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Master Thesis

POLYPHASIC SCREENING OF THE MICROBIAL QUALITY OF COMMERCIAL SHRIMP PRODUCTS FROM DIFFERENT FARMING REGIONS

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Abstract

The consumption of shrimps and related products has increased in the last 20 years. This development has led to a worldwide production of shrimp in aquacultures to a quantity of about 3.4 million t in 2008 (FAO, 2010). However, the high consumption level of crustaceans is associated with environmental problems and therefore food safety issues regarding microbial quality should not be neglected. Trade in aquaculture commodities carries an inherent risk of moving and spreading aquatic animal pathogens and this is reflected by several notifications of import alerts. A high range of broad-spectrum antibiotics are in use in shrimp farms and therefore contribute to some potential risk of increased resistance properties of food pathogens.

In this study the relevant microbial quality parameters of different shrimp products sold in Austria were examined. A polyphasic screening through autochthonous bacteria families (e.g., *Enterobacteriaceae, Pseudomonadaceae, Bacillaceae, Clostridia*) and the detection and characterisation of food-related pathogens (e.g. *Listeria*) were performed.

Identified strains of tested pathogens were subjected to antimicrobial disk susceptibility tests (NCCLS documents M2 – A7, Performance Standards for Antimicrobial disk Susceptibility Tests), in order to analyse potential resistance caused through antibiotic usage.

The study on one hand revealed important results on the overall microbial quality and diversity in commercially available shrimps. On the other hand, the outcomes from antimicrobial susceptibility testings were discussed in relation to those from other seafood studies.

Zusammenfassung

Der Konsum von Fisch und Meeresfrüchten in der EU und in Österreich stieg seit den 1990 iger Jahren stetig von Jahr zu Jahr an. Dies zeigten die Importmengen von Shrimp und Shrimpprodukten und der Umsatzsteigerung des Verkaufs von Fisch und Meeresfrüchten. Darüberhinaus sind Daten verfügbar, welche einen Anstieg der weltweiten Shrimpproduktion in Aquakulturen auf ca. 3,4 Millionen Tonnen im Jahr 2008 (FAO, 2010) angeben. Somit lässt sich aus den Zahlen der Produktion und des Imports zurückschließen, dass der Konsum von Shrimp und Shrimpprodukten in den letzten zwei Jahrzehnten sehr stark angewachsen ist.

Dieser weltweite Handel mit Fisch und Meeresfrüchten vor allem aus Aquakulturen und deren erhöhter Konsum stellt somit eine inhärente mikrobielle Gefahr dar für den Konsumenten, sowohl, als auch für die Lebensmittelproduktion im Bereich der Fisch- und Meeresfrüchte-Verarbeitung dar.

Durch den weltweiten Handel kann es zur Übertragung div. pathogener Bakterien von verschiedensten Habitaten auf die Umwelt und vor allem auf die Lebensmittelverarbeitende Industrie in Österreich bzw. der Europäischen Union kommen. Gefahr besteht insofern, dar von sämtlichen Erdteilen Produkte importiert werden welche nicht unbedingt den europäischen Anforderungen in hygienischer Produktion und mikrobiellen Status entsprechen könnten. Somit könnten verschiedene extrinsische Bakterienarten Einfluss auf die intrinsische mikrobielle Flora haben und das mikrobiologische Wachstum von in Europa hergestellten Produkten erheblich beeinflussen. Darüberhinaus besteht ebenfalls die Gefahr das Fisch- und Seetierpathogene in heimische Gewässer und Aquakulturen übertragen werden könnten, welche nicht nur wirtschaftlichen Schaden verursachen und auch die menschliche Gesundheit beeinträchtigen kann.

In dieser Masterarbeit war das Ziel den mikrobiellen Status diverser Shrimpprodukte welche am österreichischen Markt verkauft werden festzustellen. Es wurde ein Screening verschiedenster autochthoner Bakteriengattungen durchgeführt (z.B. *Enterobacteriaceae, Pseudomonadaceae, Bacillaceae, Staphylococcaceae, Clostridia* usw.) und darüberhinaus die Detektierung der Gattung *Listeria* vorgenommen. Da bei Aquakulturen verschiedenste antimikrobielle Substanzen Verwendung finden und somit die Entstehung von Resistenzen gegenüber diesen Antibiotika gegeben ist wurden weiteres isolierte *Listeria* Spezies auf Antibiotika-Resistenzen untersucht. Die Untersuchung der Antibiotikaresistenzen wurde mittels einer Disk Diffusion Methode folgend der Durchführungsmethoden der Clinical and Laboratory Standard Institute (vormals NCCLS) Dokumenten M2 – A7, Performance Standards for Antimicrobial Disk Susceptibility Tests durchgeführt.

Die Ergebnisse des allgemeinen mikrobiellen Screenings machen die mikrobiellen Qualität der Shrimpprodukte in deren verschieden Conveniencegraden deutlich und die Ergebnisse der Antibiotikaresistenz-Tests zeigen ob sich unter anderem durch den Einsatz von Antibiotika, Resistenzen entwickelt haben. Diese Ergebnisse werden mit anderen Ergebnissen diverser Studien verglichen und diskutiert.

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Abbreviations

%	percentage / percent
°C	degree Celsius
µL	microlitre
acc.	according
APC	aerobic plate count
a_w	water activity
BHI	brain heart infusion
cfu	colony forming units
E.	*Escherichia*
EC	European commission
EU	European union
FAO	Food and Agriculture Organization of the United Nations
g	gram
GHP	good hygiene practice
GMP	good manufacturing practice
h	hour
ha	hectare
HACCP	hazard analysis critical control points
IQF	individually quick frozen
kg	kilogram
L	litre
L.	*Listeria*
MC	microbiological criteria
mg	milligram
min	minute
mL	millilitre
mm	millimetre
MYP	Mannitol egg yolk polymyxin agar
OCLA	Oxoid chromogenic Listeria agar
OF	oxidation - fermentation
PCA	plate count agar

PEMBA	Polymyxin pyruvate egg yolk mannitol bromthymol blue agar
PL	postlarvae
PSP	paralytic shellfish poisoning
RTE	ready-to-eat
sp.	species
spp.	species in the plural
SSO	specific spoilage organisms
TSA	tryptic soy agar
TVBN	total volatile basic nitrogen
TVC	total viable count
VRBD	violet red bile dextrose agar
YGC	yeast extract glucose chloramphenicol agar
yr	year

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1 Introduction

1.1 Aquaculture – general description

1.1.1 Definition of aquaculture

Many different definitions are used to declare the term “aquaculture”. In the World Wide Web different simple or explicit definition are available. Aquaculture is defined on the basis of the American Heritage Dictionary as “The science, art, and business of cultivating marine or freshwater food fish or shellfish, such as oysters, clams, salmon, and trout, under controlled conditions” and in both senses also called “aquafarming” (ANONYMUS a, 2011). The Food and Agriculture Organization of the United Nations (FAO) defines aquaculture as “the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants (CHOUDHURY and JANSEN, 1999. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding and protection from predators. Farming also implies individual or corporate ownership of the stock being cultivated (CHOUDHURY and JANSEN, 1999).

Aquaculture requires access to appropriate areas of land and water. In freshwater resources these are either privately owned or in some cases are nationalised. In marine areas these are commonly held by the state and users require an appropriate lease or other legal interest to allow them to use the required area. Increasingly, the environmental fragility of coastal zones is making it necessary for the proposed use to be considered in detail from land use planning and environmental impact perspectives (ANONYMUS b, 2011).

A definition described by Stickney, R. is: *Aquaculture is the rearing of aquatic organisms under controlled or semi-controlled conditions* (STICKNEY, 2005).
This simple definition can be boiled down even more to “simply underwater agriculture”. To break the longer definition of aquaculture into its components, the term “aquatic” refers to a variety of different water environments, including freshwater, brackish water and marine water. The amount of salt in the water is defined by its salinity. Freshwater is nearly salt free (ANONYMUS c, 2011), while full seawater contains about 3.5% of salt (ANONYMUS c, 2011). Brackish water has salinities intermediate between freshwater and full seawater (ANONYMUS c, 2011). “Aquatic organisms” that are of interest with regard to human food include a wide variety of plants, invertebrates and vertebrates. Mariculture is a term reserved for the culture of organisms in saltwater (from brackish to full strength seawater) (STICKNEY, 2005).

Aquaculture organisms tend to be classified as having a preference for warm, cool and cold water. While not absolute, warm water species tend to grow optimally at or above 25°C (for example catfish and tilapia), while cold water species exhibit optimum growth at temperatures below 20°C (trout, salmon and halibut). Cool water species grow best at temperatures between 20°C and 25°C (walleye, yellow perch). Most commercially cultured species are either of the warm water or coldwater variety, while some fish species popular for sport fishing, as mentioned in the examples, fall into the mid-range group (STICKNEY, 2005).

1.1.2 Importance of aquaculture production

It was estimated many years ago that the world's oceans can produce sufficient amounts of fish and shellfish to allow for about 100 million metric tonnes (a metric tonne is 1000 kg) to be harvested annually (STICKNEY, 2005). In Figure 1 the trend of world capture production of all fish and seafood products between the years 1950 and 2009 is presented. Global capture production increased between the years 1950 with a production of over 17 million tonnes to an amount over 58 million tonnes in 1980 and reached in 1996 a level of nearly 95 million tonnes. In the last decade the production amount levelled off the amount of around 90 million tonnes (FAO a, 2011).

In fact that level of harvest was reached in the 1990s and has not increased since then. As fisheries have declined, new species have been targeted. Squid, popularly sold as calamari today, were not available in many western hemisphere markets until about the 1980s as there was no market for them, though historically, a market has existed, particularly in parts of Asia. Only by finding new target species which is suitable for human consumption, was the annual harvest from the sea maintained (STICKNEY, 2005).

Today, the situation is that capture fisheries are being fully exploited or overharvested in nearly every case, yet the demand for seafood continues to rise. That increase in demand is fuelled in part by the increasing human population but also by rising per capita consumption of seafood. Different studies were published that seem to show certain health benefits from eating fish. One recent recommendation was that everyone should eat at least two fish meals a week. So, demand is increasing, while the supplies of fish are not increasing (STICKNEY, 2005). How could this dilemma be resolved? The answer that has been widely campaigned is aquaculture. The average consumer does not see any reduction in the availability of seafood in restaurants or supermarkets and the overall amount of seafood in the markets of the world continues to increase, despite the fact that capture fishery volumes are not increasing. This is because aquaculture has been able to fill the gap. Currently, at least 20% of the fish and shellfish marketed globally are produced by aquaculturists and the percentage can only be expected to grow (STICKNEY, 2005).

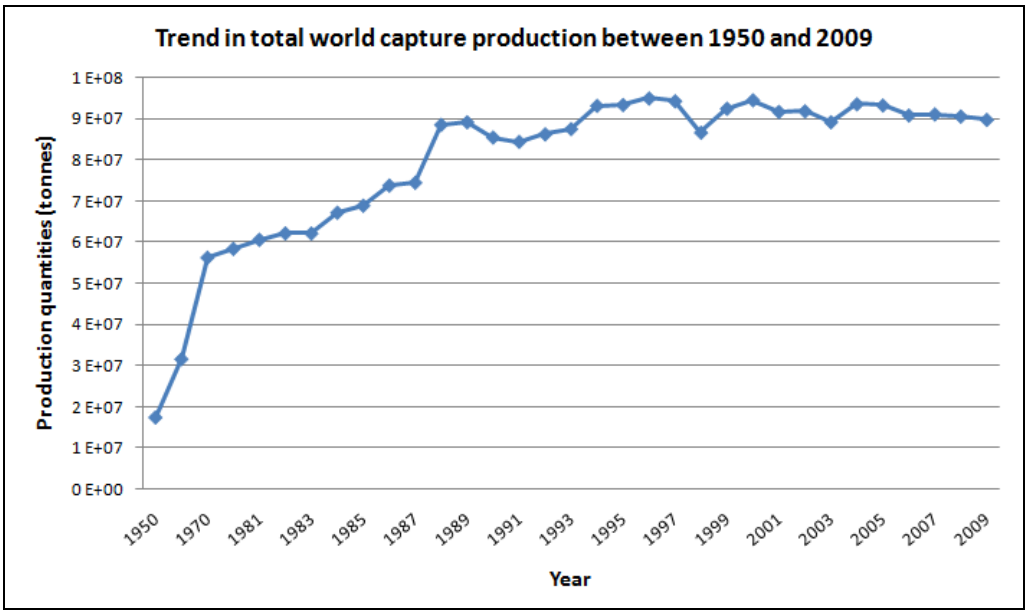


Figure 1: Trend in total world capture production between 1950 and 2004 (FAO a, 2011)

1.1.3 Forms of aquaculture

Integrated multitrophic aquaculture

This is the incorporation of species from different trophic/nutrional levels in the same system. Solid and soluble nutrients from fed organisms and their feed are converted into harvestable crops and/or extractive organisms and through the increase of economic diversification, integrated multitrophic aquaculture promote economic and environmental sustainability (FAO, 2009). The waste of one species becomes the nutritional input of another and this potential of a contamination is a concern of food safety and quality. This new practice needs research to guarantee produced fish without danger to consumers (FAO, 2009).

Organic aquaculture

This form also attracted the attention of consumers, environmental advocates and entrepreneurial innovators. The arguments are that it reduces overall exposure of toxic chemicals from pesticides, that can accumulate in the ground, air, water and food supply and hereby a decline of health risks for consumers. Other positive features include curbing topsoil erosion, improving soil fertility, protecting groundwater and saving energy (FAO, 2009). Organic standards also prohibit the use of genetically modified organisms (GMOs) for production. Meanwhile governments are working on the regulation of organic aquaculture. Standards and certification procedures are developed and tested (FAO, 2009). Syndicates in aquaculture also developed their own specific organic culture standards and accreditation bodies, which often vary significantly to international standards, to different places, certifiers and species (FAO, 2009).

GMOs in aquaculture

Supporters of GMOs claim that they are enhancing the performance and profitability of farmed aquatic resources and, hence, improve food safety. The opposite opinion is that GMOs pose significant risks to the environment and possibly have negative effects to human health (FAO, 2009). Some groups prefer a complete ban of GMOs, others call for mandatory labelling genetically modified food and other products to alert consumers to potential health effects. However, GMO products from aquaculture have not yet appeared on the market (FAO, 2009).

1.1.4 Aquaculture - growth in production

Aquaculture, probably the fastest growing food-producing sector, now accounts for almost 50 percent of the world´s food fish and is perceived as having the greatest potential to meet the growing demand for aquatic food. Given the projected population growth over the next two decades, it is estimated that at least an additional 40 million tonnes of aquatic food will be required by 2030 to maintain the current per capita consumption (FAO, 2006). In Figure 2 the estimated world population and projections to the year 2050 is shown. It shows an average population of 8.9 billion and so it could be expected that the food production has to increase so that the food supply is achieved.

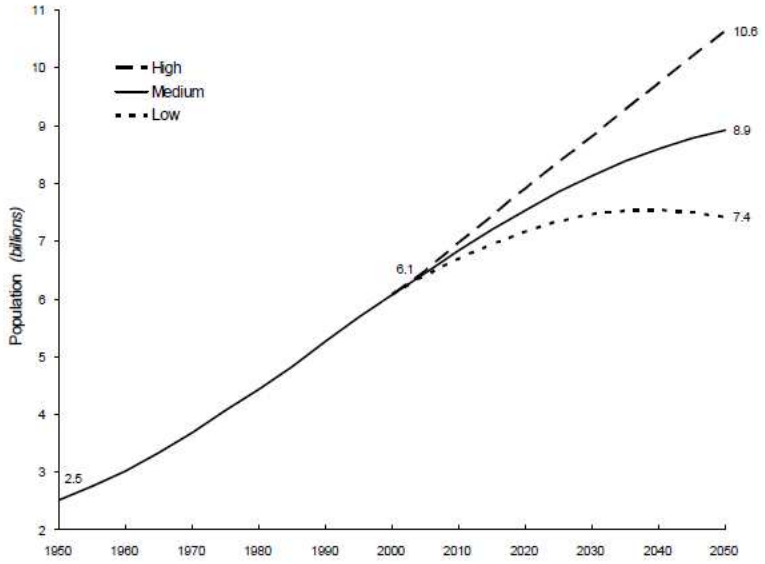


Figure 2: Estimated world population, 1950-2000, and projections: 2000-2050 (UNO, 2004)

From an activity that was primarily Asian, aquaculture has now spread to all the continents. From an activity that was focused on freshwater fish, particularly the cyprinids (finfish of the family Cyprinidae which include common carp, Chinese carps and Indian major carps (ANONYMUS c, 2011)), it now encompasses all aquatic environments and many aquatic species. Clearly, its Asian origin and its carp-focused beginnings are still evident in the present distribution and the dominance of cyprinids. The present situation in terms of natural resources, the environment, and population along with advances in biotechnology, marine engineering and in the movement of goods and services, bring with it greater potential as well as more complex challenges in the development of aquaculture (FAO, 2006).

Global aquaculture production has grown dramatically in the last 50 years (Figure 3). It has risen from about five hundred thousand tonnes in the early 1950s to the latest data, which is available from FAO, in the year 2008, to 68.3 million tonnes (Figure 3) with a value of US $ 105.9 billion, which is shown in Figure 4 (data includes aquatic plants) (FAO b, 2011).

This means that aquaculture is growing and continuous to grow more rapidly than other animal food producing sector. While capture fisheries production stopped growing in around the mid-1980, contrariwise aquaculture has maintained an average growth rate of 8.7 percent worldwide (excluding China, 6.5 percent) since 1970 (FAO, 2009) Annual growth rates in world aquaculture production between 2004 and 2006 were 6.1 percent in volume terms and 11.0 percent in value terms (FAO, 2009). If aquatic plants are included, world aquaculture production in 2006 was 66.7 million tonnes and worth US $ 85.9 billion (FAO, 2009).

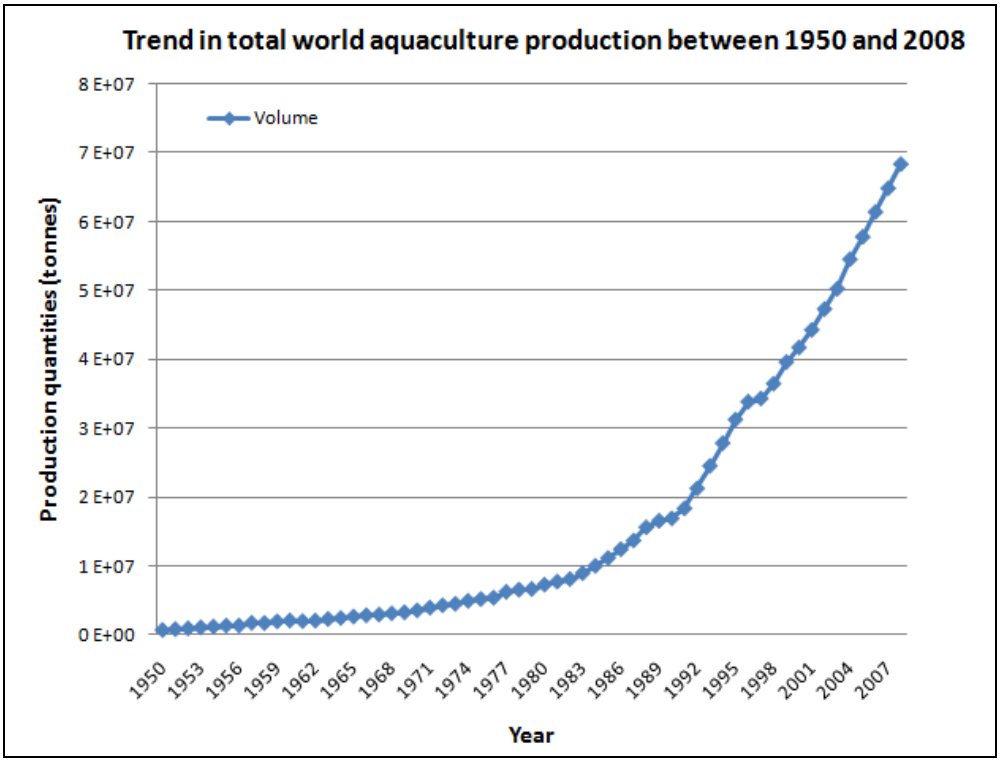


Figure 3: Trend in total aquaculture production between 1958 and 2008 (data including aquatic plants) (FAO b, 2011)

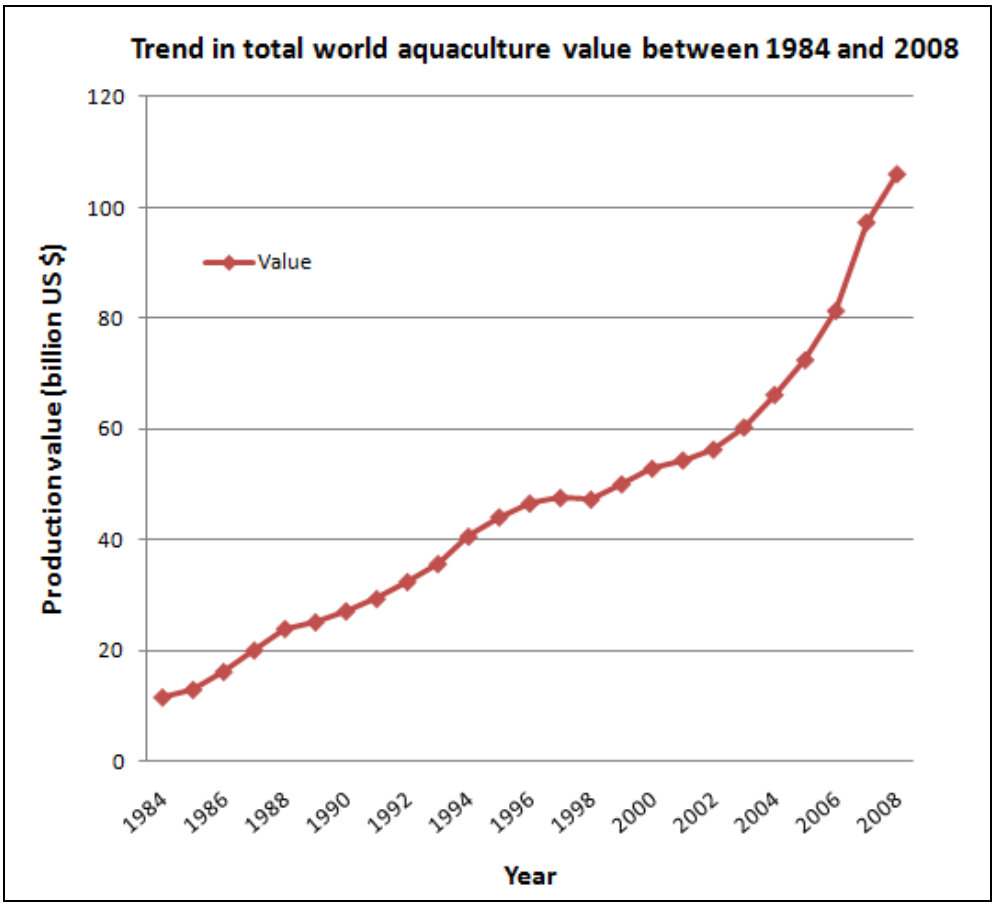


Figure 4: Trend in total world aquaculture value between 1984 and 2008 (data including aquatic plants) (FAO b, 2011)

Figure 5 shows that in the year 2006, countries in the Asian and Pacific regions accounted for 89 percent of aquaculture production by quantity and 77 percent of value. Of the world total, China is reported to produce 67 percent of the total quantity and 49 percent of the total value of aquaculture production (FAO, 2009).

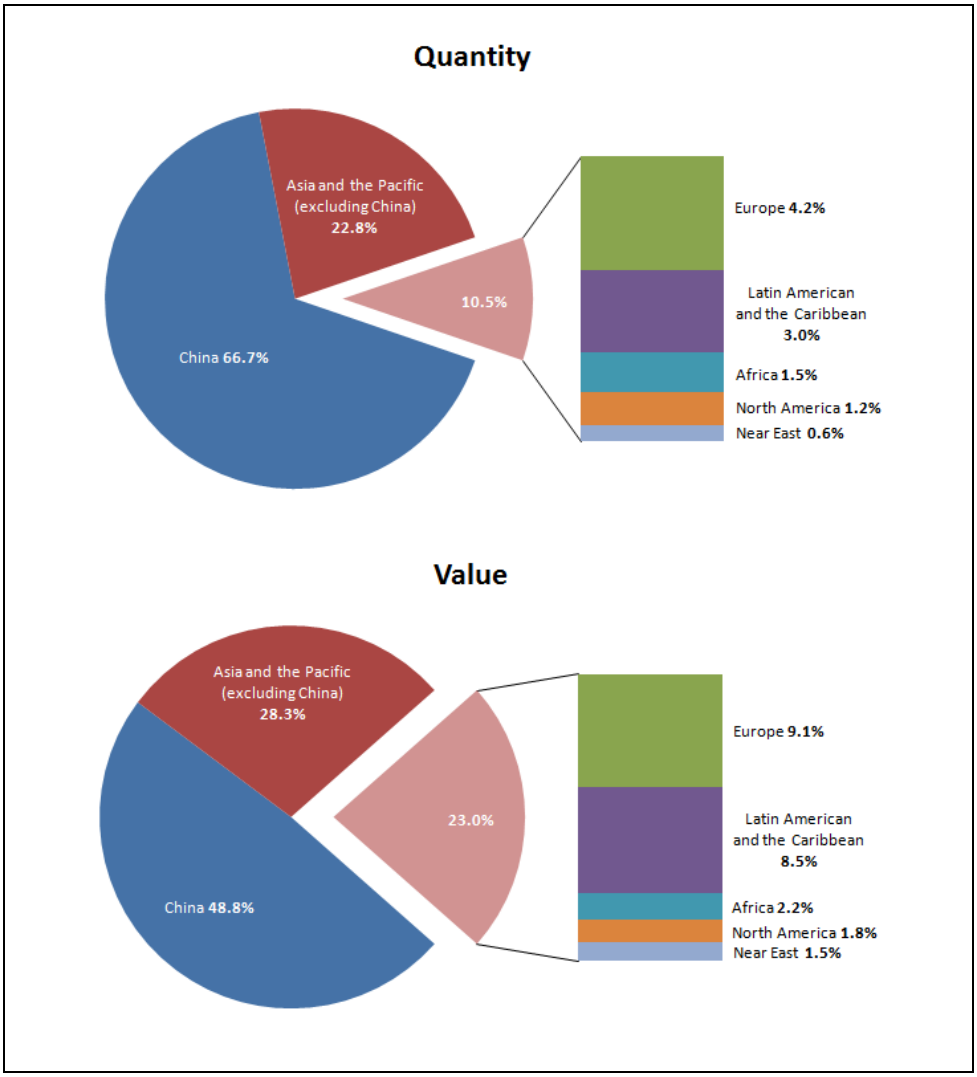


Figure 5: Aquaculture production by region in 2006 (FAO, 2009 p. 18)

An analysis of production by region for the period 1970-2006 shows that growth has not been uniform (FAO, 2009). In Figure 6 the change in growth of the world aquacultural production by region since 1970 is illustrated.

