke
- 41.7 g Lactate solution
- TSBYE until fill up 500 ml scale of the flask.
- Store at 4 °C.

9.2.3 Preparation of 6% NaCl + 0.42% K-A

- 30 g NaCl
- 2.085 g K-A
- TSBYE until fill up 500 ml scale of the flask.
- Store at 4 °C.

9.2.4 Preparation of 3% NaCl + 0.21% K-A

- 15 g NaCl
- 1.04 g K-A
- TSBYE until fill up 500 ml scale of the flask.
- Store at 4 °C.

9.2.5 Preparation of 6% NaCl

- 30 g NaCl
- TSBYE until fill up 500 ml scale of the flask.
- Store at 4 °C.

9.2.6 Preparation of 3% NaCl

- 15 g NaCl
- TSBYE until fill up 500 ml scale of the flask.
- Store at 4 °C.

9.2.7 Preparation of 0.42% K-A

- 2.085 g K-A
- TSBYE until fill up 500 ml scale of the flask.

- Store at 4 °C.

9.2.8 Preparation of 0.21% K-A

- 1.04 g K-A
- TSBYE until fill up 500 ml scale of the flask.
- Store at 4 °C.

9.2.9 Liquid smoke

Liquid smoke (Aro-smoke P-50), produced by Red arrow (Manitowoc, WI) was used as a bacteriostatic agent. The chemical properties of liquid smoke are shown in the table below.

TABLE 9.1 Components for liquid smoke Aro-smoke P-50

Parameter	**Aro-smoke P-50**
pH	**4.0-5.0**
pH(10% aqueous solution)	3.0-4.0
Total acidity (as acetic acid)	4.0%max.
Smoke flavor compounds	37.0-42.0 mg/g
Density (ave.)	1.08 kg/liter=9.0Ib./gal.

Adapted from Red arrow specification sheets (1998)

9.2.10 PURAC solution:

PURAC solution (PURASAL Opti. Form PPA PLUS) was used as preservatives. Two different salts (Potassium lactate and Potassium acetate) were present in the PURAC solution.

TABLE 9.2 Components of Purac solution

Test	Units	Specifications	Result
Potassium lactate	%	71.3-74.3	72.7
Potassium acetate	%	4.9-5.5	5.0
Colour fresh	APHA	<=50	16
Sodium	%	<=0.2	-0.1
pH(direct)		8.5-9.5	9.2
pH (12.8 g product+ 87.2g water)		6-8	7

Adapted from PURAC sheet (2008)

9.3 DNA molecular weight marker XIII (Roche).