It is recognisable that Latin America and the Caribbean region shows the highest average annual growth (22 percent) followed by the Near East region (20 percent) and the Africa region (12.7 percent) (FAO, 2009). The aquaculture production growth in Europe and North America has slowed substantially to about 1 percent per year since 2000 (FAO, 2009). In France and Japan, countries that used to lead aquaculture development, production has fallen in the last decade. It is apparent that, while aquaculture output will continue to grow, the rate of increase me be moderate in the near future (FAO, 2009).

Furthermore Figure 6 shows that the growth rate of China's aquaculture production increased at an average annual rate of 11.2 percent between 1970 and 2006 (FAO, 2009). China's growth rate has declined to 5.8 percent from 17.3 percent in the 1980s and 14.3 percent in the 1990s (FAO, 2009), so it seems that a maximum limit of a production quantity was reached and the growth rate is now present on a lower level.

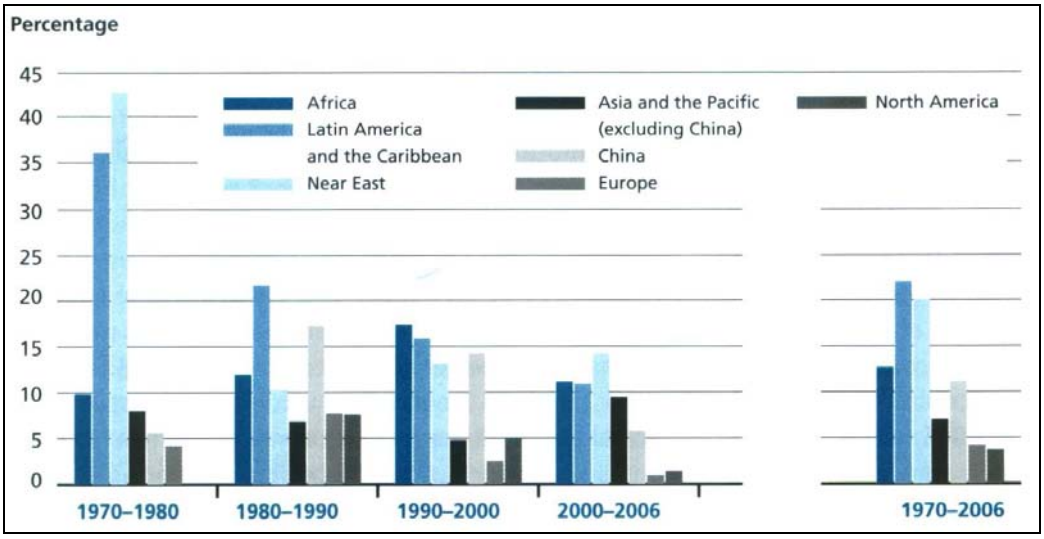


Figure 6: World aquaculture production: change in growth by reagion since 1970 (data exclude aquatic plants) (FAO, 2009 p. 18)

In Table 1 the top ten producing countries for aquatic animals in 2006 and the top ten countries in terms of annual growth in aquacultural production for the period 2004-2006 (included are just countries that reported production of more than 1000 tonnes 2006) are shown.

Table 1: Top ten aquaculture producers of food fish supply: quantity and growth (FAO, 2009 p. 19)

Top ten producers in terms of quantity, 2006				Top ten producers in terms of growth, 2004-06			
	2004	2006	APR		2004	2006	APR
	(Tonnes)		(%)		(Tonnes)		(%)
China	30614968	34429122	6.05	Uganda	5539	32392	141.83
India	2794636	3123135	5.71	Guatemala	4908	16293	82.20
Viet Nam	1198617	1657727	17.60	Mozambique	446	1174	62.24
Thailand	1259983	1385801	4.87	Malawi	733	1500	43.05
Indonesia	1045051	1292899	11.23	Togo	1525	3020	40.72
Bangladesh	914752	892049	-1.25	Nigeria	43950	84578	38.72
Chile	665421	802410	9.81	Cambodia	20675	34200	28.61
Japan	776421	733891	-2.78	Pakistan	76653	121825	26.07
Norway	636802	708780	5.50	Singapore	5406	8573	25.93
Philippines	512220	623369	10.32	Mexico	104354	158642	23.30

Notes: Data exclude aquatic plants.
APR refers to the average annual percentage growth rate for 2004-2006

In Table 1 is presented that China has the highest production by quantity and the annual percentage growth rate is stable too. While countries like Chile or the Philippines have a quite high production growth, countries like Japan and the United States of America have slipped down the list (FAO, 2009).

Most aquaculture production of fish crustaceans and molluscs continues to come from inland waters (61 percent by quantity and 53 percent by value) (FAO, 2009). In 2006 the aquaculture production by aquatic environments represented a production in freshwater environment of 58 percent by quantity (Figure 7) and 48 percent by value (Figure 8) (FAO, 2009). In marine environment 34 percent by quantity (Figure 7) and 36 percent by value (Figure 8) were produced. Brackishwater production represented only 8 percent of production by quantity but it contributed 16 percent of the total value (FAO, 2009).

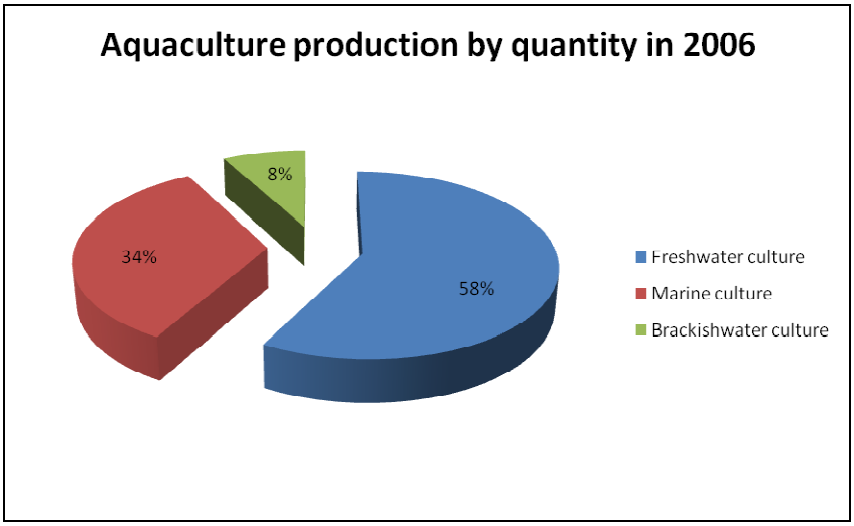


Figure 7: Aquaculture production by environment and quantity in the year 2006 (FAO, 2009)

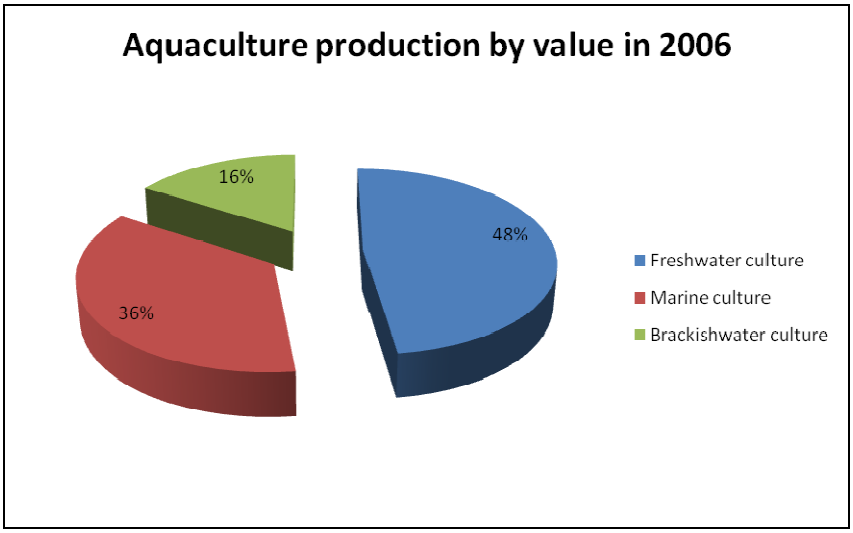


Figure 8: Aquaculture production by environment and value in the year 2006 (FAO, 2009)

In Figure 9 average annual aquaculture production growth rate by environment is presented. The production growth of 11.6 percent per year in terms of quantity since 2000 is shown from brackishwater environment, which is the highest growth by quantity (FAO, 2009). Otherwise the growth in terms of value stagnated at 5.9 percent. In the same period the average annual increases of freshwater and marine environments have been 6.5 and 5.4 percent in terms of quantity and 7.8 and 8.3 percent in value terms (FAO, 2009).

Figure 10 and Figure 11 present the global aquaculture production in the year 2006 by quantity and by value. Freshwater finfish was produced in 2006 with an amount of 54 percent (FAO, 2009). Second largest produced group were molluscs in the same year (Figure 10). The production amounts of crustaceans were 4.5 million tonnes, which is compared to the production of freshwater finfish (27.8 million tonnes, worth US$ 29.5 billion) and molluscs (14.1 million tonnes, worth US$ 11.9 billion) a much smaller amount of production but worth significantly more: US$ 17.95 billion (FAO, 2009). The production growth of the major species groups continues, but the increases of the production have been smaller than those in the 1980s and 1990s (FAO, 2009).

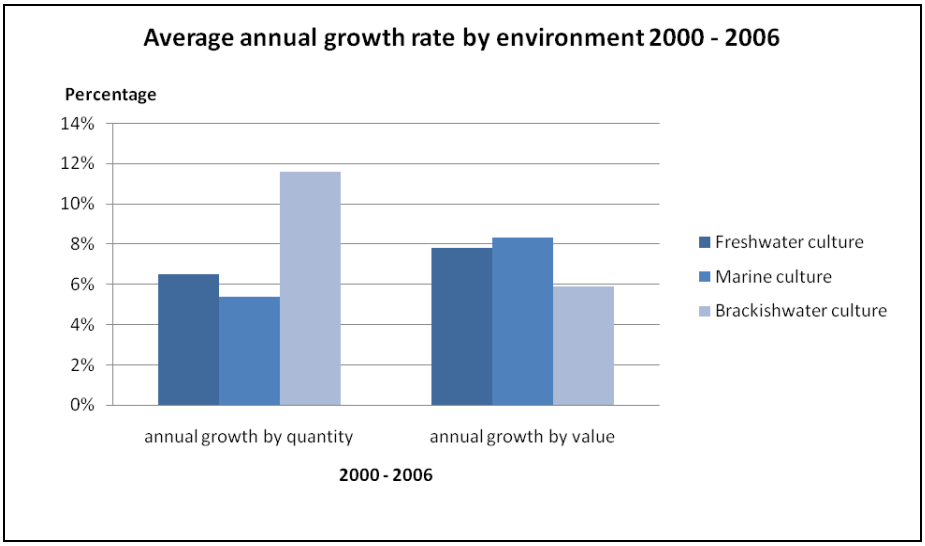


Figure 9: Average annual growth rate by environment in the years 2000 to 2006 (FAO, 2009)

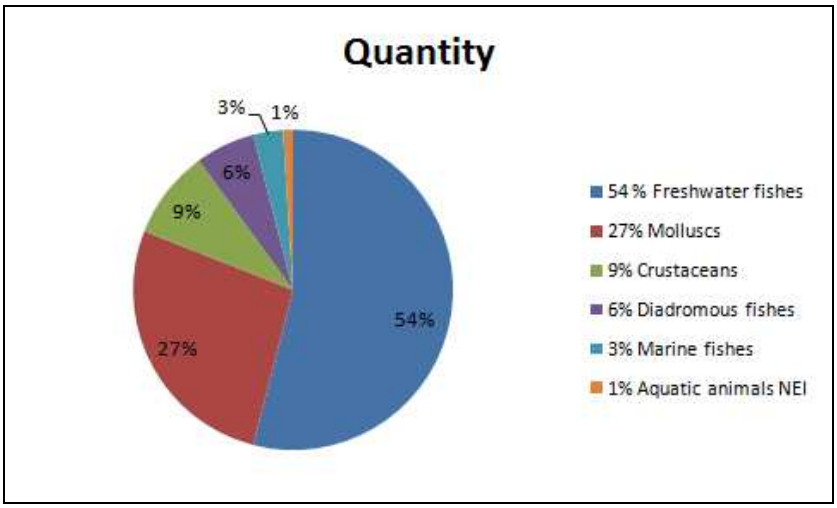


Figure 10: World aquaculture production by quantity: major species groups in the year 2006 (NEI – not elsewhere included) (FAO, 2009 p. 20)

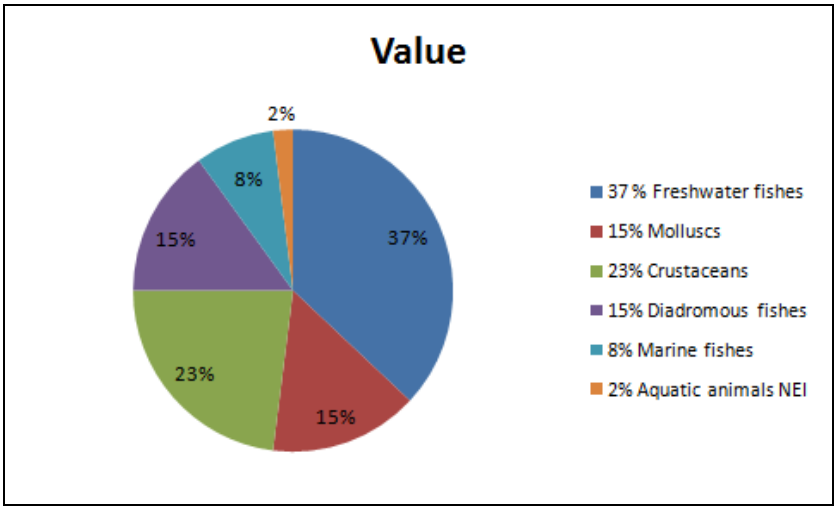


Figure 11: World aquaculture production by value: major species groups in the year 2006 (NEI – not elsewhere included) (FAO, 2009 p. 20)

The trend of world aquaculture production of the different major species groups is presented in Figure 12. In the period of 2000 to 2006 a strong growth in the production of crustaceans in particular, and in marine fish was visible (FAO, 2009).

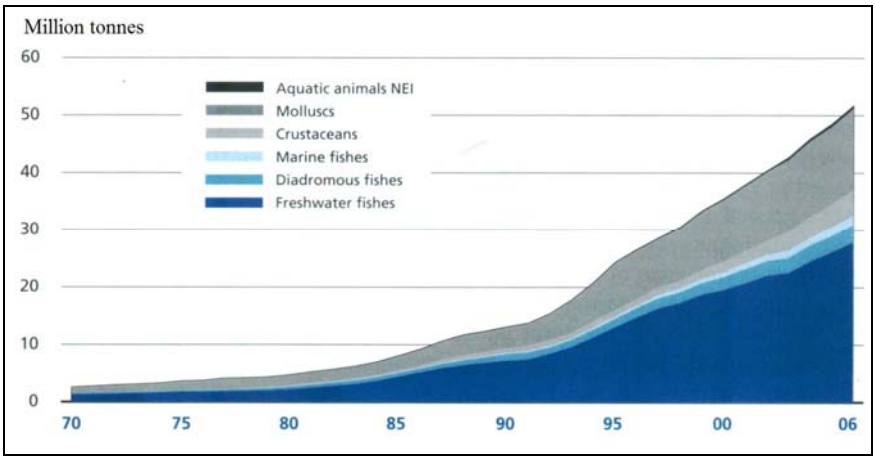


Figure 12: Trends in world aquaculture production: major species groups (NEI – not elsewhere included) (FAO, 2009 p. 21)

Aquaculture accounted in 2006 a global production of freshwater finfish of 76 percent and 65 percent of mollusc and diadromous (fish, which migrate freely between the sea and freshwater (ANONYMUS c, 2011)) fish production (FAO, 2009). The contribution to the world's supplies of crustaceans has grown rapidly in the last decade reaching 46 percent of world production in 2008 (FAO b, 2011). In the year 2006 aquaculture production accounted for as much as 70 percent of shrimps and prawns (penaeids) produced worldwide (FAO, 2009). Most marine species which are farmed in aquaculture have a relatively high commercial value, sometimes because wild stocks are small or declining (FAO, 2009). While the overall share of farmed fish in marine finfish production has stayed quite low, for species that are farmed, aquaculture frequently dominates the market (FAO, 2009). For species such as the Japanese seabass, gilthead seabream, red drum and bastard halibut, the amounts now produced by aquaculture are often substantially higher than the past highest catch recorded by capture fisheries (FAO, 2009).

1.1.5 Aquaculture production in different regions

The aquaculture production continues changing and so more differentiations between the regions are the result. In the Asian and Pacific region, in China, South Asia and most of Southeast Asia aquaculture mainly produces cyprinids, while in the rest of East Asia the production consists high-value marine fish (FAO, 2009). In the last decade in Latin America and the Caribbean, the production of salmonids has overtaken shrimp, as the top aquaculture species group as a result of outbreaks of disease in shrimp-producing areas and also the rapid salmon production in Chile (FAO, 2009).

In the sub-Saharan Area (SSA), Nigeria leads in the region with a production of 85 thousand tonnes of catfish, tilapia and other freshwater fishes and in Madagascar the farming of black tiger shrimp (*Penaeus monodon*) has started (FAO, 2009). Globally, still just a few countries are dominating the production of major species, e.g. China, which produces 77 percent of all carp (cyprinids) (FAO, 2009). All in all, the Asian Pacific region has a production of 98 percent of the carp and 95 percent of the oyster supply in the world (FAO, 2009).

Eighty-eight percent of shrimps and prawns (penaeids) are also produced in this region, with the five producers (China, Thailand, Viet Nam, Indonesia and India) accounting for 81 percent (FAO, 2009). In the year 2008 the production of shrimp and prawns in the Asian Pacific region accounted 85.5 percent and the top five producers accounted 78% (FAO c, 2011).

1.1.6 Shrimp and prawns of the world

The animal shrimp belongs to the invertebrates (*Invertebrata*), to the phylum *Arthropoda*. Shrimp belong within the Arthropods to the Crustacea (crustaceans) class and in this class in the order *Decapoda* (ten legs) (LEE & WICKINS, 2000). The Decapods order is divided into the suborders *Reptantia* (walking decapods) and *Natantia* (swimming decapods). Decapods are characterised by having five pairs of walking legs (10 legs in total), the first often bearing substantial chelae (claw, pincer) as in the case of clawed lobsters and crayfish (LEE & WICKINS, 2000).

Marine and brackish water Penaeidae are called shrimp in the USA, prawns in Australia, India and South Africa while either term may be used in Japan and Taiwan. The names “shrimp” and “prawns” have originated in Great Britain. The FAO convention is to call marine and brackish water forms, shrimp, and freshwater forms, prawns (ANONYMUS c, 2011). In North America the name “prawn” is obsolete and almost entirely replaced by the word “shrimp”, even the species of Palaemonidae, like *Palaemonetes* (“grass shrimps”) and *Macrobrachium* (“river shrimps”), are usually indicated as shrimps. “Freshwater Invertebrates of the United States” are indicated as “freshwater prawns”, in England the word “prawn” denotes the larger *Natantia*. Summarizing in Britain and North America, shrimp is the more general term and the usage of the term “prawn” is almost the direct opposite in the two regions, in Britain the larger palaemon like animals, in America the smaller ones (HOLTHUIS, 1980). In South Africa, Australia and New Zealand the terms are similar to the British form and in french the term “crevette” is generally used for shrimps and prawns and unfortunately causes no problems. In Spanish the general term for both names is “camaron”, the word “gamba” is less generally used (HOLTHUIS, 1980).

1.2 Aquaculture production and further processing of shrimp and prawns

In this chapter the production processes of the main examined shrimp species are described. Most common shrimp/prawn species, which are available on the Austrian market, are *Penaeus monodon*, *Penaeus vannamei* and the freshwater prawn *Macrobrachium rosenbergii*.

1.2.1 Aquaculture production of penaeid shrimp

There are several species of interest for the aquaculture production of the penaeid shrimp family. Giant tiger (*Penaeus monodon*), whiteleg (*P. vannamei*), green tiger (*P. semisulcatus*), greasyback (*Metapenaeus ensis*) are some important species of this family used in aquaculture production. These species are cultivated in marine and brackish water. The techniques for culturing vary greatly in their complexity in the different production phases (LEE & WICKINS, 2000).

Broodstock, spawning and hatching

Preferably wild caught broodstock (sexually mature specimens of both sexes kept for the purpose of controlled reproduction (ANONYMUS c, 2011)) is used. *P. monodon* broodstock from greater depths (60-80 m), or more than 20 miles offshore are preferred because of lower prevalence of shrimp diseases, which are higher in coastal shrimp farming areas. P. vannamei broodstock is used from three sources: sea-caught and spawned; cultured shrimp harvested from ponds: tank-reared specific pathogen free (SPF) or specific pathogen resistant (SPR) broodstock (FAO d, 2011; FAO e, 2011). Broodstock is kept for several months in maturation tanks (Figure 13) and spawning nauplii (larvae) are kept in rearing tanks afterwards (Figure 13). Important for the hatchery and nursing processing steps is to keep the environment staple by water exchange and a staple water temperature (FAO d, 2011; FAO e, 2011).

On-growing techniques

After the hatchery the postlarvae (PL) with an age of 15 days are transferred to grow out ponds (Figure 13) (FAO d, 2011; FAO e, 2011). On-growing techniques can be divided into four main categories: extensive, semi-intensive, intensive and super-intensive, which represent low, medium, high and extremely high stocking densities respectively. *P. monodon* is commercially cultured only in earthen ponds under varying salinities from 2 to 30 per mill (FAO d, 2011; FAO e, 2011).

Extensive grow-out of *P. monodon* is commonly found in Bangladesh, India, Indonesia, Myanmar, the Philippines and Viet Nam and for P. *vannamei* in Latin America. The stocking capacity is 2/m^2 for *P. monodon* and 4-10/m^2 for *P. vannamei*. The shrimp feed is mainly based on natural foods that enter the pond regularly on the tide and are subsequently enhanced by organic or chemical fertilizers. Harvesting of *P. monodon* (> 50 g) is done within six months or more (Figure 13). *P. vannamei* (11-12 g) are harvested in 4-5 months (FAO d, 2011; FAO e, 2011).

Semi-intensive ponds (Figure 13) are stocked with 5-20 PL/m^2 (*P. monodon*) or 10-30 PL/m^2. The shrimp feed is natural food enhanced by fertilizers and artificial diets are supplemented. The production amounts range from 400 to 4000 kg/ha/yr of *P. monodon* and 500-2000 kg/ha/crop with 2 crops per year for *P. vannamei* (FAO d, 2011; FAO e, 2011).

Intensive farming systems are common in Asia and Latin America. Stocking densities are 20-60 PL/m^2 (*P. monodon*) and 60-300 PL/m^2 (*P. vannamei*). Important for intensive growing is a heavy aeration to guarantee water circulation and oxygen supply. Feeding with artificial diets is carried out 4-5 times per day. The production yields of 4000-15000 kg/ha/yr of *P. monodon* and 7-20000 kg/ha/crop with 2-3 crops per year of *P. vannamei* (FAO d, 2011; FAO e, 2011). Research on super-intensive production of *P. vannamei* in super-intensive raceway systems enclosed in greenhouses, using no water exchange or discharge, is conducted in the USA. Stocking 282m^2 raceways with 300-450 0.5-2 g juveniles/m^2 has realized productions of 28000-68000 kg/ha/crop (FAO d, 2011; FAO e, 2011).

Feed supply

Each major shrimp producing country has developed its own commercial feed factories instead of importing expensive feed (FAO d, 2011; FAO e, 2011). All juvenile and adult farmed crustaceans are able to browse on organic matter and microorganisms, they scavenge non-living material of the water and they become active predators during the on-growing phase (LEE & WICKINS, 2000). Growing intensity of the shrimp production, demands increased needs to compounded feeds where the composition achieves the nutritional demands of the shrimp species, so that rapid and economical growth is conformed (LEE & WICKINS, 2000). In super-intensive systems artificial formulated diets are fed and in intensive occasional these diets are adjusted with fresh fish or mollusc flesh (LEE & WICKINS, 2000). Artificial feeds should not present any health related difficulties. Therefore high quality products should be chosen and the storage conditions have to be cool and dry. Artificial feeds include dried algae, liquid feeds, microencapsulated feeds flakes and crumbled pellets, and mineral and vitamin supplements and enrichments (FAO, 2007). Feeding also depends on the quantity and frequency of live feed offered. General guidelines for artificial feed are available from the FAO (FAO, 2007).

Harvesting techniques

Extensive and semi-intensive ponds are harvested by draining the pond at low tide or by pumping the water out of the pond. In semi -intensive systems harvesting machines pump shrimp and water up to the pond bank where they are dewatered (FAO d, 2011; FAO e, 2011). Intensive systems may also be harvested by using small seine nets, which are dragged around the pond to corral the shrimp to one side, where they are collected by cast or dip net or buckets (FAO d, 2011; FAO e, 2011). In super-intensive systems shrimp are harvested with large scoop nets (FAO d, 2011; FAO e, 2011).

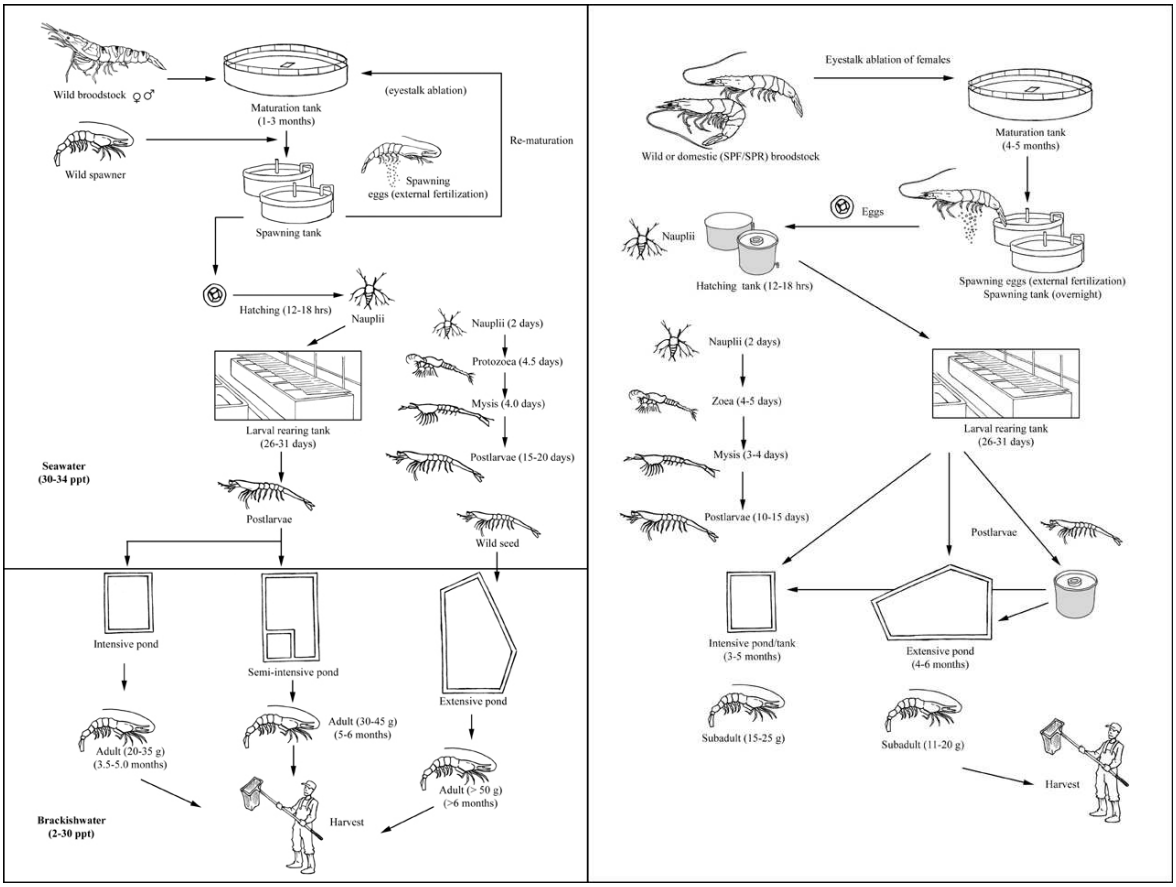


Figure 13: Production cycle of *Penaeus monodon* (FAO d, 2011) and *Penaeus vannamei* (FAO e, 2011)

Handling and processing

Shrimp which are directly sold to processing plants require special teams for harvesting and handling, to ensure a high quality of the raw material. Shrimp are roughly sorted, washed, weighted and immediately killed in iced water at 0-4°C. Careful cleaning of the shrimp bodies from mud and organic matter is very important. After these steps the shrimps are kept in ice insulated containers and transported by small pick-up trucks for short distances or by large insulated trucks over long distances (FAO d, 2011; FAO e, 2011).

1.2.2 Production of freshwater prawns

Species of freshwater prawns used for aquaculture production are giant river prawn (*Macrobrachium rosenbergii*); monsoon river prawn (*M. malcom-sonii*); African river prawn (*M. vollenhovenii*); cinnamon river prawn (*M. Acanthurus*). The species which dominates the aquaculture production is *M. rosenbergii* (LEE & WICKINS, 2000).

Summarized the production of *M. rosenbergii* is quite similar to the production of penaid shrimp species except that this animals are cultured with the use of freshwater (FAO f, 2011). On-growing is done in extensive, semi-intensive ponds and additionally intensive systems with a production of >5000 kg/ha/yr exist rarely (FAO f, 2011). Harvesting techniques and further processing steps do not differentiate significantly. Time and frequency of harvesting depends on the volume and characteristics, which is demanded by the market (FAO f, 2011).

1.3 Relevant aquaculture produced shrimp species

In the aquaculture production of shrimp three species developed to the most relevant species for production and shrimp trade in the world. These species are Penaeus monodon, Penaeus vannamei and Macrobrachium rosenbergii. Following basic facts of the species, geographical distribution and production are described. Further information about species which are used for aquaculture production in general is available from the FAO Fisheries and Aquaculture Department (ANONYMUS d, 2011).

Scientific name with original description: *Penaeus monodon* (FAO d, 2011)

FAO name: Giant tiger prawn

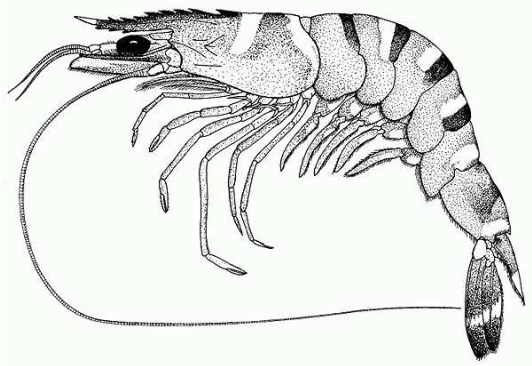


Figure 14: Giant tiger prawn (IMAGE 1, 2011)

Geographical distribution:

Indian Ocean - West Pacific: East and Southeast Africa, Pakistan to Japan, the Malay Archipelago and northern Australia (FAO d, 2011).

Global aquaculture production:

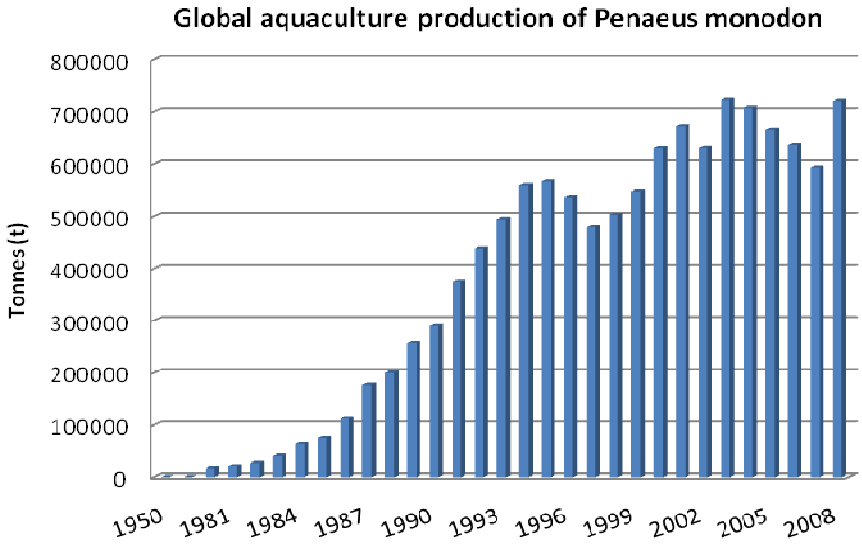


Figure 15: Global aquaculture production of *Penaeus monodon* (FAO b, 2011)

Scientific name with original description: *Penaeus vannamei* (FAO e, 2011)

FAO name: Whiteleg shrimp

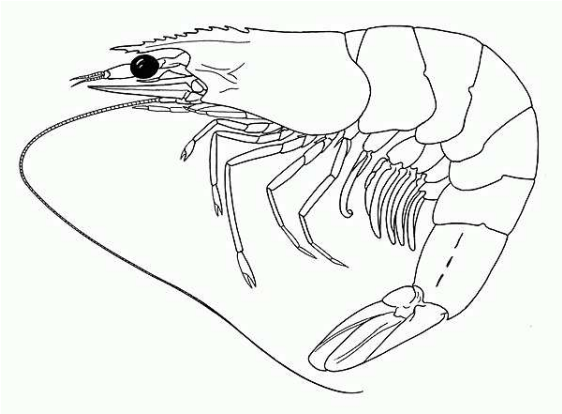


Figure 16: Whiteleg shrimp (IMAGE 2, 2011)

Geographical distribution:

Eastern Pacific: from Sonora, Mexico, south to northern Peru (FAO e, 2011).

Global aquaculture production:

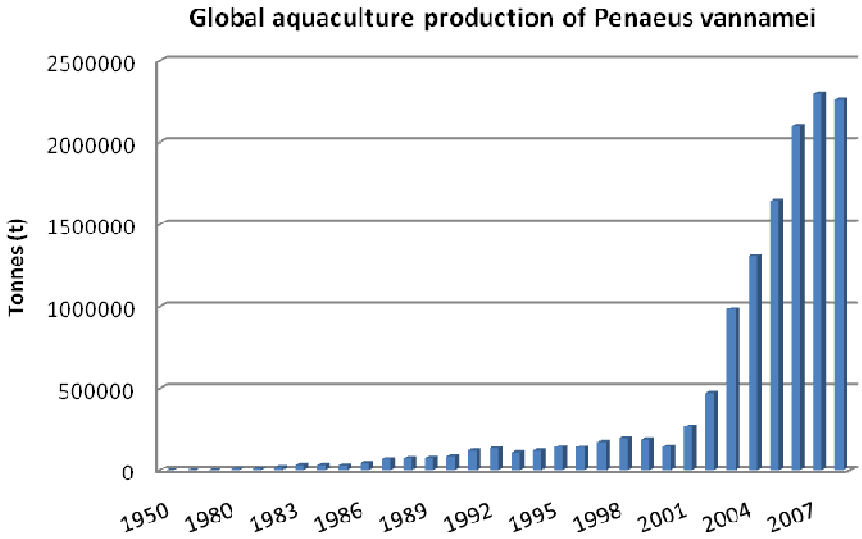


Figure 17: Global aquaculture production of Penaeus vannamei (FAO e, 2011)

Scientific name with original description: *Macrobrachium rosenbergii* (FAO f, 2011)

FAO name: Giant river prawn

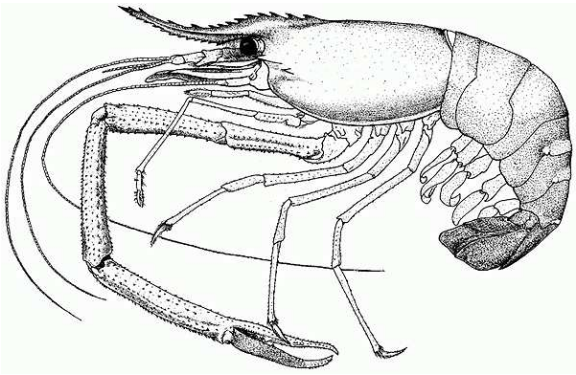


Figure 18: Giant river prawn (IMAGE 3, 2011)

Geographical distribution:

Indian Ocean - West Pacific: Northwest India to Viet Nam, the Philippines, New Guinea and Northern Australia (FAO f, 2011).

Global aquaculture production:

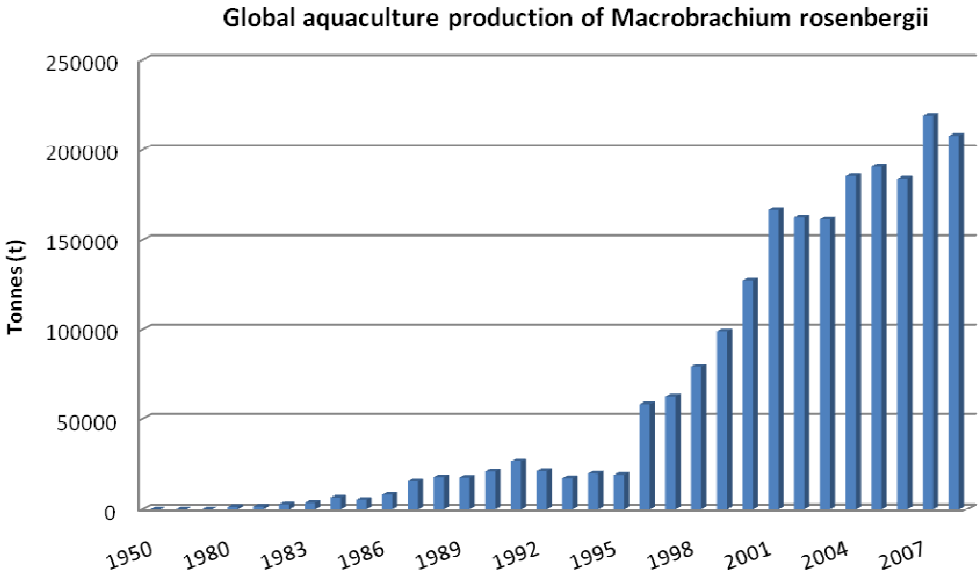


Figure 19: Global aquaculture production of Macrobrachium rosenbergii (FAO f, 2011)

1.4 Processing cycle of shrimp and prawns after the aquacultural production

The aquaculture production of and further processing of fish and fishery product has to follow defined regulations steps different. Following the processing of frozen shrimp and prawns are described according the guidelines of the FAO / WHO Food Standards (CAC, 2011). The Codex Alimentarius Commission provides the food producing industry with information material and guidelines, so that standardised production of food is guaranteed. For the aquaculture production and processing of fish and fishery products, the code of practice for fish and fishery products (CAC/RCP 52-2003) is a recommended guideline for the fish and seafood production industry.

1.4.1 Processing of frozen shrimp and prawns

The code of practice for fish and fishery products provides examples of potential hazards and defects and describes technological guidelines, which can be used to develop control measures and corrective action (CAC/RCP 52-2003).

The origin of frozen shrimp products varies widely. The different sources are deep cold seas, shallow tropical inshore waters and rivers and aquaculture in tropical and semi-tropical regions (CAC/RCP 52-2003). Wild caught and cultivated *Penaeus* species are produced in tropical and sub-tropical countries, which are the largest product line. The product line of these shrimps ranges from whole, headless (head off), peeled, peeled and deveined, raw and /or cooked products presented in different market forms (easy peel, tail-on, tail-off, butterfly, stretched, sushi shrimp) (CAC/RCP 52-2003). These products are prepared in shrimp processing plants that may be not fully automated, using manual techniques or large dimensions fully mechanised equipments. Warm water shrimp may also added to further processes, like marinating or batter and crumb coatings (CAC/RCP 52-2003). Food safety considerations are important during processing, because raw frozen, as well as cooked shrimp products may be consumed without further processing. The correct handling and process in line with the relevant sections in the code of practice takes priority in processing plants (CAC/RCP 52-2003).

Figure 20 gives an overview of the different processing steps, which have to be followed to produce shrimp and prawns into the different marketing forms. The processing flow chart starts with receiving of fresh or already frozen shrimp, delivered from aquaculture farms or from fishery companies. Following selecting and grading is performed and the production flow devises into two processing lines – one were the product is kept in the raw form and another one where the product undergoes a heat treatment (in Figure 20 the cooking of whole shrimps is illustrated). Also cooking of raw peeled and deveined shrimps can be performed. After these different processing flows the production lines are consolidated in the packing and labelling process step, followed by metal detection and storage. Specified description of the different production steps ordered in sections follow. Figure 20 shows a summary of common procedures of the shrimp production. Furthermore the processing flow allows different variations to produce a product designated.

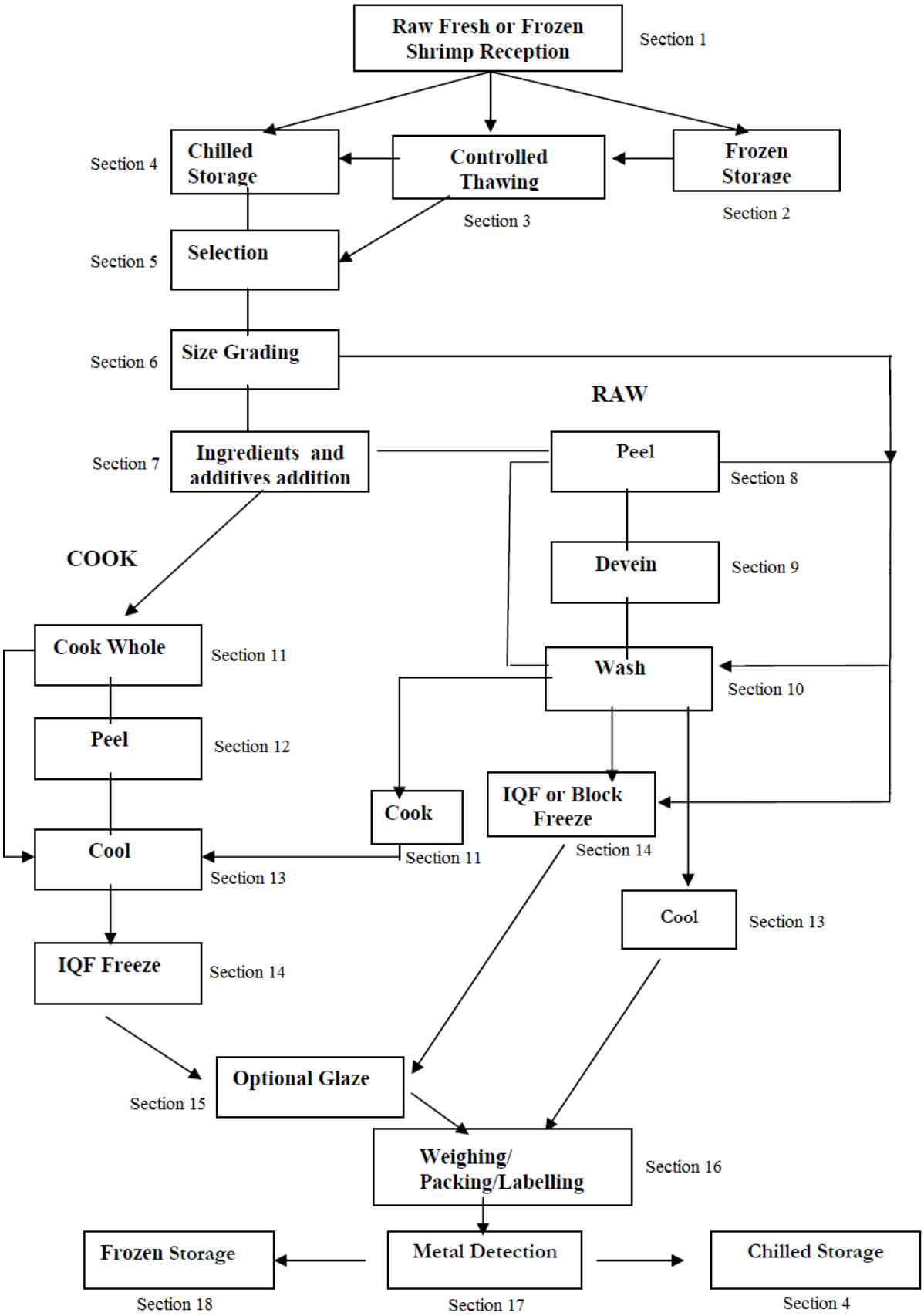


Figure 20: Flow chart of a shrimp and prawn processing line (CAC/RCP 52-2003)

1.4.2 Shrimp preparation processing steps

Following processing steps are described according to the code of practice for fish and fishery products section 14 – processing of shrimp and prawns (CAC/RCP 52-2003).

Section 1 - Raw fresh and frozen shrimp reception

Potential Hazards	Potential Defects
phytotoxins (e.g. PSP)	variable batch quality
microbiological contamination	mixed species
antioxidants	taints
sulphites	blackspot
pesticides	softening from head enzymes
fuel oil (chemical contamination)	decomposition

Raw fresh or frozen shrimps from capturing are controlled using inspection protocols following HACCP plan parameters to ensure that the product is well iced or deep frozen.
The code of practice recommends planning a level of checking that is necessary for phytotoxins is necessary for caught shrimp and the potential antibiotic presence in aquaculture shrimps. Other chemical indicators for heavy metals, pesticides and indicators of decomposition such as TVBN's may be applied and also sulphites should be monitored at harvesting. Also a sensory evaluation, to guarantee that the incoming products are of acceptable sensory quality, should be performed (CAC/RCP 52-2003). The storage is done in suitable facilities and allocated use-by times for processing to ensure quality parameters in the end products. It is necessary to wash the fresh shrimp after receiving with a series of low velocity sprays with chilled clean water (CAC/RCP 52-2003).

Section 2 - Frozen storage

Potential Hazards	Potential Defects
unlikely	Protein denaturation, dehydration

The frozen storage is done in protective packaging (damaged packages should be repacked) to exclude contamination and dehydration with cold storage temperatures with a minimum fluctuation and the storage facility should have a temperature monitoring device, optimally a continuous recording unit to record the ambient temperature. Further processing or shipping has to be done within the best before time on the package (CAC/RCP 52-2003).

Section 3 – Controlled thawing

Potential Hazards	Potential Defects
microbial contamination, contamination from wrapping	decomposition

Prior the defrosting, outer and inner packaging should be removed to prevent contamination and extra care should be taken on block frozen prawns (inner wax or polyethylene packaging may be entrapped with blocks). For thawing clean sea water or water

and ice of potable quality with a water temperature not higher 20°C should be used to achieve a defrosted product with a temperature under 4°C. After the thawing (this should be achieved as quickly as possible) a washing step, with chilled clean water, follows and immediately the shrimps should be re-iced or chilled to avoid increasing temperature (CAC/RCP 52-2003).

Section 4 - Chilled storage

Potential Hazards	Potential Defects
Microbial contamination	decomposition

Chilled storage, preferably under ice, in chill rooms at less than 4°C is proposed, which is monitored by a temperature monitoring device. Unnecessary delays during chilled storage should be avoided (CAC/RCP 52-2003).

Section 5 - Selection

Potential Hazards	Potential Defects
unlikely	decomposition

Shrimps are selected for different quality grades – this should be undertaken with a minimum of delay followed by re-icing of the shrimps (CAC/RCP 52-2003).

Section 6 - Size grading

Potential Hazards	Potential Defects
microbial contamination	decomposition

Size grading is performed through mechanical graders or manually. The “carry over” of old shrimp trapped in the bars of graders and so potential microbial contamination should be prevented by a prompt carrying out of the process step (CAC/RCP 52-2003).

Section 7 - Addition of ingredients and use of additives

Potential Hazards	Potential Defects
chemical and microbial contamination, sulphites	decomposition, improper use of additives

Different ingredients and additives are added to the product in different processing steps. It is essential to monitor the process and the product to ensure that any legislative standards are not exceeded. Additives and ingredients should be used according the manufacturer’s instructions and GMP (good manufacturing practice) (CAC/RCP 52-2003).

Section 8 – Full and partial peeling

Potential Hazards	Potential Defects
microbial cross contamination	decomposition, shell fragments, foreign matter

The assurance of clean peeling tables and tools is important. Peeling tables have to be kept clear of contaminated shrimps and shell fragments using water jets and the shrimps are rinsed after peeling to ensure no carryover of shell fragments (CAC/RCP 52-2003).

Section 9 - Deveining

Potential Hazards	Potential Defects
microbial cross contamination, metal contamination	decomposition, objectionable matter, foreign matter

The vein is the gut of shrimp which may appear as a dark line in the upper dorsal region of prawn flesh. This vein may be a source of microbial contamination. So it is important to be very careful during longitudinally cutting along the dorsal region of the shrimp with a razor slide and the removal of the vein by pulling. During that labour intensive process the cleaning and maintenance schedules should be in place and trained operatives have to clean before, during and after the processing step. The removal of damaged and contaminated shrimps from the processing line is essential to eliminate cross contamination (CAC/RCP 52-2003).

Section 10 - Washing

Potential Hazards	Potential Defects
microbial contamination	decomposition, foreign matter

Peeled and deveined shrimps have to be washed to ensure that shell and vein fragments are removed. After the washing step chilled storage after draining should follow without delay (CAC/RCP 52-2003).

Section 11 - Cooking processes

Potential Hazards	Potential Defects
survival of pathogenic micro-organisms due to insufficient cooking, microbial cross contamination	overcooking

A precisely defined cooking procedure, with particular time and temperature definition according the specification requirements of the final product is required. For the cooking process just drinkable water is allowed to use. The review of the cooking schedule has to be done before each batch - if continuous cookers are used constant logging of process parameters is needed. The cooking operation has to be monitored and the maintenance and cleaning schedules should be available for the stuff which should be trained in the operations.

To ensure no cross contamination an adequate separation of cooked shrimps by different equipment is essential (CAC/RCP 52-2003).

Section 12 - Peeling of cooked shrimps

Potential Hazards	Potential Defects
microbial cross contamination	presence of shell

The cooked shrimps are peeled through mechanical or manual peeling in line with cooling and freezing processes. Cleaning and maintenance schedules should be available so that efficient and safe processing is ensured (CAC/RCP 52-2003).

Section 13 - Cooling

Potential Hazards	Potential Defects
microbial cross contamination and toxin formation	Unlikely

After cooking shrimp should be cooled immediately to decrease the products temperature that limits bacterial growth or toxin production and, most important, the separation of raw and cooked products is essential. For that cooling schedules are needed to enable the time-temperatures requirements. Furthermore only cold or iced potable water should be used for cooling and not be used for further batches and for continuous operations a top-up procedure and maximum run-length will be defined. The freezing step should immediately follow the cooling and draining to avoid any environmental contamination (CAC/RCP 52-2003).

Section 14 - Freezing processes

Potential Hazards	Potential Defects
microbial contamination	slow freezing – textural quality and clumping of shrimps

The freezing process varies according to the type of product and is done by block or plate freezing (raw whole or head-off shrimps) in purposed designed cartons into potable water is poured to form a solid block with protective ice. Warm water shrimp are individually quick frozen (IQF) either on trays in blast freezers or in continuous belt freezers. It is necessary to ensure that the freezing conditions specified generate no clumping, e.g. pieces frozen together especially for IQF products. When the freezing process is started before the freezer reached the operating temperature glazed, slow frozen products and eventually contamination is the result. Fully trained stuff should operate the cleaning of the freezers following cleaning and maintenance schedules (CAC/RCP 52-2003).

Section 15 - Glazing

Potential Hazards	Potential Defects
microbial cross contamination	Inadequate glaze, too much glace, spot welding, incorrect labelling

Glazing protects the frozen shrimps against dehydration and maintains quality during storage and distribution. The simplest form of glazing is done by block freezing followed by dipping and draining frozen shrimps in chilled water. To receive even and calculable glaze covers a more sophisticated process is to pass frozen size graded shrimps under cold-water sprays on vibratory belts at a steady state rate. Glazed shrimp products should receive a secondary re-freezing prior to packing, if not, they should be packed as quickly as possible and moved to cold storage, otherwise shrimps my freeze together and clump as the glaze hardens (CAC/RCP 52-2003).

Section 16 - Weighing, packing and labelling of all products

Potential Hazards	Potential Defects
sulphites	incorrect labelling, decomposition

The material used for wrapping and packaging including glues and inks should be specified to be food grade, odourless and with no risk of transferring of substances to the packed food, which may be harmful to the health being. The products should be weighed in packaging with scales appropriately tared and calibrated to ensure correct weight. Glazed products should be checked to ensure the correct compositional standards to comply with legislation and packaging declarations. The ingredients list should declare presence of ingredients in the product in descending order by weight, including any additives used and still present in the food. The packaging process should be carried out in a manner to ensure that the frozen products remain frozen. If sulphites are used, it should be done according the manufacturer's instructions and the GMP and care should be taken that they are properly labelled (CAC/RCP 52-2003).

Section 17 - Metal detection

Potential Hazards	Potential Defects
presence of metal	unlikely

In final pack the products should be metal detected through machines set to the highest sensitivity possible. Larger packs will be detected at a lower sensitivity than smaller packs, so it should be considered to test these products prior the packing (CAC/RCP 52-2003).

Section 18 - Frozen storage of end product

Potential Hazards	Potential Defects
unlikely	texture and flavour deviations due to fluctuations in temperature, deep freezer burn, cold store flavour, cardboard flavour

The frozen products should be stored at or below -18°C in a facility which is clean, sound and hygienic with minimal temperature fluctuations (± 3°C). It is recommended to have a temperature recording in the storage facility. The products should be properly protected from dehydration, dirt and other forms of contamination and a systematic stock rotation plan should be developed and maintained (CAC/RCP 52-2003).

1.4.3 Food additives used for quick frozen shrimp and prawns

In Table 2 permitted additives are listed which are approved for the use in quick frozen raw, partially or fully cooked shrimp and prawns according to the codex standard for quick frozen shrimp or prawns (CODEX STAN 92-1981).

Table 2: Food additives permitted to use for frozen shrimp products (CODEX STAN 92-1981)

E-No.	Additive	Description	Maximum level in the final product
Acidity Regulators			
330	Citric acid		acc. GMP
450 (III)	Tetrasodium diphosphate	increasing water hydration in the product frozen products **(**SMITH & HONG-SHUM, 2007)	10 g/kg expressed as P_2O_5, singly or in combination (includes natural phosphate)
450 (IV)	Tetrapotassium diphosphate		
451 (I)	Pentasodium triphosphate		
451 (II)	Pentapotassium triphosphate		
Antioxidant			
300	Ascorbic acid (L-)	antioxidant activity, colour stabilisation **(**SMITH & HONG-SHUM, 2007)	acc. GMP
Colour			
124	Ponceau 4 R	Red colour, also available as Cochinal Red A	30 mg/kg in heat-treated products only
Preservatives			
221	Sodium sulphite	antimicrobial preservative, antioxidant activity **(**SMITH & HONG-SHUM, 2007)	100 mg/kg in the edible part of the raw product, or 30 mg/kg of the edible part of the cooked product, singly or in combination, expressed as SO_2
223	Sodium metabisulphite		
224	Potassium metabisulphite		
225	Potassium sulphite		

1.5 Antibiotics in aquacultural production

In industrial livestock farming in water (aquaculture), the implementation of different antibiotic agents is needed, to treat bacterial diseases and for prophylactic inhibition of diseases (GESAMP, 1997). Antibiotics are drugs of natural or synthetic origin that have the capacity to kill or inhibit the growth of micro-organisms (SERRANO, 2005). Antibiotics which are not toxic to the host are used for the treatment of infectious diseases of humans, animals and plants (SERRANO, 2005). Antibiotics revolutionized the treatment of infectious diseases, leaded a dramatic reduction in morbidity and mortality and the health status of the general population improved (SERRANO, 2005).

Most of the classes of antimicrobial drugs used for humans are also used in animals. In the EU in 1997, an estimated 10493 tons of active antimicrobial substance was used: 52% for humans, 33% for therapeutic use in animals, and 15 % as feed additives for growth promotion (ASM, 1999). Furthermore different antibiotics are used as anti-infectious agents and also for growth promotion (SERRANO, 2005). In Table 3 typical mechanisms of action of antibiotics are listed.

Table 3: Typical modes of action of common antibiotics (SERRANO, 2005)

Mechanism	Comments	Examples
Damage cell membrane, allowing contents to leak out. Bactericidal	High toxicity to animals and humans; topical use only	polymixins
Inhibitors of bacterial cell wall synthesis	Animals and humans not affected because their cells do not have walls	penicillins; aminopenicillins; cephalosporins (cephalexin); bacitracin (topical); vancomycin
Inhibitors of folic acid synthesis. Folic acid is needed to make RNA and DNA for growth and multiplication, and bacteria must synthesize folic acid, bacteriostatic	Animals and humans obtain folic acid from their diets, so they are not affected	sulphonamides; sulfasalazine; trimethoprim; co-trimoxazole
Inhibitors of DNA function. DNA is needed for cell growth and division. Most are bactericidal	Drugs used affect bacterial (or fungal) cells more than animal or human cells	nalidixic acid; ofloxacin; metronidazole; rifampin; enrofloxacin; sarafloxacin
Inhibitors of protein synthesis. Proteins are synthesized on cell structures called ribosomes, bactericidal or bacteriostatic.	High doses can affect animals and humans because some ribosomes are similar to those in bacteria	tetracyclines; aminoglycosides; chloramphenicol; florfenicol; macrolides; spectinomycin; lincosamides

Antibiotics are used for growth promotion because feeding of antibiotics is associated with decreases in animal gut mass, increased intestinal absorption of nutrients and energy sparing. So a larger portion of consumed nutrients can be used for growth and production – the efficiency of nutrient use is improving (SERRANO, 2005). By altering the non-pathogenic intestinal flora, beneficial effects on digestive processes are produced and nutrients are more efficient utilised. Moreover antibiotics inhibit the activities of intestinal bacteria to inactivate pancreatic enzymes and the metabolism of dietary protein, so that the digestibility of dietary protein is increasing (SERRANO, 2005).

To control infectious diseases in aquaculture, similar strategies used in other areas of animal production are employed. Whenever antibiotics are used, they should be strictly controlled. For aquaculture no specific antibiotics are designed, so authorized products developed for other areas of veterinary medicine are used (SERRANO, 2005).
To control aquatic animal pathogen bacteria, formally in aquaculture production of shrimp and prawns and for salmon production, a high number of different antibiotics is used. The

prophylactic use of different agents is common and also the advantage of growth promotion is a reason to use antibiotic agents (GP, 2001).

Therefore the use of antimicrobials as growth promoting agents, the use of agents from classes which are or may be used in human or veterinary medicine (i.e. where there is a risk of selecting for cross-resistance to drugs used to treat bacterial infections) are not allowed in the EU since January 1st, 2006 (EC 1831, 2003).

In many countries the use of antibiotics is under veterinary medicine control (SERRANO, 2005). In Table 4 antibiotics which are authorized for use in aquaculture are listed. In Table 5 the antibiotics used for aquaculture purposes in some countries are listed. A similar range of antibiotics is permitted in most European countries for aquaculture purposes (FAO/NACA/WHO, 1999).

Table 4: Antibiotics authorized for use in aquaculture (SERRANO, 2005)

Antibiotic	Treatment of
Oxytetracycline	Furunculosis in salmonids (salmon trout) caused by *Aeromonas samonicida*
	Gafkemia in lobsters (*Aerococcus viridians*)
	Hemorrhagic septicaemia due to *Aeromonas hydrophila*, *A. sobria* and *Pseudomonas*
	Cold water disease in salmonids (*Chondrococcus columnaris*)
	Columnaris disease in salmonids, caused by susceptible *Chondrococcus (Flexibacter) columnaris*
	Enteric redmouth disease, caused by susceptible *Yersinia ruckeri*
	Indicated for the control of *Pseudomonas* disease in catfish and salmonids.
	Indicated for the control of ulcer disease caused by susceptible *Haemophilus piscium* in salmonids (salmon, trout).
Florfenicol	Indicated in the treatment of furunculosis caused by susceptible strains of *Aeromonas salmonicida*
Sarafloxacin	Indicated in the treatment of furunculosis, vibriosis and enteric redmouth in Salmonidae.
Erythromycin	In the treatment of bacterial kidney disease (*Renibacterium salmoninarum*) and streptococcosis in yellowtail in Japan.
Sulphonamides potentiated with trimethoprim or ormethoprim	Against furunculosis, enteric redmouth disease and vibriosis

Wider ranges of antibiotics are approved in Asian countries. On intensive shrimp farms in some countries antibiotics like oxtetracycline or oxolinic acid are widely used (SERRANO, 2005). When no regulatory regime existed, farmers used any antibiotic they might obtain (FAO/NACA/WHO, 1999).

For finfish and for crustaceans, antibiotics are usually administered in the feed, either compounded during the process or as surface-coated feed pellets. Oil is frequently used as vehicle, either by the feed manufacturer or at the farm (SERRANO, 2005).

In the shrimp industry, antibiotics are mainly used as a bath medication in the hatchery. For finfish or for juvenile shrimp, the antibiotic is used as an oil-based coating. The use of antibiotics in older shrimps is increasingly restricted due to concerns about antibiotic residues (SERRANO, 2005).

Table 5: Antibiotics used for aquaculture purposes in certain countries (SERRANO, 2005)

Country	Antibiotic	Purpose
United Kingdom		specific purpose is not mentioned
	oxytetreacycline	see Table 4
	oxolinic acid	against Gram-negative bacteria
	amoxicillin	against alpha- and beta-haemolytic *Streptococci*, some *Staphylococci*, and *Clostridia* species and Gram-negative bacteria (*E. Coli*, *Pasteurella multocida* and *Salmonella*)
	co-trimazine (trimethoprim-sulfadiazine)	against furunculosis, enteric redmouth disease and vibriosis
Norway		specific purpose is not mentioned
	benzylpenicillin+dihydrostrepto-mycin	benzylpenicillin is mainly active against Gram-positive where dihydrostreptomycin is active against Gram-negative bacteria
	florfenicol	treatment of furunculosis caused by susceptible strains of *Aeromonas salmonicida*
	flumequine	against Gram-negative bacteria
	oxolinic acid	see United Kingdom
	oxytetracycline	see Table 4
	co-trimazine (trimethoprim-sulfadiazine)	see United Kingdom
USA (FDA approved)	sulfadimethoxine and ormethoprim	to control furunculosis (*Aeromonas samonicida*) in salmonids, to control enteric septicaemia (*Edwardsiella icttaluri*) in catfish
	oxytetracycline	to control ulcer disease, furunculosis, bacterial haemorrhagic septicaemia and Pseudomonas disease in salmonids and catfish
Mexico		specific purpose is not mentioned
	oxytetracycline	see Table 4
	enrofloxacin	broad spectrum, bactericidal in relatively low concentrations, highly bio-available following either oral or parenteral administration in most species, and achieves good penetration of body tissues and fluids
India	oxytetracycline	*Myxobolus* spp., columnaris disease
	sulfadiazine-trimethoprim	ulcerative and systemic type (*Aeromonas hydrophila*)
	chlor-tetracycline	not defined
		broad spectrum against Gram-positives and Gram-negatives, including anaerobics
Indonesia	oxytetracycline	bacterial fish and shrimp disease
	chloramphenicol (1)	bacterial disease in shrimp and ornamental fish
	erythromycin	bacterial fish and shrimp disease
	streptomycin	bacterial disease in shrimp and ornamental fish
	neomycin	bacterial disease in shrimp and ornamental fish
	enrofloxacin	high skills required
		effects see Mexico
People's Republic of China	sulfonamid	oral administration
	nystatine	fish or egg disinfection
	terramycin	fish or egg disinfection, bath treatment, oral administration
	aureomycin	fish or egg disinfection, oral administration
	penicillin	bath treatment and injection
	streptomycin	fish or egg disinfection, bath treatment, injection
	doxycycline	oral administration
	erythromycin	bath treatment, oral administration
	chloramphenicol (1)	bath treatment and injection
	oxolinic acid	oral administration

Notes: (1) currently banned for use in aquaculture in Indonesia and China

Consumers have a critical opinion to fish and shrimp products, which were produced in aquaculture, because an abusing use of antibiotic agents is assumed (MRI, 2008). Different studies have shown that the increase of the shrimp production in South-East-Asian countries was not just achieved by enlarging the production areas, but also by increasing the stocking density of the farming ponds. Thereby the water contamination increased and therefore the

risk of disease outbreaks. Hence, an increased use of antimicrobial agents occurred (MRI, 2008).

In a study about antibiotic use in shrimp farms in Thailand (HOLMSTRÖM et al., 2003) seventy-six farmers, which cultivated in intensive or semi-intensive form *P. monodon*, were interviewed. The results showed that 74% of the farmers used antibiotics in the pond management. A minimum of thirteen different known antibiotics were used and about ten were documented but not identified. Most commonly used antibiotics were norfloxacin, oxytetracycline, enrofloxacin and different sulphonamides. 86% of the antibiotic using farmers used them in preventive management, as well as treat diseases when symptoms had arisen (HOLMSTRÖM et al., 2003).

In the annual report 2009 of the Rapid Alert System for Food and Feed of the European Union (RASFF, 2010), veterinary drug residues in different foods are listed. In shrimp samples from Bangladesh, India and Sri Lanka nitrofuran residues were significantly high. Meanwhile crustacean consignments from Bangladesh presented for import into the EU must be analysed at origin for nitrofurans and some other substances. From October 2009, the same measure applies to India (CD, 2009).

Table 6 shows a list of pharmacologically active substances which are banned to use in the European Union for animal production. Still chloramphenicol and nitrofurans which are banned in the EU are used in the aquaculture animal production.

Table 6: List of banned pharmacologically active substances in the EU (EEC 2377/90)

chloramphenicol	used in aquaculture
chloroform	
chlorpromazine	
colchicine	
dapsone	
dimetridazole	
metronidazole	
nitrofurans (including furazolidone)	used in aquaculture
ronidazole	

1.6 Antibiotic resistance development in aquaculture

The use of antimicrobial substances for aquaculture animal production has high risks. The present knowledge about influences of antibiotics used in aquaculture production to the human health or to the environment is marginal (GP, 2001). The growing aquaculture industry started to use pharmaceuticals which were used in agriculture. The problem is that these products are used now in aquatic environment without some knowledge of effects e.g. to coastal waters (GP, 2001).

1.6.1 Impact of antibiotic use to the environment

Impacts of antibiotics to the environment are the formation of resistant bacteria species and damage of different organisms (GP, 2001). Formally prophylactic use of antibiotics in the aquatic environment of rivers, lakes and oceans has resulted in an increased antibiotic resistance of bacteria in this environment. Moreover the resistance of fish pathogens to antibiotics increased too (CABELLO, 2006).

The effectiveness of prophylactic use of antibiotics in aquaculture becomes dubiously because of the emergence of antibiotic resistance of pathogens. Antibiotic use increases the possibilities for the passage of antibiotic resistant bacteria (and pathogens) and additionally also antibiotic resistance determinants to bacteria of terrestrial animals and human beings, including pathogens (CABELLO, 2006). By prophylactic use of antibiotics the effectiveness of the immune system of fish to clear up bacterial infections is decreasing and therefore an increased use of antibiotics is required. Antibiotics are given to aquatic animals by feed, in baths and by injections. Unconsumed feed and fish faeces containing antibiotics reach the sediments of production ponds (CABELLO, 2006) and residual antibiotics which are spread to the aquatic environment can be ingested by vertebrates and macro-invertebrates, potentially increasing the risk of antibiotic entering the human food supply (SERRANO, 2005).

Substances in inhibitory concentrations affect the environmental microflora, altering the microbial composition with the result that bacteria can develop antibiotic resistances (CABELLO, 2006; SERRANO, 2005). This is confirmed by different studies in which the bacterial flora in the environment surrounding aquaculture sites contains an increased number of resistant bacteria (CABELLO, 2006).

The problem with antibiotic residues in sediments is that most substances appeared to have a half-life in soil of about 2-3 weeks at 20°C, while lower temperatures generally cause a slower degradation (SERRANO, 2005).

The environmental microflora is also affected in that way that e.g. the soil nitrogen mineralisation is reduced. Different studies suggested that antibiotic residues might be removed through microbial degradation, but it has yet to be determined if environmental microorganisms can degrade these compounds and whether aerobic or anaerobic condition is required (SERRANO, 2005).

Furthermore if municipal solid waste and agricultural waste is used for biogas production, the presence of substances which have an effect to anaerobic bacteria responsible for biogas production affect the whole process. Also antibiotic residues in the environment affect the treatment of wastewater which generally involves microbial processes (SERRANO, 2005). Summarized there are several effects of antibiotics in the environment. The problem is that still not all influences are known and studies are just done after effects appeared.

1.6.2 Effects of antibiotics to human health

Potential hazards affected by antibiotic residues in food are:

- Toxic effects
- Allergies
- Effects on human intestinal flora
- Transfer of resistant bacteria to humans
- Transfer of resistance genes from non-pathogen bacteria to human pathogens

Toxic effects could be observed in target animal species, or non-target (incidental intake), or in humans. Animals and humans may be exposed via residues in animal products and humans handling products containing residues. Both ways could be harmful if the product has organ toxicity, mutagenicity or allergenicity (SERRANO, 2005).

Effects of antibiotics on target species can be nephrotoxic (e.g. bacitracin), macrolides can cause diarrhoea, other cause hormonal disturbances. The likelihood of direct toxicity from antibiotics or their metabolites in animals is extremely low. The exception could be chloramphenicol, which produces toxic aplastic anaemia not related to the dose used (SERRANO, 2005).

Potential toxic effects of drugs which are released to the aquatic environment, because of use in terrestrial and aquaculture farming, are not completely understood, although consequences such as chemical contamination, bioaccumulation and induction of microbial resistance have been described (SERRANO, 2005).

Several antibiotics (e.g. macrolides, quinoxalines and bacitracin) are potent antigens and exposure on daily basis can lead to sensitization.

Allergies can be caused by intake of antibiotic residuals from food or can be created by the intake of antibiotics of unprotected workers in the aquaculture industry and in the production of animal feed. In this production sites humans are exposed to large amounts of antibiotics, which come in contact with the skin, the intestinal and the bronchial tracts (SERANNO, 2005; CABELLO, 2006).

Most reported allergic reactions are related to β-lactam antibiotic residues in milk or meat and the allergic reaction has been associated with exposure to antibiotic residues in foods – but many cases refer to people previously treated with antibiotics and hypersensitized to a degree that subsequent oral exposure evoked a response. Other allergic reactions (e.g. streptogramins, bacitracin) have been reported in association with clinical therapy.

Some studies considered that the risk to have allergic response to antibiotic residues intake by food is small for humans: the molecular weights of the antibiotics are too low to cause reactions by themselves and when complexed to proteins of larger molecular weight the probability of hypersensitivity reaction is also minimized (SERRANO, 2005).

Effects on the human intestinal flora

Bacteria as a part of the human body's internal and external environment can be influenced by antibiotic use for food animals. The microbial populations in the body normally compete with foreign bacteria within a stable internal environment, which is important for maintaining health (SERRANO, 2005). Foreign bacteria and residues of antimicrobial agents may have different influences to the human intestinal flora and may affect the human health too. The principal concern is how antibiotic residues may affect the human intestinal flora either by:

- exerting a selective pressure on the dominant intestinal flora
- favouring the growth of bacteria with natural or acquired resistance
- promoting the development of acquired resistance in pathogenic enteric bacteria
- impairing colonization resistances
- altering metabolic enzyme activity of the intestinal flora

In different studies it has been demonstrated that therapeutic dosages of β-lactams, tetracyclines and macrolides have a distinct impact on the number of enterobacteria, Enterococci, anaerobic bacteria and the development of resistant strains of the intestinal flora and also at a low level of exposure, effects on the intestinal microflora might occur (SERRANO, 2005).

Resistance transfer

Using antimicrobial agents in agriculture and aquaculture causes the development of resistant bacteria. As more antimicrobials are used, as higher will be the frequency of resistant microorganisms in that environment and also the emergence of resistant pathogens is higher (FAO/NACA/WHO, 1999). Two risks of resistance transfer have to be considered independently:

- the transmission of resistant bacteria from aquaculture environments to humans – a risk is the increase of resistance of human pathogens as a direct consequence of antibiotic use in aquaculture (SERRANO, 2005)
- the introduction of non-pathogenic bacteria containing antimicrobial resistance genes in the human environment and the subsequent transfer of such genes to human pathogens (SERRANO, 2005)

Resistant pathogens, causing zoonoses, have significant importance for the human health. Zoonoses causing pathogens can be transferred over food-intake to humans. For instance the Salmonella typhimurium strain DT104, sourced from England, developed resistance to tetracycline, chloramphenicol, sulphonamides, ampicillin, streptomycine and latest to fluorchinolones (BARLAGE, 2007). Another possibility of bacteria transfer is drinking water. In developing countries drinking water is not treated. In these cases pathogenic fish bacteria may reach the drinking water supply and may infect the human body (SERRANO, 2005). Antibiotic resistant bacteria, which can reach the human body by water or food intake, can transmit and share determinants for resistance to antibiotics with pathogens such as *E. coli* (CABELLO, 2006). Moreover other studies showed that resistance to different antibiotics were transferred from fish pathogens such as *Aeromonas* to human pathogens such as *E. coli*, *Salmonella* and *Vibrio cholera* (CABELLO, 2006).

Mechanisms of resistance transfer

The phenotypic characteristic of resistance is determined by the bacterial genome. The resistance profile can change by mutation or by acquisition of new genetic material. Resistant bacteria are able to transfer the resistance to other bacteria (even to bacteria of different genera) that have never been exposed to the antibiotic. This phenomenon is known as horizontal gene transfer (SERRANO, 2005).

Different examples of horizontal gene transfers appeared in the last few years. Horizontal gene transfer indicates the risk that resistance from bacteria, which were exposed to antibiotics in aquaculture moves to human or animal pathogens. The use of one antibiotic can cause resistance to itself but also to another unrelated antibiotic. This is confirmed in the Swedish Veterinary Antimicrobial Resistance Monitoring Report (SVARM, 2001). A single transfer of linked resistance genes conveys resistance to several antimicrobials in the recipient bacteria.

In bacteria parts of the bacterial DNA (plasmids) or portions of their chromosomes, which may contain the resistance codifying genes, may exchange and resistance can occur (SERRANO, 2005). The new genetic material may be acquired by three different mechanisms:

Conjugation → A plasmid is passed between the organisms through a pilus. It may occur between the same species and between different genera or families. This mechanism is also called "infectious drug resistance" (MADIGAN et al., 2001).

Transformation → DNA is assimilated from the external environment (released by a bacterium) and incorporated into the chromosome of the host cell. (MADIGAN et al., 2001).

Transduction → Genetic material can be acquired from an infecting bacteriophage, hence DNA is transferred into the hosts chromosome (MADIGAN et al., 2001).

Determinants of antibiotic resistance which come from aquatic environments have the potential of being transmitted by horizontal gene transfer to bacteria of the terrestrial environment, including human and animal pathogens (CABELLO, 2006).

1.6.3 Control strategies to avoid the development of antibiotic resistance

As it is well established that antibiotics given to animals have resulted in the emergence of resistant bacteria which have the potential to infect humans via the food chain, the hazard of the development of antimicrobial resistance in seafood products and in the environment surrounding aquacultural farms is existing (HUSS et al., 2003). Additionally illegal residues have been reported in aquaculture products.

Antibiotics should never be used as an alternative to a good aquaculture practice. Therefore national governments created control programmes respectively governments need to put in place control programmes for the use of antimicrobials in aquaculture production. Additionally it is required that at national level has a up-to-date legislation and standards based on actual science, monitoring programmes adequate resources for enforcement of the legislation (HUSS et al., 2003).

Consumers can protect themselves against antibiotic resistant bacteria. These bacteria are just as susceptible to heat and hygiene as their non-resistant counterparts, so that a proper hygiene practice during preparation and cooking of seafood like frequent hand washing, prevention of cross contamination by separation of raw products from other foods and proper storage can minimise the incidence of seafood poisoning (HUSS et al., 2003).

2 Marine microbial diversity

2.1 Seafood microflora

2.1.1 General fish microflora

The majority of literature on the microbiology of seafood products is concerned with marine finfish species, because these constitute the majority of commercially important products (WARD & HACKNEY, 1991). Meanwhile different studies on the microbiology of crustaceans have been performed. However, many similarities exist between the spoilage of finfish and crustaceans (WARD & HACKNEY, 1991).

Fish meat with a high protein and vitamin content has a high water activity and a small amount of carbohydrates. The occurrence of spoilage at cooling temperatures is mainly caused by *Shewanella* and *Pseudomonas* species (KRÄMER, 2002). The most important spoilage bacterium in fish is *Shewanella putrefaciens*, causing an intensive formation of hydrogen sulphide and trimethylamine, which is already noticeable in small concentrations. Other bacteria, such as *Moraxella-Acinetobacter* strains show growth either, without causing the same level of spoilage malodour and at higher temperatures *Bacillus* and *Micrococcus* species show increased growth (KRÄMER, 2002).

Fish spoilage occurs, compared to meat, faster and shows significant malodour, caused by the degradation of soluble nitrogen compounds and fish protein and the formation of intensive olfactory compounds such as ammonia, hydrogen sulphide, methylmercaptan, dimethyl sulphide, dimethylamine, trimethylamine and other amines (KRÄMER, 2002).
Spoilage causing bacteria in fish are able to degrade amino acids to biogenic amines, such as histamine, which can cause fish poisoning at higher concentrations. Further *Clostridium botulinum* type E is able to grow and produce toxins at 3°C (KRÄMER, 2002).

Faecal contamination through animal excretions and polluted water during processing, can be caused by *Salmonella* spp., *Staphylococcus aureus* or with *Erysipelothrix* sp. (KRÄMER, 2002).

2.1.2 General microflora of crustaceans

Meat from crustaceans has a similar composition as fish meat, although it contains an higher amount of available aminoacids and other nitrogen compounds. This causes the problem that crustaceans deteriorate much faster compared to fish, formally caused by *Pseudomonas* and other gram-negative bacteria (KRÄMER, 2002).

In different studies differences in the “normal” microflora for various shrimp species due to the differences of the ecology such as surrounding waters, sediment and postharvest handling procedures, as well as the analysis methods, were found (WARD & HACKNEY, 1991). In most cases different spoilage causing bacteria such as *Moraxella*, *Acinetobacter*, *Microccoccus* and *Pseudomonas* species were found. Many studies suggested that changes in the nutritional properties (and/or salinity) on the surface of shrimp, or interactive microbial activities, are involved in the changes of the microbial flora of shrimp during iced storage (WARD & HACKNEY, 1991).

Most investigations resulted that marine shrimp from warm waters, such as finfish, carry a microbial population composed primarly of gram-positive bacteria such as *Micrococcus*, *Bacillus* and coryneforms, whereas cold-water species harbour predominantely gram-negative microbes, including *Moraxella*, *Acinetobacter*, *Pseudomonas*, *Flavobacterium* and *Vibrio* (WARD & HACKNEY, 1991). In Table 7 the dominant bacterial genera found in marine shrimp from different regions are listed.

Table 7: Dominant bacterial genera in fresh marine shrimp (WARD & HACKNEY, 1991)

***Shrimp Genus* / Location**	**Dominant bacterial genera**
Penaeus / Gulf of Mexico	*Aeromonas, Achromobacter, Acinetobacter, Bacillus, Coryneforms, Flavobacterium, Micrococcus, Moraxella, Pseudomonas, Vibrio*
Sicyonia / Gulf of Mexico	*Flavobacterium, Cytophaga*
Pandalus / Pacific	*Acinetobacter, Arthrobacter, Bacillus, Flavobacterium, Micrococcus, Moraxealla, Pseudomonas*
Metapenaeus	*Coryneforms, Micrococcus*
Parapeneopsis	*Achromobacter, Micrococcus*
Pandalus	*Achromobacter, Acinetobacter, Coryneforms, Flavobacterium, Moraxella, Pseudomonas*

Also yeasts, such as *Rhodotorula*, *Candida* and *Torulopsis* and molds of the genus *Pulluleria* (*Aureobasidium*) were isolated from fresh marine shrimp (WARD & HACKNEY, 1991).

The microbial composition of crustaceans depends on the region of production or capturing and has significant differences between the different climatic zones. The microbiota of warm water species is differing from the cold water species and during storage the microbial composition may change too. At cold (melting ice) temperatures the outgrowth of psychrotrophic microorganisms such as *Pseudomonas* spp. may occur (WARD and HACKNEY, 1991).

2.2 Microorganisms associated with seafood spoilage

Spoilage is defined as the sensory changes resulting in a fish product being unacceptable for human consumption (REHBEIN & OEHLENSCHLÄGER, 2009).
Spoilage of shrimp and prawns happens although through bacterial action, but primarily autolytic reactions controlled by naturally occurring enzymes in muscle tissue are responsible. Different studies found that the nucleotide catabolism correlates with the loss of freshness. The activity of the enzymes adenosine deaminase and adenosine monophosphate deaminase gave a good indication of postharvest storage time and temperature (WARD & HACKNEY, 1991).

Figure 22 shows the sequence of nucleotide degradation: The first five reactions proceed relatively fast as a result of the actions of endogeneous enzymes in muscle tissue, whereas the oxidation of hypoxanthine to xanthine and finally to uric acid is slower and considered to be due primarily to bacterial enzymes (WARD & HACKNEY, 1991).

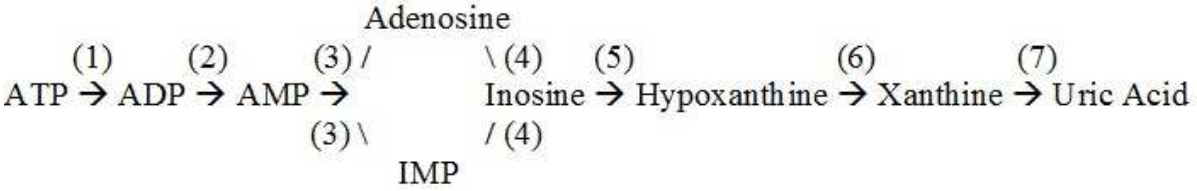


Figure 21: Sequence of nucleotide catabolism in penaeid shrimp (WARD & HACKNEY, 1991)

Bacterial spoilage of crustaceans under chill temperatures is a surface problem. Generally crustaceans exhibit a reasonably long chill storage shelf life, which is related to the following points: (WARD & HACKNEY, 1991).

- Initial bacterial flora, i.e., the number and types of spoilage organisms and subsequent contamination as a result of further processing steps.
- Certain enzymes that continue to operate after death, thus effectively controlling the outgrowth of spoilage bacteria.
- Low-molecular-weight muscle extractives in shrimp and prawns that are quantitatively and qualitatively different from those in more rapidly spoiling seafood products.
- The integrity of the chitinous exoskeleton, i.e. how well it protects the underlying muscle from invasion by surface bacteria. The exoskeleton offer one extreme of protection from mechanical damage compared i.e. to fish skins.

Spoilage of seafood is caused by autolytic and chemical changes or off-odours and off-flavours due to bacterial metabolism (REHBEIN & OEHLENSCHLÄGER, 2009). Typically just a few species of the group of bacteria called specific spoilage organisms (SSO), several are listed in Table 8, cause the production of metabolites associated with the off-flavours and off-odours (REHBEIN & OEHLENSCHLÄGER, 2009).

Table 8: Specific spoilage organisms (SSO) (REHBEIN & OEHLENSCHLÄGER, 2009)

Photobacterium phosphoreum
Shewanella putrefaciens
Pseudomonas spp.
Lactic acid bacteria (LAB)
Enterococci
Brochothrix thermosphacta
Enterobacteriaceae

Summarised the general scheme of spoilage of seafood products including shrimp, prawns and crawfish is shown below (WARD & HACKNEY, 1991):

- Spoilage bacteria are naturally present on fish.
- Amino acid and other non-protein nitrogen (NPN) substrate pools are present.
- There is selective growth of organisms (mostly *Pseudomonas*) which actively oxidatively deaminate amino acids.
- Repression of proteinase production is derepressed by selective use of amino acids by *Pseudomonas* bacteria.
- Amino acids are recruited to substrate pool by bacterial hydrolysis of protein and ammonia and volatile fatty acid production sharply increases due to it.
- Specific “spoiler” types of bacterial produce sulphur-containing and other odorous compounds.

2.3 Pathogens associated with illness due to the consumption of seafood

Disease or illness resulting from consumption of shrimp, prawns or crawfish can generally be attributed to product contamination from one or more of the following three sources listed in Table 9 (WARD & HACKNEY, 1991). Further, in Table 9 the pathogens which appeared as contaminants in shrimp, prawns and crawfish products are listed.

Table 9: List of pathogens contaminants of shrimp, prawns and crawfish (WARD & HACKNEY, 1991)

1. Naturally occurring pathogens	*Clostridium botulinum* Type E, *Vibrio cholerae*, *V. Parahaemolyticus*, *V. vulnificus*
2. Pathogens introduced into the aquatic environment as a result of improper disposal of human waste and/or land runoff	*Clostridium perfringens*, *Staphylococcus*, *Erysipelothrix*, *Edwardsiella*, *Salmonella*, *Shigella, Franciscella*, *Vibrio* species
3. Pathogens introduced via product handlers – during harvesting, through processing plant and food service handlers, and ultimately the consumer	Coliforms, faecal coliforms including *E. coli*, *Staphylococcus*, *Salmonella* and *Listeria*

In an article about cooking parameters for safe seafood for consumers (NACMCF, 2008) also other foodborne pathogens associated with illness attributed to the consumption of seafood are described. In Table 10 these pathogens are listed.

Table 10: Bacterial foodborne pathogens associated illness outbreaks from seafood consumption (NACMCF, 2008)

Aeromonas hydrophila
Bacillus cereus
Campylobacter jejuni and *Campylobacter coli*
Clostridium botulinum
Listeria monocytogenes
Plesiomonas
Salmonella
Shigella
Staphylococcus aureus
Vibrio cholerae
Vibrio parahaemolyticus
Vibrio vulnificus

2.3.1 Taxonomy and special properties of *Listeria monocytogenes* in shrimp products

The genus *Listeria* has eight species: *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, *L. masthii* and *L. rocourtiae*. Among these *L. monocytogenes* is pathogenic to humans and animals while *L. ivanovii* is pathogenic to animals and others are considered non-pathogenic (BHUNIA, 2008). Although *L. seeligeri* is considered non-pathogenic, it possesses a part of the virulence gene cluster, which is present in pathogenic *L. monocytogenes* and *L. ivanovii* (BHUNIA, 2008). The non-pathogenic species are also of interest since their presence indicates the potential for the presence of the pathogenic *L. monocytogenes* (LABBÉ & GARCÍA, 2001).

Listeria species are small (1.0-2.0 x 0.5 µm), gram-positive, facultatively anaerobic, rod-shaped and nonspore-forming bacteria (BHUNIA, 2008; LABBÉ & GARCÍA, 2001). *Listeria* is ubiquitous in nature and can be isolated from soil, water, sewage, green plant material, decaying vegetation and numerous species of birds and mammals including humans (LABBÉ & GARCÍA, 2001). The bacteria survive in extreme environments, including broad pH ranges (4.1-9.6), high salt concentrations (10%), and in presence of antimicrobial agents. They are psychrophilic and grow at a wide temperature range (1 - 45°C) (BHUNIA, 2008). *L. monocytogenes*, *L. ivanovii* and *L. seeligeri* are haemolytic and produce a zone of β-haemolysis on blood agar plates (BHUNIA, 2008).

Pathogenesis of *L. monocytogenes*

L. monocytogenes is responsible for a rare but fatal systematic disease called listeriosis (BHUNIA, 2008). It is the most known form of invasive disease transmitted by food products. Listeriosis mostly affects people in particular risk groups where the immune defence system is reduced such as infants, elderly people, pregnant women, people with HIV infection, and transplant patients (BHUNIA, 2008; HUSS et al., 2003). The disease may infect the central nervous system and often manifests itself as meningitis. In infected pregnant women, listeriosis typically results in abortion. The fatality rate in the risk group is high, typically 20 - 40 % (HUSS et al., 2003). The incubation period ranges from one to 91 days and so it is often difficult to trace the food that was the source of the pathogen because most people do not remember their food consumption three months ago (HUSS et al., 2003). *L. monocytogenes* may also cause non-invasive febrile gastroenteritis in otherwise healthy people. The incidence of this type of listeriosis is not known (HUSS et al., 2003).

Listeriosis is typically caused by processed, industrialized foods that are ready-to-eat (RTE) and which have extended shelf life at chill temperatures, because there is no final heat treatment by the consumer. Hence *L. monocytogenes* is easily isolated from several types of RTE food products (HUSS et al., 2003).

Prevalence of *L. monocytogenes* and *Listeria* spp. in shrimp and shrimp products

Environments with decaying plant material are a typical indigenous biotope of *L. monocytogenes*. The bacterium also occurs in the gastrointestinal tract and 2-6% of humans are healthy carriers. Several studies approved that *Listeria* spp. are not typical in aquatic and marine environments (HUSS et al., 2003). Unlike other studies describe that *Listeria* spp. are indigenous to marine and estuarine environments (REILLY & KÄFERSTEIN, 1997) and therefore the association with shrimp is expected (WAN NORHANA et al., 2009). In the study of WAN NORHANA et al., 2009 the prevalence of *Listeria* spp. in the shrimp production chain is described. This article lists several studies where the presence of *Listeria* spp. and *L. monocytogenes* in aquatic samples of shrimp ponds such as water, sediment and fresh shrimp was determined (Table 11). Beyond that the article lists studies reporting the

prevalence of *Listeria* spp. and *L. monocytogenes* in processed shrimp and shrimp products from distribution companies, retail markets and shrimp processing companies and it is described that the prevalence of *Listeria* spp. and *L. monocytogenes* in shrimp pond samples is very low (WAN NORHANA et al., 2009).

Table 11: Prevalence of Listeria in the shrimp production chain (modified from WAN NORHANA et al., 2009)

Point of sampling	Type of sample	Origin of sample	No. of samples	Positive for (%)		References
				Listeria spp.	*L. monocytogenes*	
Shrimp pond	During farming					
	Sediment	India	30	0	0	Bhaskar et al. (1998)
	Water		30	0	0	
	Fresh raw shrimp		18	0	0	
	Clam meat		30	10.0	0	
	Formulated feed		30	0	0	
	At harvest					
	Sediment		6	50.0	0	
	Water		6	0	0	
	Shrimp		18	16.6	0	
Wholesalers/distributors/ importers	Raw shrimp	Imported products (US)	7	n.s.	28.5	Weagant et al. (1988)
	Cooked and peeled shrimp		8		25.0	
	Frozen shrimp (for export)	Brazil	45	6.6	8.8	Hofer and Ribeiro (1990)[a]
	Raw shrimp	US	49	n.s.	9.0	Farber (1991)
	Frozen raw shrimp	Imported products to US	30	16.7	6.7	Berry et al. (1994)
	Fresh and frozen shrimp	Imported into US	205	6.8	n.s.	Gecan et al. (1994)
	Frozen raw shrimp	Imported into US	74	20.0	5.0	Jinneman et al. (1999)
	Cooked shrimp	Imported into Canada	274	n.s.	1.5	Farber (2000)
	Peeled shrimp		4			
Retailers	Fresh raw (Metapenaeus sp.)	Cochin, India	5	40.0	n.d	Fuchs and Surendran (1989)
	Frozen peeled prawn		4	50.0		
	Fresh and frozen shrimp	US	4	25.0	0	Buchanan, Stahl, Bencivengo, and Dell Corral (1989)[b]
	Fresh shrimp	Costa Rica	12	n.s.	33.0	Ellner et al. (1991)[a]
	Frozen shrimp		2	n.s.	50	
	Cooked shrimp	n.s.	20	n.s.	20.0	Farber (1991)
	Frozen semi-ready foods[c]	Taiwan	68	n.s.	34.0	Wong et al. (1990)
	Shrimp salad	Iceland	13	23.0	23.0	Hartemink and Georgsson (1991)
	Fresh raw		11	9.0	9.0	
	Fresh and frozen shrimp	India	19	10.5	0	Manoj et al. (1991)
	Freshly caught shrimp	US Gulf Coast	74	n.s.	11.0	Motes (1991)
	Raw seafood[d]	US	59	28.8–44.1	n.s.	Noah et al. (1991)
	Peeled shrimp in brine	Norway	16	n.s.	18.0	Rørvik and Yndestad (1991)
	Frozen shrimp	Japan	70	8.6	1.4	Masuda et al. (1992)[b]
	Raw shrimp	Japan	38	n.s. 15.8	2.6	Ryu et al. (1992)
	Fresh seafood[e]	Trinidad	102	n.s. 10.8	2.0	Adesiyun (1993)
	Fresh raw shrimp	France	17	23.5	11.8	Ravomanana et al. (1993)[b]
	Cooked shrimp		35	54.3	11.4	
	Fresh raw shrimp	Malaysia	16	n.s.	44.0	Arumugaswamy et al. (1994)
	RTE food[f]		27	n.s.	22.0	
	Cooked, peeled, frozen shrimp	England	30	n.s.	6.0	McLauchlin and Nichols (1994)
	Fresh raw shrimp	Mangalore, India	28	3.6–46.4	10.7	Jeyasekaran et al. (1996)
	Cured seafood[g]	Denmark	191	n.s.	4.0	Jørgenson and Huss (1998)
	Fresh shrimp	Chile	59	n.s.	28.8	Cordano and Rocourt (2001)
	Cooked-peeled and shell-on	Australia	380	n.s.	3.0	Anon. (2002c)
	Fresh shrimp	India	11	9.1	n.d	Dhanashree et al. (2003)
	Dried shrimp		27	11.1		
	Fresh shrimp (*P. monodon*)	India	30	73.3	6.7	Moharem et al. (2007)
	Fresh shrimp and frozen	Iran	12	8.3	n.d	Jalali and Abedi (2008)
	Fresh shrimp	Goa, India	10	30.0	n.d	Parihar et al. (2008)
Manufacturer/processors	Frozen shrimp (*P. brasiliensis*)	Brazil	178	47.2	18.0	Destro, Piva, Leitao, and Landgraf (1994)[a]
	Processed seafood[d]	US	152	5.9–18.4	n.s.	Noah et al. (1991)
	Plant environment	Brazil	56	n.s.	25.0	Destro et al. (1996)
	Water		21		23.8	
	Utensils		33		24.2	
	Shrimp		178		17.4	
	Cooked-peeled shrimp	Iceland	3331	8.1	26.5[h]	Valdimarsson et al. (1998)
	Raw material (fresh shrimp)	Iceland	43	23.2	20.9	Gudmundsdottir et al. (2006)
	Shrimp shell		18	16.7	16.7	
	Plant environment		552	13.4	12.0	
	Cooked-peeled shrimp (end product)		82	n.d	n.d	

n.s. – not specified n.d. – not detected.
[a] Cited from Destro (2000).
[b] Cited from Ben Embarek (1994).
[c] Various types of dumplings including shrimp.
[d] Including shrimp, prawns and breaded shrimp.
[e] Including fresh shrimp.
[f] Including shrimp dishes.
[g] Including brined shrimp and oil-marinated shrimp.
[h] Species identification was done on 49 of the 270 (8.1%) positive samples.

According the results listed in Table 11 (modified from WAN NORHANA et al., 2009) *L. monocytogenes* was absent from all samples taken from shrimp ponds. Prevalence was detected in clam meat, in the sediment and in shrimp samples at harvest in India. The presence of *L. monocytogenes* at retailers, wholesalers and importers in fresh and raw frozen

shrimp is quite common (Table 11). The prevalence varies from low to almost 50 % in raw frozen shrimp (WAN NORHANA et al., 2009). *L. monocytogenes* infection from fresh and raw frozen shrimp is not given because these products are processed before the consumption, even though that the prevalence of *L. monocytogenes* is high. Infections may be caused by cross contaminations in the processing plant, kitchen or food service establishment and so a risk to susceptible populations is given (WAN NORHANA et al., 2009).

L. monocytogenes has also been isolated from ready-to-eat (RTE) shrimp products such as cooked shrimp and shrimp salad (Table 11). The prevalence in cooked products ranges from 1.5 – 25.0 % and in shrimp salads up to 23.0 % (Table 11). Also occurrence in frozen semi-ready foods (e.g. dumplings including shrimp) (34.0 %) and cured seafood (4.0 %) such as brined shrimp and oil-marinated shrimp was documented. Furthermore *L. monocytogenes* occurred in shrimp processing plant environments (25.0 %), in water used for the shrimp processing (23.8 %), in utensils (24.2 %) and in shrimp products (17.4 – 26.5 %) (Table 11). With the high convenience degree of shrimp products, *L. monocytogenes* is more likely to occur. *L. monocytogenes* from raw products may contaminate final products (WAN NORHANA et al., 2009) and it is hypothesized that the presence of *Listeria* in cooked products is due to undercooking of the products (WAN NORHANA et al., 2009).

Growth and survival of *Listeria* in shrimp and shrimp products

Different factors including the availability of nutrients, pH, temperature, water activity, competitive microflora and the presesence may influence the growth of *Listeria* in shrimp (WAN NORHANA et al., 2009). *L. monocytogenes* is halo- (survives at salt concentrations up to 30 %) and psychrotolerant, thus it is able to survive for long periods in processing plants, household refrigerators and freezers (WAN NORHANA et al., 2009; HUSS et al., 2003). Furthermore it was observed that the growth rate of *L. monocytogenes* in cooked crustaceans is reported to be higher compared to other RTE foods (WAN NORHANA et al., 2009). Optimum growth conditions, such as the pH range from 6.8 to 7.0; a_w 0.99 and salt content from 1 to 2 % are factors which promote the growth of *L. monocytogenes* in cooked shrimp (WAN NORHANA et al., 2009). Several studies observed high growth rates of *L. monocytogenes* in shrimp products stored at chill temperatures. Even if *L. monocytogenes* is present in cooked shrimp in low numbers significant levels may be reached during chilled storage and even higher numbers could be reached in the case of temperature abuse of the products (WAN NORHANA et al., 2009). Shrimp products consisting cooked and peeled shrimp in brine containing salt and combinations of benzoic, citric and sorbic acids are popular. Also in these products growth and survival of *L. monocytogenes* was observed (WAN NORHANA et al., 2009).

The application of heat is the simplest and most effective method to reduce the number of *Listeria* in food (WAN NORHANA et al., 2009; HUSS et al., 2003), even though the heat resistance of certain *Listeria* strains is different. Other methods to control the presence of *L. monocytogenes* such as irradiation, modified atmosphere packaging (MAP), high-pressure processing (HPP), high-pressure carbon dioxide (CO2), the use of chlorine, ozone, phosphates or quaternary ammonia compounds are further described and summarised by WAN NORHANA et al. (2009).

Prevention and control

To control contaminations with *Listeria* during processing of food in achievable, the implementation of Hazard Analysis of Critical Control Points (HACCP) strategies and Good

Hygiene Practice (GHP) are mandatory for processing plants that produce RTE foods (BHUNIA, 2008). If any products carry *L. monocytogenes* food producers are asked to recall these products. For consumers it is recommended to heat certain products of processed foods before consumption. Heating of food at 71°C for 1 min will kill *L. monocytogenes* (BHUNIA, 2008; KRÄMER, 2002). For several RTE seafood products critical control points cannot be identified (HUSS et al., 2003). Therefore routine surveillance of production facilities, an effective sanitation scheme (GHP) are practices to reduce *L. monocytogenes* in products (BHUNIA, 2008). Furthermore microbiological levels of *L. monocytogenes* in food products were suggested. According the regulation EC 2073/2005 a level of *L. monocytogenes* of 100 cfu/g in ready-to-eat food products placed on the market during the shelf-life (food unable to support growth of *L. monocytogenes* and food which is not intended for infants and special medical purposes) was suggested. Absence of *L. monocytogenes* is requested for RTE food products intended for infants or medical purposes and for the immediate control of the food producer also the absence of *L. monocytogenes* is required (EC 2073/2005). Guidelines for ready-to-eat foods according Food Standards of Australia and New Zealand (FSANZ, 2001) suggest a RTE product as satisfactory, when *L. monocytogenes* is not detected in 25g. A maximum marginal level of 10^2 cfu/g is specified, but it is considered that products stored under refrigeration should have no *L. monocytogenes* detected and that a bacterial count of 10^2 cfu/g is potentially hazardous to the risk groups (FSANZ, 2001).

3 Microbial criteria of seafood and shrimp products

3.1 Regulations of the microbial quality of seafood and shrimp

Different countries or economic communities defined different regulations and standards for fish and fishery products. Following the some examples of regulations and standards for the import of seafood products are listed. The FAO recommended microbial limits for seafood according to ICMSF, 1986. In Table 12 recommended microbial limits for seafoods are listed (ICMSF, 1986). Table 13 shows a list of additional microbiological examinations which are carried out if further examinations are required.

Table 12: Recommended microbiological limits for seafoods (ICMSF, 1986)

				Limit per gram or per cm^2	
Product	**Test**	**n**	**c**	**m**	**M**
Fresh and frozen fish and cold-smoked	APC *E. coli*	5 5	3 3	5×10^5 11	10^7 500
Precooked breaded fish	APC *E. coli*	5 5	2 2	5×10^5 11	10^7 500
Frozen raw crustaceans	APC *E. coli*	5 5	3 3	10^6 11	10^7 500
Frozen cooked crustaceans	APC *E. coli* *Staph. aureus*	5 5 5	2 2 0	5×10^5 11 10^3	10^7 500 -
Cooked, chilled, and frozen crabmeat	APC *E. coli* *Staph. aureus*	5 5 5	2 1 0	10^5 11 10^3	10^6 500 -
Fresh and frozen bivalve molluscs (These criteria are to be used only for molluscs from approved harvesting areas where waters are free from enteric bacteria or virus contamination and there is no significant contamination by toxic metals or toxic and carcinogenic chemicals which may be accumulated by animals. Molluscs from non-approved areas should enter trade only after processing by a treatment to destroy enteric bacteria and viruses and may be tested for dangerous chemicals before distribution)	APC *E. coli*	5 5	0 0	5×10^5 16	- -

Table 13: Additional tests to be carried out when appropriate (ICMSF, 1986)

				Limit per gram or per cm^2	
Product	**Test**	**n**	**c**	**m**	**M**
Fresh and frozen fish and cold-smoked (For fish known to derive from inshore or inland waters of doubtful bacteriological quality, particularly in warm-water areas and where fish are to be eaten raw, it may be desirable to test for *Salmonella* and *V. parahaemolyticus*. Smoked fish may be tested for Staph. aureus)	*Salmonella* *V. parahaemolyticus* *Staph. aureus*	5 5 5	0 2 2	0 10^2 10^3	- 10^3 10^4
Precooked breaded fish (*Staph. aureus* may also be tested for cooked fish products likely to be mishandled)	*Staph. aureus*	5	1	10^3	10^4
Frozen raw crustaceans (*Staph. aureus* may be tested in breaded products. *Salmonella* and *V. parahaemolyticus* tests may be applied to products from warm-water areas and *V. parahaemolyticus* tests in products from temperate regions in summer, if likely to be eaten raw)	*Salmonella* *V. parahaemolyticus* *Staph. aureus*	5 5 5	0 1 2	0 10^2 10^3	- 10^3 10^4
Frozen cooked crustaceans (*Salmonella* and *V. parahaemolyticus* may be tested for in cooked crustaceans originally harvested from waters and processed in regions of known high environmental hazard)	*Salmonella* *V. parahaemolyticus*	10 5	0 1	0 10^2	- 10^3
Cooked, chilled, and frozen crabmeat (Crabmeat from animals harvested from waters above 15°C may be tested for *V. parahaemolyticus*)	*V. parahaemolyticus*	10	1	10^2	10^3
Fresh and frozen bivalve molluscs (*Salmonella* should be tested for when there is concern for bacteriological safety and *V. parahaemolyticus* should be tested for in molluscs from endemic areas and harvested from warm waters)	*Salmonella* *V. parahaemolyticus*	20 10	0 1	0 10^2	- 10^3

n = Number of representative sample units.
c = Maximum number of acceptable sample units with bacterial counts between m and M.
m = Maximum recommended bacterial counts for good quality products.
M = Maximum recommended bacterial counts for marginally acceptable quality products.
Plate counts below "m" are considered good quality. Plate counts between "m" and "M" are considered marginally acceptable quality, but can be accepted if the number of samples does not exceed "c." Plate counts at or above "M" are considered unacceptable quality (ICMSF, 1986).

Microbiological criteria (MC) applied by EU and other countries are described by HUSS et al. (2004) in the FAO Fisheries Technical Paper 444. Food safety and hygienic practices throughout the EU is controlled by “Vertical Directives” dealing with specific products of animal origin or “Horizontal Directives” which cover all foodstuffs entering the market (HUSS et al., 2004). Directives that include MC for fish are:

- Live Bivalve Molluscs Directive (91/492/EEC) (EC, 1991a)
- Fishery Products Directive (91/493/EEC) (EC, 1991b)
- Commission Decision on the microbiological criteria applicable to the production of cooked crustaceans and molluscan shellfish (93/51/EEC) (EC, 1993)

In addition a number of Directives have provision for MC to be added in the future (e.g. Council Directive 93/43/EEC on the hygiene of foodstuff) (HUSS et al., 2004).

Additionally following guidelines and recommendations were used to compare the results of the microbiological examination of the shrimp samples for the microbial estimation of the microbial quality of the samples:

- Guidelines for the microbiological examination of ready - to - eat foods according the Food Standards Australia and New Zealand (FSANZ, 2001)
- Published microbiological guidelines and critical limits for the evaluation of food (Veröffentlichte mikrobiologische Richt- und Warnwerte zur Beurteilung von Lebensmitteln) (DGHM, 2010)

4 Aim of the thesis

The main target or idea of the thesis was based on the raising worldwide consumption of seafood and therefore an increased development of the importance of the seafood market. This global development could also be noticed in smaller local regions, for instance in an obvious increase of the availability of seafood in the Austrian market in the last decade. This increase is partly caused by advertised health benefits of fish and seafood e.g. seafood is low in fat and cholesterol; high in protein, vitamins and minerals and the abundance of polyunsaturated omega -3 fatty acids. Consequently, because of this development, the seafood trade worldwide needs to be controlled. Due to the fact that most of the seafood is produced in aquacultures, the focus was set on the aquaculture production.

In this study relevant microbial quality parameters of shrimp products at retail level were examined in order to obtain information about the microbial status of the different products on the market. After the general screening of the microbial quality the detection of pathogens was performed. Specifically the detection of *Listeria monocytogenes* and other *Listeria* species was performed.

In aquaculture production the prophylactic use of antibiotics is common to inhibit bacterial (microbial) growth therefore outbreaks of different diseases in the production units are averted. That induces the question if antibiotic resistance of the pathogen *Listeria monocytogenes* is developed through antimicrobial residues in shrimp.

Therefore, isolates of *Listeria monocytogenes* and other *Listeria* species obtained were submitted to antibiotic susceptibility testing, because the importance of antibiotic resistance and its transfer within the food supply chain was an important parameter to be checked.

5 Materials and Methods

5.1 List of materials for the microbial examinations

Agar Bacteriological	Agar No. 1 (Merck, Germany)
Anaerocult® A	Anaerobic Atmosphere Bag (Merck, Germany)
Anaerotest	(Merck, Germany)
Antibiotic Discs	(Oxoid, UK)
Api Test Kit	API® Listeria (bioMérieux, France) plus reagents
Baird Parker Agar	Staphylococcus Selectivity Agar Base according to BAIRD-PARKER (Merck, Germany) plus Egg-Yolk-Tellurite Emulsion (Oxoid, UK)
Beta Lysine Discs	(Remel, USA)
BHI Broth	Brain Heart Infusion Broth (bioMérieux, France)
Bromthymolblue	(Merck, Germany)
CASO Agar	Caseinpeptone Soypeptone Agar (Merck, Germany)
Columbia Agar	+ 5 % Horseblood Muttonblood (bioMérieux, France)
D-Glucose	(Merck, Germany)
DILUCUP Peptone 9.0	(LabRobot Products AB, Sweden)
Fluorocult® Laurylsulfate Broth	(Merck, Germany)
Fraser Broth	Listeria Selective Enrichment Broth (Merck, Germany)
Gram Staining Reagents	(Division of Food Quality Assurance, BOKU Vienna)
GSP Agar	Pseudomonas Aeromonas Selective Agar Base according to KIELWEIN (Merck, Germany) plus Pimaricine (Merck, Germany) and Penicilline G Sodium (Merck, Germany)
KOVÁCS Reagent	Indole Reagent (bioMérieux, France)
LMX Bouillon	Listeria enrichment bouillon for VIDAS® LMX tests (bioMerieux, France)
MRS Agar	Lactobacillus Agar according to DE MAN, ROGOSA and SHARPE (Merck, Germany)
Mueller Hinton Agar	Merck, Germany

MYP Agar	Cereus-Selective Agar Base according to MOSSEL (Merck, Germany) plus Egg Yolk Emulsion (Oxoid, UK) and Polymyxin B Sulfate (Sigma-Aldrich, Germany)
OCLA Agar	Chromogenic Listeria Agar, ISO (Oxoid, UK) plus OCLA Selective Supplement (Oxoid, UK) and OCLA Differential Supplement (Oxoid, UK)
Oxidase Reagent	(bioMérieux, France)
PALCAM Agar	PALCAM Agar Base (Merck, Germany) plus PALCAM Selective Supplement (Oxoid, UK)
Paraffine	(Oxoid, England)
PEMBA Agar	Bacillus Cereus Agar Base (Oxoid, England) plus Egg Yolk Emulsion (Oxoid, UK) and Polymyxin B Sulfate (Sigma-Aldrich, Germany)
Peptone Salt Solution	0.1% Peptone plus 0.85% Sodiumchloride
Photobacterium Agar	Photobacterium Broth (Fluka, Switzerland) plus Agar No. 1 (Merck, Germany)
Plate Count Agar	Caseinpeptone-Glucose-Yeastextract Agar (Merck, Germany)
Puffered Peptone water	(Merck, Germany)
STAA Agar	STAA Agar Base plus STAA Selective Supplement (Oxoid, UK)
Tryptone	(Oxoid, UK)
TSC Agar	Tryptose-Sulfite-Cycloserine Agar Base (Merck, Germany) plus D-Cycloserine pure USP (AppliChem GmbH)
Universal-Peptone M66	(Merck, Germany)
VRBD Agar	Violet-Red-Bile-Dextrose Agar according to MOSSEL (Merck, Germany)
Yeast Extract	(Oxoid, UK)
YGC Agar	Yeastextract-Glucose-Chloramphenicol Agar (Merck, Germany)

5.2 Chemicals / Reagents

Acriflavine-Hydrochloride

Ammonium Ferric Citrate

API® Listeria (bioMérieux, France)

Crystal violet solution

di-Potassium-Hydrogen-Phosphate waterfree p. A. (Merck, Germany)

Ethanol 75 %

Ethanol 96%

Glycerine 87% (AppliChem GmbH)

Hydrogen peroxide Solution,

Lugol's solution (Division of Food Quality Assurance, BOKU Vienna)

Microbact™ (Oxoid, UK)

Nalixidine Nalixidineacid-Salt

Potassiumhydroxide - Solution (3%)

Safranine solution

Sodium hydroxide, NaOH, (0,05 mol/L)

Sodiumchloride pure (Sigma-Aldrich, Germany)

Sodiumchloride solution (0,85%)

5.3 Equipment and other materials

Anaerobe pot: GasPak® System (BD Diagnostics, USA)

Bunsen burner (Division of Food Quality Assurance, BOKU Vienna)

Dilushaker II Variosensor (LabRobot Products AB, Sweden)

Disc Dispenser (90 mm) for 8 disc tubes (Oxoid, England)

Disc dispenser (Oxoid, UK)

Durham test tubes

Filter papers

Forceps

Freezer: -20°C (Elektra Bregenz AG)

Glass flasks: 2 L, 1 L, 0.5 L, 0.25 L (Schott AG, Switzerland)

Graduated cylinder

Heating plate: IKA RCT basic (IKA Labortechnik Staufen, Germany)

Incubator (25°C); Heraeus

Incubator (30°C, 37°C): J.P. Selecta Spain

Knife

Lab balance: type Sartorius GP 4102 (Sartorius AG, Germany)

Laminar flow workbench: Laminar MSC 12 EN-NF G 2 x ELALL Jouan (Thermo Electron Corporation)

Laminator Sealbag 235 A-2, Serial No. 9704-01563 (W. Kopp, Verpackungsmaschinen, Germany)

Magnetic stirrer bar

Microscope: Olympus BX 41

Microwave

Mini VIDAS®: Model Vidas 12, Serial No. ITV 1216143 (bioMérieux, USA)

Object plates (Division of Food Quality Assurance, BOKU Vienna)

Pipette Controller: BRAND macro

PSIpette (bioMérieux, France)

Psipipettes: api®PSIpettes

Refrigerator: 4°C (Electrolux GmbH)

Scalpel

Scissor

Smasher AES Chemunex, Serial No. 01030478

Steam autoclave: Certo Clav CV-EL 10 LO/12 LO 18 LO (Certo Clav Sterilizer GmbH, Austria)

Sterile cotton swabs (Sterilin, England)

Sterile Eppis: 1.5 mL, 2 mL, (VWR International GmbH, Austria)

Sterile inoculating loops single-use (Greiner Bio-one GmbH. Austria)

Sterile petridishes: 94/16 (Greiner Bio-one, Austria)

Sterile pipet tips: 20-300 µL, 50-1000 µL (Eppendorf AG, Germany)

Sterile pipettes: 1 mL, 5 mL, 10 mL, 25 mL (Falcon®, USA)

Sterile spatula (Applicators): with L form (VWR International GmbH, Austria)

Sterile stomacher bags (Greiner Bio-one GmbH, Austria)

Sterile swabs

Sterile syringe filters: 0.2 µm cellulose-acetate (VWR International GmbH, Austria)

Sterile syringes: 5 mL Terumno® (Terumno Europe N.V., Belgium), 10 mL Omnifix® (B. Braun Melsungen AG, Germany)

Sterile test tubes: Cellstar®Tubes, 15 mL (Greiner Bio-one GmbH)

Stomacher: Stomacher Lab Blender 400, Model No. BA 6021, Serial No. 12990 (Seward Medical UAC House, London)

Test tubes plus aluminium capsules (Cap O Test)

UV-lamp: ChemImager 5500 (Alpha Immotech, Alpha Ease FC)

VIDAS® Heat and Go: TECHNE, DRI-BLOCK® DB-3D, Cat. No. 93555, Serial No. R00100130

VIDAS® Listeria monocytogenes Xpress test kit (bioMérieux, France)

Volumetric flasks

Vortex: MS2 Minishaker (IKA Works Incorporation, USA)

Water bath: type W22, No. 90004, Class 1 (Medingen GmbH, Germany)

5.4 Reference Strains used and their incubation conditions

In Table 14 different reference strains and their incubation conditions are presented. These strains were used for quality control of divers media used for the microbial examination and for the quality control of different identification procedures. The different *Listeria* strains in Table 14 were used as reference strains for the antibiotic susceptibility tests for comparing test results of the isolated strains from the samples with references.

Table 14: Reference strains used during the microbial examinations

Genus	Species	Reference number	Growth medium	Incubation conditions		Quality control of
				h	T [°C]	
Bacillus	*cereus*	LMG 8221 ATCC 9634 ATCC 11779	Nutrient broth	3	37	PEMBA / MYP
Bacillus	*cereus*	LMG 6910 ATCC 7004	Nutrient broth	3	37	PEMBA / MYP
Bacillus	*cereus*	DSM 31 ATCC 14579	Nutrient broth	3	37	PEMBA / MYP
Pseudo-monas	*fluorescens*	DSM 50090 ATCC 13525	TSA	48	25	O / F tests
Pseudo-monas	*aeruginosa*	LMG 6395 ATCC 27853	TSA	48	25	O / F tests
Escherichia	*coli*	ATCC 25922	Brain heart infusion (BHI)	24	37	Fluorocult Laurylsulfat broth and antibiotic susceptibility tests
Staphylo-coccus	*aureus*	ATCC 25923	BHI	24	37	antibiotic susceptibility tests
Strepto-coccus	*pneumoniae*	ATCC 49619	BHI	24	37	antibiotic susceptibility tests
Listeria	*grayi*	ATCC 25401	BHI	24	37	antibiotic susceptibility tests
Listeria	*grayi*	ATCC 700545	BHI	24	37	antibiotic susceptibility tests
Listeria	*innocua*	LMG 11387 ATCC 33090	BHI	24	37	antibiotic susceptibility tests
Listeria	*ivanovii*	LMG 11388 ATCC 19119	BHI	24	37	antibiotic susceptibility tests
Listeria	*ivanovii subspecies ivanovii*	DSM 20750 ATCC 19119	BHI	24	37	antibiotic susceptibility tests
Listeria	*monocytogenes*	ATCC 19111	BHI	24	37	antibiotic susceptibility tests
Listeria	*monocytogenes*	NCTC 10890	BHI	24	37	antibiotic susceptibility tests
Listeria	*monocytogenes*	ATCC 13932	BHI	24	37	antibiotic susceptibility tests
Listeria	*monocytogenes*	SLR 2249	BHI	24	37	antibiotic susceptibility tests
Listeria	*monocytogenes*	LMG 13305 ATCC 11994	BHI	24	37	antibiotic susceptibility tests
Listeria	*monocytogenes*	ATCC 19114	BHI	24	37	antibiotic susceptibility tests
Listeria	*monocytogenes*	ATCC BAA 751	BHI	24	37	antibiotic susceptibility tests
Listeria	*monocytogenes*	ATCC 19118	BHI	24	37	antibiotic susceptibility tests
Listeria	*monocytogenes*	NCTC 13726	BHI	24	37	antibiotic susceptibility tests
Listeria	*monocytogenes*	ATCC 19112	BHI	24	37	antibiotic susceptibility tests
Listeria	*monocytogenes*	ATCC 19115	BHI	24	37	antibiotic susceptibility tests
Listeria	*monocytogenes*	ATCC 7644	BHI	24	37	antibiotic susceptibility tests
Listeria	*seeligeri*	DSM 20751 ATCC 35967	BHI	24	37	antibiotic susceptibility tests
Listeria	*welshimeri*	20650	BHI	24	37	antibiotic susceptibility tests

5.5 Samples

5.5.1 Sample description

All examined shrimp samples were bought in the Austrian market at retailers or at warehouses. Shrimp products were either bought in different Austrian retail markets (14 products), in a warehouse (Metro) (11 products) and received from two companies (33 products). All products were sold deep frozen with a recommended storage temperature of -18°C. In total 58 shrimp samples were used for the microbiological examination.

The products were transported immediately to the laboratory, using isolated bags or boxes to avoid an increase of the product temperature. All products were packed in synthetic material (Figure 22). Many products were additionally packed in coated or uncoated paperboard boxes (Figure 22).

The shrimp products were stored in the examination lab at deep frozen condition at a minimum temperature of -18°C. Before the samples were transferred to the frozen storage, the products were labelled with a random identification number and the shelf live was noted to prepare the examination plan, in order to analyze the samples before the end of shelf life.

Figure 22: Examples of different shrimp samples (raw frozen and cooked shrimps)

5.5.2 Convenience degree and retail market forms of commercial shrimp and prawn products

According to the code of practice for fish and fishery products (CAC/RCP 52-2003) the term shrimp (includes the frequently used term “prawn”) refers to the species which are covered in the FAO listing of shrimps (HOLTHUIS, 1980). Furthermore the code defines different forms of convenience degrees respectively retail stages. Retail stages are defined as the different forms as shrimps and prawns are produced form the commercial global market. According the Codex Alimentarius Commission following definitions of shrimp products (Table 15) are produced:

Table 15: Different retail market forms of shrimps and prawns (CAC/RCP 52-2003)

Dehead	means to remove the head from the entire shrimp or prawn
De-veined shrimp	Means all the shrimp which have been peeled, the back of the peeled segments of the shrimp have been open out and the gut ("vein") removed.
Fresh shrimp	Are freshly caught shrimp which have received no preserving treatment or which have been preserved only by chilling. It does not include freshly cooked shrimp.
Peeled shrimp	are shrimps with heads and all shell removed
Raw headless shrimp	are shrimps with heads removed and the shell on

Shrimp and prawn samples which were analysed for this study were available on the retail market in three different convenience degrees:

Raw frozen shrimps are freshly caught shrimp which have not received any treatment, except freezing to -18°C by IQF or block freezing process (IQF - individually quick frozen). The quick freezing process shall be carried out in such a way that the range of temperature of maximum crystallisation is passed quickly (CODEX STAN 92-1981).

Blanched shrimps (parboiled) are shrimp which are plunged into boiling water for a few seconds to a few minutes and afterwards cooled in ice water. After the heat treatment a freezing process follows. Another definition explains blanching as a heating treatment (steam or hot water) for a period of time such that the surface of the product reaches a temperature adequate to coagulate the protein (CAC/RCP 17-1978; NOAA, 1996).
The Austrian Codex Commission defines blanching as a heating treatment to fresh or frozen products, which undergo a heat treatment at temperatures under 100°C, but with a minimum core temperature of 60°C (CODEX B 35, 2007)

Cooked shrimps are products which were heated until an internal temperature of 65 - 70°C without overcooking the product. The cooking times vary according to the size of the product and the temperature used for the process. Exact times and conditions for cooking of the product should be determined by prior experimentation (CODEX STAN 92-1981). After the heat treatment a freezing process follows. According the recommended international code of practice for shrimps or prawns cooking is explained as the heating (in boiling potable water, clean seawater or brine or heating in steam) for a period of time such that the thermal centre of the product reaches a temperature adequate to coagulate the protein (CAC/RCP 17-1978; NOAA, 1996).

5.5.3 Retail market forms of shrimps and prawns

Shrimps and prawns available on the retail market were produced to different market forms. Table 16 shows a list of these market forms.

Table 16: Market forms of shrimps and prawns (NOAA, 1996)

Heads on	head, shell, tail fins on
Headless	only head removed; shell, tail fins on
Peeled, undeveined, tail on	all shell removed except last shell segment and tail fins, with segment unslit
Peeled, undeveined, tail off	all shell and tail fins removed, with segments unslit
Peeled and deveined, tail on	all shell removed except last shell segment and tail fins, with segments shallowly slit to last segment
Peeled and deveined, tail off	all shell and tail fins removed, with segments shallowly slit to last segment
Peeled and deveined, fantail or butterfly, tail on	all shell removed except last shell segment and tail fins, with segments deeply slit to last segment
Peeled and deveined, fantail or butterfly, tail off	all shell and tail fin removed, with segments deeply slit to last segment
Peeled and deveined, western	all shell removed except last shell segment and tail fins, with segments split to fifth segment and vein removed to end of cut

Following figures (Figure 23 to Figure 27) present the most common market forms of shrimps and prawns available on the European market:

Head-On Shell-On is a frozen shrimp which is available in the original shape (no processing steps in changing the shape were performed)

Figure 23: Head-On Shell-On shrimp (IMAGE 4, 2011)

Headless Shell-On is a frozen shrimp which was not peeled but the head was removed

Figure 24: Headless Shell-on shrimp (IMAGE 5, 2011)

Peeled Deveined Tail-On is a frozen shrimp without head and which was peeled and deveined

Figure 25: Peeled Deveined Tail-On shrimp (IMAGE 6, 2011)

Peeled Deveined Tail-Off is a frozen shrimp which was peeled, deveined and the head and tail was removed

Figure 26: Peeled Deveined Tail-Off shrimp (IMAGE 7, 2011)

Peeled Undeveined is a frozen shrimp which was peeled and the head and tail was removed

Figure 27: Peeled Undeveined shrimp (IMAGE 8, 2011)

Count – weight specification

Besides the market forms, traded shrimps and prawns are declared by size respectively weight specifications. The count declares the quantity of shrimp per kilogram or per English pounds (lbs, 454 g). Shrimps and prawns are customarily graded by size counts. Raw, head-off shrimp or tails are conventionally counted in pieces to the pound (454 g). Raw, head-on shrimp are counted in pieces to the kilogram (1000 g) (ITC, 1983). These count groups may vary slightly between countries. The tail weight and counts of freshwater prawns and marine shrimp will differ because freshwater prawns have a larger head relative to the tail (LEE & WICKINS. 2000). In Table 17 the weight specifications of head-off and head-on shrimps and prawns are presented. The weight specification was important for the sampling procedure for the microbiological examination. As higher the count number as more singular shrimp bodies were used for each first dilution step for the examination procedure.

Table 17: Weight specification of head-off and of head-on shrimp and prawns (LEE & WICKINS. 2000)

Head-off shrimps or prawns			Head-on shrimps or prawns	
No. of tails per pound (454 g)	approximate weight [g] of			
	Tail	Whole shrimp	No. of whole shrimp per kg	Approximate weight of whole shrimp [g]
> 70	< 6	< 10	71-90	11-13
61-70	6-7	10-11	61-70	14-16
51-60	7-9	12-13	51-60	17-20
41-50	9-11	14-16	41-50	21-24
36-40	11-12	17-18	31-40	25-32
31-35	13-14	19-21	21-30	33-49
26-30	15-17	22-26	16-20	50-62
21-25	18-21	27-32	11-15	63-91
16-20	22-27	33-42	< 11	> 91
11-15	28-40	43-65		
< 10	> 40	> 65		

6 Methods

6.1 Sample preparation

The sample preparation was performed according to the specific rules for fish and seafood according § 64 Lebensmittel und Bedarfsständegesetz L 10.00-10, December 2004 (ANONYMUS f, 2004) which correlates with the norm EN ISO 6887-3: 2003 (modified norm of the international published norm ISO 6887-1: 1999) (ISO 6887-1: 1999; ISO 6887-3: 2003). All examinations were done before the end of the shelf-life.

Frozen samples, were thawed at room temperature for a maximum of six hours. Afterwards from each sample two parallel samples were weighted (two times 10 g sample) in a sterile stomacher bag and 90 mL of a peptone-salt-solution was added aseptically into the stomacher bag and homogenised in a smasher for one minute. Figure 28 and Figure 29 show the sample preparation. For the microbiological examination two dilution series were set to get replicated results. This first dilution 1:10 (10^{-1}) was used to prepare the following dilution steps to the maximum dilution, which was 1:1000000 (10^{-6}).

In the beginning of the work a new kind of dilution series system was used to prepare the dilution steps, called “Dilucup” from the Swedish company LabRobots Products. Figure 30 shows a schematic illustration how the dilution series and further procedure are performed using the “Dilucup” system (LABROBOTS, 2011).

Figure 29: Sample preparation

Figure 28: Smasher and laboratory balance

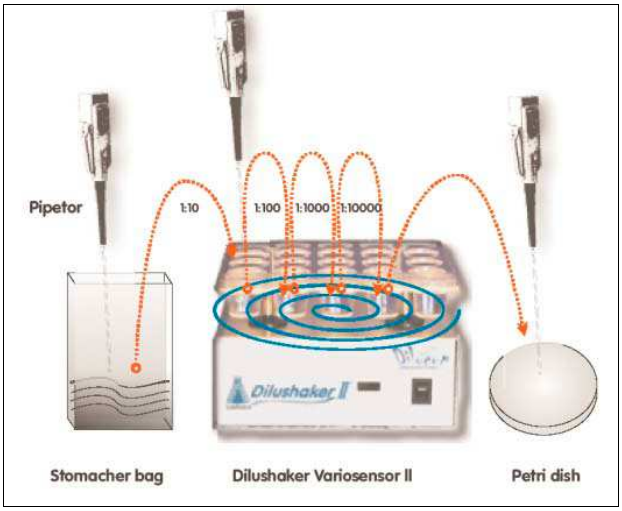


Figure 30: Principle of the dilution step procedure by use of the Dilushaker system (IMAGE 9, 2011)

Figure 31 presents a schematic illustration of performing a pour plate method. The pour plate method is performed by adding one mL of a particular dilution step into an empty petridish and in the next step about 15 to 20 mL of liquid agar (temperature about 48°C) are mixed with the dilution aseptically. To guarantee a complete homogenisation of the sample with the agar, a mixing step is performed, following an eight loop, about three to five times.

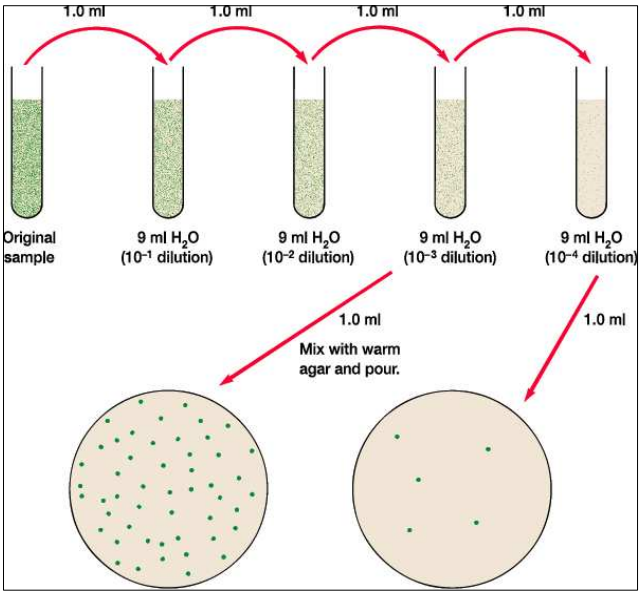


Figure 31: Principle of the pour plate method (IMAGE 10, 2011)

As a second method for the microbiological examination the spread plate method was performed. This method is easier to perform because agar plates are already prepared and it is not necessary to prepare fresh at 48°C temperate agar solution to perform the intermixture of the sample dilution with the specific agar type. An aliquot of 0.1 mL of dilution is transferred with a pipette on a prepared agar surface and spread over the entire surface using a single-use L-shaped sterile spatula. The spreading is done until the agar surface absorbs the liquid, visible through a dry surface.

6.1.1 Calculation of the colony count

For the evaluation and counting of the colonies after incubation, just agar plates with colony counts between 10 and 300 cfu are consulted (cfu – colony forming unit). Colonies spread together are counted as one colony. The colony counts from countable dilution steps are consulted for the calculation of the weighted arithmetic mean of the colony count.

Weighted arithmetic mean:

$$\bar{c} = \frac{\Sigma c}{n_1 * 1 + n_2 * 0{,}1} * d$$

c: weighted arithmetic mean of the colony count
Σc: total of colonies in all plates chosen for enumeration
n1: number of plates of the lowest dilution step
n2: number of plates of the next dilution step
d: dilution factor of the lowest counted dilution step

6.2 Microbiological screening methods

In the discussion which different microbial species are important to detect, it was decided to start a screening with performing the enumeration of the total viable count and other different bacteria types which have a high prevalence in fish and seafood and otherwise have a significant influence in the microbial spoilage of fish and seafood.

The total viable count (TVC) respectively aerobic plate count (APC) is the number of bacteria (cfu/g) in a food product obtained under optimal conditions of culturing (HUSS, 1993). The TVC is not an examination of the "total" bacterial population, but a measure of the fraction of the microflora able to produce colonies in the medium used under the conditions of incubation (HUSS, 1993). Additionally the TVC is also influenced by the temperature of incubation of the plates (HUSS, 1993). In the case of the study it was decided to incubate the plates at a temperature of 30°C up to 72 hours. The examination of TVC was performed, to get an overview over the bacterial load of the different shrimp samples. During freezing and cold storage an uncontrolled kill or damage of bacteria may take place (HUSS, 1993). Furthermore the measuring of TVC is useful to examine the conditions of the raw material, the effectiveness of procedures such as heat treatment and hygiene conditions during processing, sanitary conditions of equipment and time-temperature profile during storage and distribution (HUSS, 1993).

To get an overview of the bacterial status of all seafood spoiling microorganisms the examination of lactic acid bacteria (LAB), *Pseudomonas* spp., *Photobacterium* spp. and *Brochothrix thermosphacta* was performed. These bacteria species are members of the dominating microflora in fish and seafood products and it is expected to detect relevant bacterial counts of these species in the shrimp products. Moreover *Enterobacteriaceae* also may act as specific spoilage organism (SSO) for these products, but primarily those were examined because they occur in fish and fishery products as a result of contamination from the animal/human reservoir (HUSS, 1993). This contamination has normally been associated with faecal contamination or pollution of natural waters or aquacultures (the organisms may survive for months) or through direct contamination of products during processing (HUSS, 1993). Therefore a good personal hygiene and health education of food processors is essential to control the diseases caused by *Enterobacteriaceae* (HUSS, 1993). It is expected that the bacterial counts between the convenience degrees will have significant differences in their value because the processing procedures with heat treatment can minimize or eliminate the *Enterobacteriaceae* content.

Further *Staphylococcus aureus*, *Clostridium perfringens* and *Bacillus cereus* were examined to identify specific hazardous species which may cause food poisoning by intoxication of the shrimp products. Staphylococci are ubiquitous organisms which survive very well in the environment (HUSS, 1993). The main reservoir and habitat of these bacteria is the animal/human nose, throat and skin (HUSS, 1993). Thus the hazard of a contamination with *Staphylococcus aureus* during processing of seafood exists. Certain *Bacillus* and *Clostridium* species also may cause intoxications and so a legitimated interest of the examination of these species given. Furthermore, the detection of the foodborne pathogen *Listeria monocytogenes* has also been performed.

6.2.1 Determination of the total viable count (TVC)

In Table 18 the composition of plate count agar is shown. The composition equates the composition according to the standard regulations from "§64 LMBG zur Untersuchung von Lebensmitteln". This agar is free of indicators and inhibitors and the growth of all microbial species is possible. The total viable count was examined because it gives an overview about the microbial contamination of the sample and is an important factor in the microbial estimation of examined food samples. Visible colonies between 10 and 300 are counted to calculate the number of colony forming units (cfu).

Table 18: Composition and incubation conditions of plate count agar (Merck, Germany)

Composition	Conc. g/L	Preparation	Incubation conditions			Method
			Time [h]	Temp. [°C]	Environment	
Peptone from casein	5.0	manufacturer instructions	72	30	aerobic	pour plate
Yeast extract	2.5					
D(+) Glucose	1.0					
Agar	14.0					
pH: 7.0 ±0.2 at 25°C						

6.2.2 Determination of lactic acid bacteria (LAB)

The culture medium for the determination of lactic acid bacteria (LAB) is MRS Agar (DE MAN, ROGOSA and SHARPE). The composition and incubation conditions are listed in Table 19. The medium is used to enrich, cultivate and isolate Lactobacilli from different sample material. The composition of the medium, which is optimized to guarantee the growth of Lactobacilli, includes appropriate amounts of nutrients such as sorbates, acetate, magnesium and manganese. Furthermore the marginal selectivity supports the growth of other lactic acid bacteria like *Pediococcus* and *Leuconostoc*. For the enumeration colony counts between 10 and 300 are consulted.

Table 19: Composition and incubation conditions of MRS agar (Merck, Germany)

Composition	Conc. g/L	Incubation conditions		Method
Peptone from casein	10.0	Time [h]	72	pour plate plus overlay
Meat extract	10.0			
Yeast extract	4.0			
D(+) Glucose	20.0			
di-Potassium-hydrogen-Phosphate	2.0	Temp. [°C]	30	
Tween®80	1.0			
di-Ammonia-hydrogen-citrate	2.0			
Sodium-acetate	5.0			
Magnesium-sulphate	0.2	Environment	aerobic	
Manganese-sulphate	0.04			
Agar	14.0			
pH: 5.7 ±0.2 at 25°C				

6.2.3 Determination of yeasts and moulds

Yeast Extract Glucose Chloramphenicol Agar (YGC) is used to perform the examination of yeasts and moulds. The composition is listed in Table 20. This medium is used for isolating and counting yeasts and moulds. YGC Agar includes the antibiotic chloramphenicol to eliminate bacteriological growth.

Table 20: Composition and incubation conditions of YGC agar (Merck, Germany)

Composition	Conc. g/L	Preparation	Incubation conditions			Method
			Time [h]	Temp. [°C]	Environment	
Malt extract	3.0	manufacturer instructions	72	25	aerobic	pour plate
Yeast extract	3.0					
Peptone	5.0					
Dextrose	10.0					
Agar	20.0					
pH: 6.2 ±0.2 at 25°C						

6.2.4 Determination of *Enterobacteriaceae*

Table 21 presents the composition of Violet Red Bile Dextrose Agar (VRBD). This culture medium according to MOSSEL et al., 1962 provides the isolation and enumeration of *Enterobacteriaceae* in food stuffs.

Table 21: Composition and incubation conditions of VRBD agar (Merck, Germany)

Composition	Conc. g/L	Preparation	Incubation conditions			Method
			Time [h]	Temp. [°C]	Environment	
Peptone from meat	7.0	manufacturer instructions	24 - 48	37	aerobic	pour plate plus overlay
Yeast extract	3.0					
Sodium chloride	5.0					
D(+) Glucose	10.0					
Bile salts	1.5					
Neutral-red	0.03					
Crystal-violet	0.002					
Agar	13.0					
pH: 7.3 ±0.2 at 25°C						

The composition (Table 21) conforms the suggestion of the international Organisation for Standardisation (ISO) and regulations from "§64 LMBG zur Untersuchung von Lebensmitteln". Background growth of gram-positive bacteria is inhibited by crystal-violet and bile salts. Glucose degradation with acid accumulation is visible through colour change to red-violet of the colonies and bile salt precipitation around it. The enumeration is done by counting red colonies with and without a reddish precipitation halo around the colonies. Colony numbers between 10 and 300 are counted.

Determination of coliforms and *Escherichia coli*

Different colonies from VRBD Agar are isolated, using a single-use sterile inoculation loop, by spreading on a neutral nutrient culture media (Tryptic Soy Agar (TSA) composition listed in Table 22). After isolation the plates are incubated for 24 h at 37°C at aerobic conditions.

Table 22: Composition of tryptic soy agar (Merck, Germany)

Compostion	Conc. g/L	Preparation
Peptone from casein	15.0	manufacturer instructions
Peptone from soymeal	5.0	
Sodium chloride	5.0	
Agar	15.0	
pH: 7.3 ±0.2 at 25°C		

Colonies growing on tryptic soy agar are tested on their gram characteristic by performing the potassium-hydroxide-test and the oxidase-test (BAUMGART, 2004). KOH-positive (gram-negative), oxidase-negative colonies are used to continue the identification process.

Presumptive colonies from tryptic soy agar are inoculated to the culture medium Fluorocult® Laurylsulfat Broth (LST-MUG medium) to examine the presence of *E. coli* and coliform bacteria. Fluorocult® Laurylsulfate Broth is a selective culture media for testing coliforms and *E. coli*. The composition of this medium is listed in Table 23.

Sodiumlaurylsulfate inhibits the growth of other unrequested bacteria. Gas formation through anaerobic lactose degradation (fermentation) from coliforms including *E. coli* is visible after growth by a gas bubble in the Durham-tube which resides in the broth in the test tube. MUG is degraded by E. coli through the enzyme β-glucuronidase. The product of this degradation is visible by a blue-green fluorescence under UV-light with 366 nm wavelength. The degradation of the aminoacid tryptophan, which is converted to indole by *E. coli*, can be verified by a red colour formation after adding KOVÀCS reagent to the broth.

Table 23: Composition and incubation conditions of Fluorocult® Laurylsulfat broth (Merck, Germany)

Composition	Conc. g/L	Preparation	Incubation conditions			Method
			Time [h]	Temp. [°C]	Environment	
Tryptose	20.0	manufacturer instructions	24 - 48	35	aerobic	Inoculation of the test tube with the broth
Lactose	5.0					
Sodium chloride	5.0					
Sodiumlaurylsulfate	0.1					
di-Potassium-hydrogen-phosphate	2.75					
Potassium-di-hydrogen-phosphate	2.75					
L-Tryptophan	1.0					
4-methylumbelliferyl-β-D-glucuronide (MUG)	0.1					
pH: 6.8 ±0.2 at 25°C						

The evaluation is done after 24 hours of incubation. The test-tubes are transferred into a dark room, respectively a dark box and the fluorescence under the UV-light is tested. Is this test negative, the culture medium is incubated for further 24 hours. By positive fluorescence after 24 h respectively 48 h incubation KOVÀCS reagent is added to the broth (layer about 5

mm thick). The alcoholic solution appears on the surface of the broth and changes it colour to cherry-red, if the test is positive. The presence of coliforms is approved by the gas formation in the Durham-tube. Presence of *E. coli* is approved through gas formation, fluorescence and a positive indole reaction (BAUMGART, 2004) (red colour of the KOVÀCS reagent).

6.2.5 Determination of *Bacillus cereus*

To enumerate *Bacillus cereus* two types of selective culture media were used. The first medium of choice was Polymyxine pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) (Table 24) and the second mannitol egg yolk polymyxine agar (MYP) (Table 25). Both culture media are specified and are allowed to use for food examination according to the regulations of "§64 LMBG zur Untersuchung von Lebensmitteln".

Table 24: Composition and incubation conditions of PEMBA agar (Oxoid, UK)

Composition	Conc. g/L	Preparation	Incubation conditions			Method
			Time [h]	T [°C]	Environment	
Peptone	1.0	manufacturer	48	30	aerobic	spread
Mannite	10.0	instructions				plate
Sodium chloride	2.0					
Magnesium sulfate	0.1					
di-Sodium-hydrogen-phosphate	2.5					
Potassium-di-hydrogen-phosphate	0.25					
Bromothymol blue	0.12					
Sodium pyruvate	10.0					
Agar	14.0					
pH: 7.0 ±0.2 at 25°C						
additional						
Egg yolk emulsion	50 mL/L					
Polymyxin B sulphate	0.00124					

Table 25: Composition and incubation conditions of MYP agar (Merck, Germany)

Composition	Conc. g/L	Preparation	Incubation conditions			Method
			Term [h]	T [°C]	Environment	
Peptone from casein	10.0	manufacturer	48	30	aerobic	spread
Meat extract	1.0	instructions				plate
D(-)Mannite	10.0					
Sodium chloride	10.0					
Phenol red	0.025					
Agar	12.0					
pH: 7.2 ±0.2 at 25°C						
additional						
Egg yolk emulsion	100 mL/L					
Polymyxin B sulphate	0.00124					

Bacillus cereus is not able to degrade mannite, consequently has a mannite-negative reaction on the culture medium. The mannite content in both culture media makes it possible to separate mannite-positive bacteria, which are identifiable through the alteration of the pH-

indicator colour to yellow (PEMBA – bromothymol blue: colour change from green to yellow; MYP – phenol red: colour change from orange to yellow). The additional polymyxin B sulphate inhibits the growth of accompanying bacteria.

For the differentiation of *Bacillus* species from other bacteria the lecithinase activity of *Bacillus* is examined by the degradation of lecithin (in the case of the used media lecithin in the added egg yolk), which is observable by a precipitation zone surrounding the *Bacillus* colonies. Typical colonies within the range of 10 to 300 are counted to enumerate the number of colony forming units.

Typical *Bacillus cereus* colonies appear on PEMBA as irregular, scraggy, 5 mm in diameter big, turquoise to blue coloured colony, surrounded by a egg-yolk precipitation zone with same colour. On MYP agar *Bacillus cereus* appears as rough, dry colony with a pink to purple base which are surrounded by a ring of dense precipitate. Colonies surrounded by a yellow or clear zone are not *Bacillus cereus*.

6.2.6 Determination of *Staphylococcus aureus*

The selective culture medium (Table 26) according to BAIRD-PARKER (1962) is used for isolation and differentiation of staphylococci in food and pharmaceuticals. The media complies with ISO 6888-1:2003 and is used for examination according the food law "§ 64 LFBG" (ANONYMUS f, 2004). Baird-Parker agar is formally used as a selective medium to identify *Staphylococcus aureus*. Additional tellurite and lithium chloride makes the agar highly selective and inhibits most coliforms. Staphylococci colonies show following characteristics: black colour formation as a result of the tellurite-reduction (tellurium is formed) and clear proteolyse zones surrounding the colony (clear halo around the colony). These two characteristics are typically for *Staphylococcus aureus*. After additional incubation to 48 hours opaque rings (lipase activity) within the clear zones are formed but certainly not all strains of *St. aureus* show these two reactions. Evaluation is done by counting typical colonies within the range of 10 to 300 colonies.

Table 26: Composition and incubation conditions of BAIRD-PARKER agar (Merck, Germany)

Composition	Conc. g/L	Preparation	Incubation conditions			Method
			Time [h]	T [°C]	Environment	
Peptone from casein	10.0	manufacturer instructions	48	37	aerobic	spread plate
Meat extract	5.0					
Yeast extract	1.0					
Sodium pyruvate	10.0					
Glycine	12.0					
Lithium chloride	5.0					
Agar	15.0					
pH: 6.8 ±0.2 at 25°C						
additional						
Egg yolk tellurite emulsion	50 mL/L					

Staphylococcus aureus appears as black, shiny, convex colonies, 1 - 5 mm in diameter with a narrow, white edge surrounded by a clear zone. Opaque rings within the clear zone appear after 48 h of incubation.

Staphylococcus epidermis appears as black, shiny colonies with an irregular shape. An opaque zone develops around the colony after 24 h.

Micrococci grow sometimes as very small, brown to black colonies without a clear zone. Dark brown coloured, dull and low shaped colonies characterise the presence of Bacillus and white colonies are typical for yeasts.

6.2.7 Determination of *Clostridium perfringens*

The culture media tryptose sulfite cycloserine agar (TSC) (Table 27) is used for isolation and enumeration of vegetative and spores of *Clostridium perfringens* in food, clinical samples and other examination material. It follows the recommendations, for the examination of food (APHA, 1992) and of the International Organisation for Standardisation (ISO, 2004).

Table 27: Composition and incubation conditions of TSC agar (Merck, Germany)

Composition	Conc. g/L	Preparation	Incubation conditions			Method
			Time [h]	T [°C]	Environment	
Tryptose	15.0	manufacturer instructions	24	37	anaerobic	pour plate
Peptone from soy	5.0					
Yeast extract	5.0					
Sodium-di-sulphite	1.0					
Ammonium ferric citrate	1.0					
Agar	15.0					
pH: 7.6 ±0.2 at 25°C						
additional						
D-Cycloserine	0.4					

TSC agar is prepared for *Clostridium* to get the optimum supply of nutrients. Ammonium ferric citrate and sodium-di-sulphite effect the production of hydrogen sulphide (H_2S) which characterises the bacteria and cause a colour formation to black colonies. Cycloserine inhibits other microbial growth and has influence in forming small colonies. It also reduces a diffuse and thus disturbing black colour formation surrounding the colonies of *Cl. perfringens*. To evaluate the growth all black colonies in the range of 10 to 300 are counted.

6.2.8 Determination of *Brochothrix thermosphacta*

Streptomycin sulphate thallous acetate actidione (STAA) culture medium (Table 28) including the selective supplement according to GARDNER (1966) which contains streptomycin sulphate, thallous acetate and actidione (cycloheximide) makes the medium highly selective against the growth of yeasts and moulds, as well as aerobic and facultative anaerobic bacterial growth. The medium follows the recommendations of ISO 13722:1996 for the examination of meat and meat products.

The evaluation of *Brochothrix thermosphacta* colonies is done by counting, straw-coloured, size of 1 mm diameter colonies which have a growth on agar plates between 10 and 300. *Pseudomonas* species which are able to show growth can be differentiated by oxidase-reaction (BAUMGART, 2004). *Brochothrix thermosphacta* shows a negative reaction.

Table 28: Composition and incubation conditions of STAA agar (OXOID a, 2010) (Oxoid, UK)

Composition	Conc. g/L	Preparation	Incubation conditions			Method
			Time [h]	T [°C]	Environment	
Peptone	20.0	manufacturer instructions	48	25	aerobic	spread plate
Yeast extract	2.0					
di-Potassium-hydrogen-phosphate	1.0					
Magnesium sulphate	1.0					
Agar	13.0					
pH: 7.0 ±0.2 at 25°C						
additional						
Glycerine	7.5					
Streptomycin-sulphate*	0.5					
Thallous acetate*	0.05					
Cycloheximide*	0.05	*: STAA Selective Supplement				

6.2.9 Determination of *Photobacterium* spp.

Photobacterium broth according to DAUDOROFF (1942) and GIESE (1943) is used for cultivation and demonstration of the luminescence of photobacteria. The composition, shown in Table 29 is arranged for the optimum cultivation of photobacteria. Luminescence is affected through chlorides, sulphates, carbonates and glycerophosphate. To create a possibility to isolate and count the photobacteria agar agar is added according BAUMGART (2004) to the photobacterium broth. The evaluation is performed by counting bacteria showing luminescence in the range between 10 and 300.

Table 29: Composition and incubation conditions of Photobacterium agar (FLUKA, 2011)

Composition	Conc. g/L	Preparation	Incubation conditions			Method
			Time [h]	T [°C]	Environment	
Enzymatic hydrolysate of casein	5.0	manufacturer instructions	48	25	aerobic	spread plate
Yeast extract	2.5					
Sodium chloride	30.0					
Ammonium chloride	0.3					
Magnesium sulphate	0.3					
Ferric chloride	0.01					
Calcium carbonate	1.0					
Monopotassium phosphite	3.0					
Sodiumglycero-phosphate	23.5					
Agar No. 1	13.0					
pH: 7.0 ±0.2 at 25°C						

6.2.10 Determination of *Pseudomonas* spp. and *Aeromonas* spp.

Glutamate starch phenol red (GSP) agar (pseudomonas aeromonas selective agar base) according to KIELWEIN (1969; 1971) is used for the determination of *Pseudomonas* and *Aeromonas* in food, materials and processing water. The composition in Table 30 shows glutamate and starch available as nutrients, which are not utilized by other bacteria. Starch is degraded by *Aeromonas* with accumulation of acid, which changes the indicator colour of the culture media from red to yellow (phenol red). Pimaricin and penicillin supplements are added as inhibitors. Pimaricin inhibits the growth of yeasts and is added if the sample is highly contaminated with yeasts, because it could handicap the differentiation between yeasts and *Pseudomonas*. Evaluation is done by counting typical colonies in the range between 10 and 300.

Table 30: Composition and incubation conditions of GSP agar (Merck, Germany)

Composition	Conc. g/L	Preparation	Incubation conditions			Method
			Term [h]	T [°C]	Environment	
Sodium-L(+) - glutamate	10.0	manufacturer instructions	72	25	aerobic	spread plate
Starch soluble	20.0					
Potassium-di-hydrogen-phosphate	2.0					
Magnesium sulphate	0.5					
Phenol red	0.36					
Agar	12.0					
pH: 7.0 ±0.2 at 25°C						
additional:						
Pimaricin	0.01					
Penicillin G Sodium salt	0.0628					

Pseudomonas spp. → 2-3 mm diameter, red-violet colonies with red-violet surrounding area
Aeromonas spp. → 2-3 mm diameter, yellow colonies surrounded by a yellow zone

Isolation and differentiation of colonies from GSP

Different but characteristic colonies are again inoculated on GSP agar and incubated for 72 h at 25°C. A further inoculation on the unselective culture medium TSA follows and after incubation for 48 h at 25°C the confirmation procedure of the colonies is started. Colonies from TSA agar are tested on their oxidase-reaction. *Pseudomonas* and *Aeromonas* show oxidase-positive reaction (BAUMGART, 2004). Certainly some *Pseudomonas* show a negative oxidase-reaction. Consequently oxidase-positive and -negative colonies are used for further identification.

The oxidase-reaction test is followed by gram-staining (BAUMGART, 2004) to determine the morphology of the presumptive colonies. Both bacteria families are gram-negative rods.

Furthermore a test on oxidative and fermentative glucose degradation is carried out. This is done by the so called oxidation-fermentation test (OF-test) according to HUGH and LEIFSON (1953). *Pseudomonas* species are not able to ferment glucose compared to *Aeromonas* species. Table 31 shows the composition of the oxidation-fermentation culture medium which is used for differentiation and classification of gram-negative intestinal bacteria.

Table 31: Composition and incubation conditions of OF medium acc. HUGH and LEIFSON (Merck, Germany)

Composition	Conc. g/L	Preparation	Incubation conditions			Method
			Term [h]	T [°C]	Environment	
Peptone from casein	2.0	manufacturer instructions	48	25	aerobic	stab culture
Yeast extract	1.0					
Sodium chloride	5.0					
di-Potassium-hydrogen-phosphate	0.2					
Bromothymol blue	0.08					
Agar	2.5					
pH: 7.1 ±0.2 at 25°C						
additional:		D(+)-glucose solution is autoclaved and added to 50°C culture medium aseptically				
D(+)-glucose	10.0					

The degradation of glucose is shown by accumulation of acid, which changes the colour of bromothymol blue from green to yellow. The degradation is performed under presence of oxygen (aerobic – oxditative degradation) and hermetically sealed using paraffin oil (anaerobic – fermentative degradation).

Pseudomonas shows glucose degradation only under aerobic conditions, consequently oxidative glucose degradation on the surface of the culture medium. *Aeromonas* shows fermentative glucose degradation – change to yellow colour in the test tube culture medium sealed with paraffin oil.

6.3 Detection of the foodborne pathogen *Listeria monocytogenes*

For the detection of the pathogenic species *Listeria monocytogenes* two different methods were performed. As an alternative method for the detection of *Listeria monocytogenes* VIDAS® LMX – *Listeria monocytogenes* Xpress (BioMerieux, 2009) was performed.

VIDAS® *L. monocytogenes* Xpress (BioMérieux, 2009) is an automatically qualitative analysis of the VIDAS® product line for the detection of *Listeria monocytogenes* in food and environmental samples based on the ELFA technique (Enzyme Linked Fluorescent Assay). This test can be used for the direct screening of *L. monocytogenes* in food (meat, milk products, seafood and vegetables) and environmental samples. Test results can be received within 30 hours.

In parallel the horizontal method for the qualitative detection of *Listeria monocytogenes* in food and feed (EN ISO 11290-1:1996 + AMD 1:2004, Version January, 2005) was performed to ensure the method accordance of the VIDAS® LMX test. This method is used as the reference method for the qualitative examination of *Listeria monocytogenes* in food and feed products with a guaranteed reliability of the results.

6.3.1 Description of the used selective enrichment media and agar based culture media for the detection of *Listeria* spp.

Selective enrichment culture media

Table 32 shows the different enrichment media for the reference and the VIDAS® method performed. For enrichment of *Listeria* from the shrimp samples Fraser-Listeria-Selective-Broth with the composition listed in Table 33 was used.

Table 32: Selective enrichment culture media for Listeria spp. detection method

Enrichment media	Used for method
Half-Fraser broth	Horizontal method
Fraser broth	Horizontal method
Listeria monocytogenes Xpress broth - bottle (225 mL)	VIDAS® *Listeria monocytogenes* Xpress

Table 33: Composition of Fraser-Broth (MERCK, Germany)

Composition	Conc. g/L
Proteose peptone	5.0
Peptone from casein	5.0
Yeast extract	5.0
Meat extract	5.0
Sodium chloride	20.0
Di-sodium-hydrogen-phosphate	9.6
Potassium-di-hydrogen-phosphate	1.35
Aesculin	1.0
Lithium chloride	3.0

Preparation of Half-Fraser and Fraser enrichment broth

55 g Fraser-Listeria-Selective-Broth base is weighted in to prepare one litre of broth. The weighed base is dissolved in 1 L distilled water (UHQ-water) stirred under heating and sterilized under a temperature of 121°C for 15min. The supplements for Half-Fraser and Fraser broth were prepared following the composition listed Table 34. Supplements were added to the broth after cooling down to 50°C, right after autoclaving, by sterile filtration of the supplement solutions.

Table 34: Supplements for the preparation of Half-Fraser and Fraser broth (ISO 11290-1:1996 Appendix B)

Supplement	Preparation		1 L Half-Fraser	1 L Fraser
Nalidixic acid sodium salt solution	Nalidixic acid sodium salt NaOH	0.1 g 10.0 mL	1 mL	2 mL
Acriflavine-hydrochloride-solution	Acriflavin-hydrochloride UHQ water	0.25 g 100.0 mL	5 mL	10 mL
Ammonium ferric citrate solution	Ammonium ferric citrate UHQ water	5.0 g 100.0 mL	10 mL	10 mL

Selective and chromogenic media on agar base

In Table 35 selective and chromogenic media (in form of agar plates) according to the recommendation of the reference method ISO 11290-1:1996 are listed. This media were used for the confirmation procedure of *Listeria* species after the completed enrichment procedure.

Table 35: Selective and chromogenic media for confirmation procedures of *Listeria* species

Culture media	Used for method
Oxoid Chromogenic *Listeria* agar (OCLA)	confirmation of positive samples - VIDAS® and horizontal method
PALCAM agar	confirmation of positive samples - VIDAS® and horizontal method
Columbia agar with 5% sheep blood	Confirmation of haemolysis

Preparation of Oxoid Chromogenic *Listeria* agar (OCLA)

To prepare OCLA 34.5 g of agar base is mixed with 480 mL distilled water, dissolved under heating and sterilized at 121°C for 15 minutes in an autoclave. Two mL sterile distilled water is added to the chromogenic *Listeria* selective supplement and the solution is added aseptically to the sterilized agar base at the temperature of 50°C which follows the Chromogenic *Listeria* Differential Supplement. In Table 36 the composition of OCLA agar base is listed.

Table 36: Composition of OCLA agar base (OXOID b, 2010)

Composition	Conc. g/L	Preparation	
Enzymatic digest of animal tissue	18.0	manufacturer instruction	
Enzymatic digest of casein	6.0		
Sodium pyruvate	2.0	**Selective supplement**	**Conc.[per litre]**
Glucose	2.0	Nalidixic acid	20.0 mg
Magnesium glycerophosphate	1.0	Polymyxin B	76,700 IU
Magnesium sulphate	0.5	Ceftazidime	20.0 mg
Sodium chloride	5.0	Amphotericin	10.0 mg
Yeast extract	10.0		
Lithium chloride	10.0	**Differential supplement**	**Conc.[per litre]**
Di-sodium-hydrogen-phosphate	2.5	Lecithin solution	40 mL
X-glucoside chromogenic mix	0.05		
Agar	12.0		
pH: 7.2 ± 0.2 at 25°C			

The standard method according to EN ISO 11290-1 dictates the use of ALOA agar (Agar Listeria OTTAVIANI and AGOSTI) or similar media. OCLA agar was used because the agar has the same recovery rates as ALOA agar has and its common to use it as alternative (WILLIS et al., 2006). OCLA uses the chromogen X-glucoside for presumptive identification of *Listeria* spp. and it is cleaved by β-glucosidase which is common to all *Listeria* species. Selective agents in the medium such as lithium chloride, polymyxin B and nalidixic acid inhibit other organisms which also possess this enzyme. Amphotericin inhibits the growth of yeasts and molds that may be present in the sample. *Listeria monocytogenes* and pathogenic *Listeria ivanovii* are able to produce the phospholipase enzymes PIPLC (phosphoinositide-phospholipase C) and PCPLC (phosphatidylcholine-phospholipase C) which hydrolyse phosphotidyliositol or lecithin in the medium, producing an opaque halo around the colony to differentiate from other *Listeria* species. In OCLA lecithin is used as supplement, which is detected by PCPLC (OXOID b, 2010).

Preparation of PALCAM agar as a second selective plating medium

To prepare PALCAM agar 34.5 g agar base is mixed with 500 mL distilled water, dissolved and autoclaved at 121°C for 15 minutes. After cooling to 50°C 2 mL sterile distilled water are added to PALCAM Selective Supplement and the solution is added aseptically to the sterilized agar base. In Table 37 the composition of PALCAM agar base is listed.

Table 37: Composition of PALCAM agar base (OXOID c, 2010)

Composition	Conc. g/L	Preparation	
Columbia blood agar base	39.0	manufacturer instruction	
Yeast extract	3.0		
Glucose	0.5	**Selective supplement**	**Conc. [per litre]**
Aesculin	0.8	Polymyxin B	10.0 mg
Ammonium ferric citrate	0.5	Acriflavine HCl	5.0 mg
Mannitol	10.0	Ceftazidime	20.0 mg
Phenol red	0.08		
Lithium chloride	15.0		
pH: 7.2 ± 0.2 at 25°C			

PALCAM agar is based on the formulation described by VAN NETTEN et al. (1989). The agar is highly selective due to the presence of lithium chloride, ceftazidime, polymyxin B and acriflavine hydrochloride. The diagnosis of *Listeria monocytogenes* is utilized by the double indicator system aesculin - ferrous iron and mannitol and phenol red.

Listeria monocytogenes hydrolyses aesculin, which forms a black halo around colonies. Enterococci and staphylococci are able to ferment mannitol, which causes a colour change from red to yellow of the pH indicator phenol red and therefore it is possible to differentiate them from *Listeria monocytogenes*. The influence of incubation and microaerophilic conditions inhibits the growth of strict aerobes, such as *Bacillus* spp. and *Pseudomonas* spp. that are able to grow on the medium (OXOID c, 2010).

Culture media for further examination and for strain storage

For further examination procedures such as gram-staining, oxidase-tests, API® Listeria, Microbact™ tests and for further storage, presumptive colonies were inoculated on following agar media:

Tryptic Soy Agar (TSA)	Inoculation medium for different tests and storage
Tryptic Soy Yeast Extract Agar (TSYEA)	Inoculation medium for different tests and storage

In Table 38 and Table 39 the composition and a description of these agar preparations is presented.

Table 38: Composition and preparation procedure of TSA (Merck, Germany)

Composition	Conc. g/L	Preparation
Peptone from casein	15.0	40 g of agar base are suspended in 1000 mL distilled water, dissolved under heating and autoclaved at 121°C for 15 minutes.
Peptone from soymeal	5.0	
Sodium chloride	5.0	
Agar-agar	15.0	
pH: 7.3 ±0.2 at 25°C		

Table 39: Composition and preparation procedure of TSYEA (ISO 11290-1:1996)

Composition	Conc. g/L	Preparation
Tryptone	17.0	Ingredients are dissolved under heating in 1000 mL distilled water and autoclaved at 121°C for 15 min.
Peptone from soymeal	3.0	
Sodium chloride	5.0	
Di-potassium-phosphate	2.5	
Glucose	2.5	
Yeast extract	6.0	
Agar-agar	13.0	
pH: 7.2 ±0.2 at 25°C		

6.3.2 Detection of *L. monocytogenes* by VIDAS® *L. Monocytogenes* Xpress (LMX)

Test principle

The test is an enzyme immune assay to detect *Listeria* antigens by enzyme linked fluorescent assay (ELFA) method (BioMérieux, 2009). A solid phase receptacle (SPR®) is used as solid phase and pipetting system. The inner surface of the SPR® is coated with anti *L. monocytogenes* antibodies. Testing reagents for the immune reactions are ready to use available in form of reagent locking bar. All reaction steps are performed automatically from the VIDAS® apparatus. The reaction medium is aspirated from the SPR® for several times. A partial amount of enrichment broth is transferred into the reagent locking bar. Antigens from the sample are bonding the anti *L. monocytogenes* antibodies which are fixated on the inner surface of the SPRs (Figure 32). Unbounded sample components are removed by washing. Biotin marked antibodies bond *L. monocytogenes* antigens, which are linked to the antibodies of the SPR® inner surface (BioMérieux, 2009) (Figure 32).

Biotin is detected by incubation with streptavidin which is marked with alkaline phosphatase. Further washing steps remove unlinked parts and during the final washing step the substrate 4-methyl-umbelliferyl-phosphate is aspired by the SPR® and released. The enzyme catalyses the hydrolysis of this substrate to a fluorescing product (4-methyl-umbelliferon) and this fluorescence is measured at a wavelength of 450 nm. Each sample gives a test value, which is compared with saved reference data and the result is displayed as a positive or a negative result (BioMérieux, 2009). After termination of the test results are automatically calculated. For each sample two fluorescence measurements are performed, one to measure the background value of the cuvette and substrate and the second measurement is performed after the incubation of the substrate with the enzyme. The difference of both measurements yields in the relative fluorescence value (RFV). For each sample VIDAS® is calculating the test value with following formula:

$$\text{test value} = \frac{\text{RFV sample}}{\text{RFV standard}}$$

This test value is compared with a limiting value. Is the test value lower than the limit value of 0.05, the sample contents no *L. monocytogenes* antigens. A test value which gives the same or higher result as limit value is contaminated with *L. monocytogenes*.

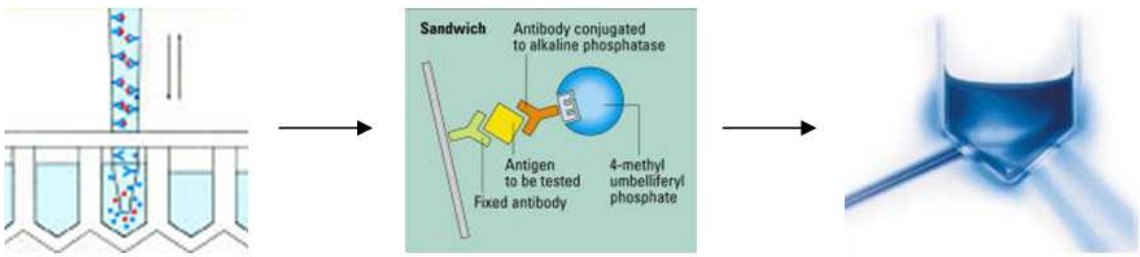


Figure 32: Main principle of the VIDAS® test (BioMérieux, 2009)

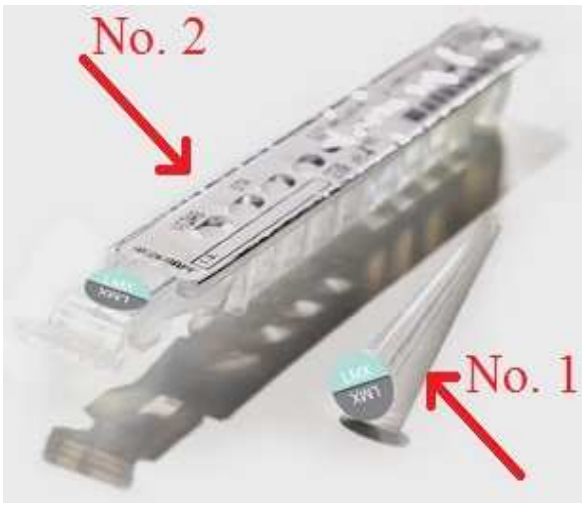


Figure 33: Solid Phase Receptacle and reagent locking bar (BioMérieux, 2009)

Solid Phase Receptacle (SPR®)

The inner surface of an SPR is coated with antibodies, which are vectored against L. monocytogenes antigens (Figure 33, No. 1).

Reagent locking bar

The locking bar has 10 foil sealed cuvettes (vessels, cells). The first cuvette is open for an easier transfer of sample liquid. In the last cuvette the photometric measuring is performed. The inner cuvettes contain different testing reagents (Figure 33, No. 2). In Table 40 the contents of the different cuvettes in the LMX locking bar are described.

Table 40: LMX locking bar contents (BioMérieux, 2009)

Cuvette	Reagent
1	Sample cuvette: 250 µL enrichment broth, standard or control
2	Pre-washing buffer solution (600 µL): sodium chloride solution buffered with TRIS (TBS) (150 mmol/L) – Triton X100 pH 7.6 + preservative
3 – 4 – 7 – 8 - 9	Washing buffer solution (600 µL): sodium chloride solution buffered with TRIS (150 mmol/L) – Tween pH 7.6 + preservative
5	Biotine-marked anti-*L. monocytogenes* antibodies + preservative
6	Streptavidin – AP (400 µL)
10	Measuring cuvette + substrate (300 µL): 4-methyl-umbelliferyl-phosphate (0.6 mmol/L) + diethanolamine (DEA) (0.62 mol/L, pH 9.2) + preservative

VIDAS® *L. Monocytogenes* Xpress procedure steps

The frozen shrimp samples were thawed over night at a temperature of 4°C (12 h) – already prepared stomacher bags with 25 g of shrimp were used for the examination.
Bottles with LMX broth (225 mL) were equilibrated in a waterbath to a temperature of 37 ± 1°C. One bottle (225 mL) of LMX broth was added aseptically to 25 g of shrimp sample, which was already prepared in a stomacher bag and afterwards 500 µL of LMX supplement were added to the broth. From each sample two stomacher bags with 25 g sample were used to perform the test.

The stomacher bag was transferred in a smasher and the sample was mixed for one minute followed by incubation at 37 ± 1°C for 27 ± 1 h. When the incubation was finished the stomacher bags were mixed again and afterwards 0.25 mL of enrichment broth were transferred into the sample cuvette of the reagent locking bar.
Afterwards the locking bars were heated for 5 ± 1 min at 95°C in the VIDAS® Heat and Go apparatus. After the heating step the locking bars were removed from the heating block and cooled for about 10 min. Afterwards the VIDAS® test was performed. Per testing phase 12 samples were tested, which takes about 45 minutes.

The enrichment broth was stored at 4°C meanwhile and for the confirmation of positive results, after the VIDAS® test. Before the test start of mini VIDAS® master lot entry (MLE) has to be entered by MLE card. Afterwards the calibration mini VIDAS® was performed, which is needed every 28 days. A standard reagent S1 has to be tested twice.

Controls C1 and C2 are measured once. The standard value has to be in the range of the relative fluorescence value (RFV). The performing procedure of the VIDAS® test is described in the VIDAS® *L. monocytogenes* Xpress (LMX) protocol REF 30 123 14226 - de - 2009/11.

Confirmation procedure

All enrichment broths from every single sample were used for the confirmation procedure, even though there was just one positive result of the VIDAS® tests. One OCLA and one PALCAM agar was inoculated with enrichment broth from every sample and incubated for 48 hours at 37 ± 1°C.

Following identification steps of positive growth on the selective media, which were performed, are specified in the description of the standard method according to EN ISO 11290-1:1996.

6.3.3 Detection of *L. monocytogenes* according to the horizontal method (ISO 11290-1:1996)

The frozen shrimp samples were thawed over night at a temperature of 4°C (12 h) – already prepared stomacher bags with 25 g of shrimp were used for the examination. For this detection method two weighted samples were used.

Pre-enrichment

25 g (1 part) of shrimp sample in a stomacher bag were blended with 225 mL (9 parts) Half-Fraser broth (lower concentration of selective compounds – see Table 33), which was aseptically added to the sample. Afterwards the stomacher bag with the dilution was mixed for 1 min in the smasher. The stomacher bags were closed with clips and incubated for 24 ± 2 hours at 30°C.

Selective enrichment

After incubation of the first enrichments for 24 hours, the stomacher bags were again mixed in the smasher and 0.1 mL of broth was transferred in a culture tube containing 10 mL Fraser broth. The inoculated Fraser broth tubes were incubated for 48 ± 2 hours at 37°C.

Inoculation on selective culture media

An inoculation on OCLA and PALCAM medium using an inoculation loop from the pre-enrichment broth (Half-Fraser) was performed. The agar plates were incubated for 48 hours at 37°C. Also from the second enrichment step (Fraser broth) broth was transferred by an inoculation loop and streaked on OCLA and PALCAM plates. These plates were incubated for 48 h at 37°C as well.

Evaluation of the agar plates

Listeria spp. appear on OCLA as blue-turquoise colonies with and without opaque white halo around the colony. On PALCAM typical *Listeria* form after 48 h incubation colonies that are approximately 2 mm in diameter, have a grey green colour with a black sunken centre and a black halo against a cherry-red medium background. *Enterococci* or *Staphylococci* form on PALCAM medium grey colonies with a brown-green halo or yellow colonies with a yellow halo. *L. monocytogenes* and *L. ivanovii* form on OCLA blue-turquoise colonies with an opaque halo around the colony.

Isolation and evaluation of presumptive *Listeria* spp.

For the confirmation of *Listeria* species five, or if there are less colonies all, colonies are transferred with an inoculation loop to tryptic soy agar (TSA) or tryptic soy yeast extract agar (TSYEA) and incubated for 24 h at 37°C.

After 24 hours of incubation, the colonies were used to perform oxidase and catalase tests followed by gram staining of the bacteria. *Listeria* spp. are gram positive short rods. The oxidase reaction is negative and catalase test shows a positive reaction. Furthermore a haemolysis test on Columbia sheepblood agar medium with beta lysin discs (Remel, USA) was performed instead of a CAMP test which is recommended in EN ISO 11290-1:1996.

The final identification step was done by biochemical systems, such as API® Listeria (BioMérieux, France) and Microbact™ Listeria 12L (Oxoid, UK).

Oxidase test: The procedure of this test was performed according BAUMGART, 2004. *Listeria* spp. are oxidase negative bacteria.

Catalase test: One *Listeria* colony is picked up with an inoculation loop from TSA and transferred on a microscope slide into a drop of hydrogen peroxide (3%). The catalase reaction is positive when gas bubbles are formed (O_2) (BAUMGART, 2004). *Listeria* spp. are catalase positive.

Gram staining: The procedure was performed according BAUMGART (2004).

Haemolysis test with beta lysin discs

One β-lysin disc (Remel, USA) is placed in the middle of a plate of Columbia agar with 5% sheep blood (BioMérieux, France). The plate should be allowed to equilibrate at room temperature before placing the disc. Afterwards the colonies which are tested are streaked on the agar plate star-shaped around the β-lysin disc with 2-3 mm distance to the disc. After all colonies were streaked on the plate, the medium was incubated at 35°C for 24 h. After the incubation the plates were observed. A crescent-shaped zone of haemolysis at the intersect point of the disc and the test isolate indicates a positive test.

6.3.4 API® Listeria (BioMérieux, France)

API® Listeria is a standardized system for the identification of *Listeria*, using miniaturized tests and a database. The strip consists of 10 microtubes containing dehydrated substrates which allow the performing of enzymatic tests and sugar fermentations.

Proceeding steps

- the incubation box is prepared and about 3 mL distilled water is distributed to the honeycombed wells of the tray to create a humid atmosphere
- the API strip is placed into the incubation box
- 24 h old well-isolated colonies are transferred with an inoculation loop in 2 mL API suspension medium to prepare a suspension with a turbidity equivalent to 1 McFarland
- with a PSIpette (BioMérieux, France) the bacterial suspension is distributed into each tube of the API strip avoiding the formation of bubbles
- at the DIM test the tube and the cupule is filled avoiding a convex meniscus of the bacterial suspension – in all other test just the tube is filled
- the incubation box is closed and incubated for 18 to 24 h at 37°C

Before reading the strip a drop of ZYM B reagent is added to the DIM test and then all reactions are read within 3 minutes by referring to the reading table which is included in the API® Listeria manual. The results are listed in a result sheet. The identification is performed using the apiweb™ (Apiweb™, 2011) identification software, where the 4-digit numerical profile is entered manually. Figure 34 shows the reading table of an API® Listeria test.

READING TABLE

TESTS	ACTIVE INGREDIENTS	QTY (mg/cup.)	REACTIONS	RESULTS	
				NEGATIVE	POSITIVE
				ZYM B / < 3 min	
DIM	Enzymatic substrate	0.106	Differentiation *L. innocua* / *L. monocytogenes*	pale orange pink beige grey beige	orange
ESC	Esculin Ferric citrate	0.16 0.024	hydrolysis (ESCulin)	pale yellow	black
αMAN	4-nitrophenyl-αD-mannopyranoside	0.045	α-MANnosidase	colorless	yellow
DARL	D-ArabitoL	0.4	acidification (D-ARabitoL)	red / orange-red	yellow / yellow-orange
XYL	D-Xylose	0.4	acidification (XYLose)		
RHA	L-Rhamnose	0.4	acidification (RHAmnose)		
MDG	Methyl-αD-glucopyranoside	0.4	acidification (Methyl-αD-Glucopyranoside)		
RIB	D-Ribose	0.4	acidification (RIBose)		
G1P	Glucose-1-Phosphate	0.4	acidification (Glucose-1-Phosphate)		
TAG	D-Tagatose	0.4	acidification (TAGatose)		

- The quantities indicated may be adjusted depending on the titer of the raw materials used.
- Certain cupules contain products of animal origin, notably peptones.

Figure 34: Reading table of the API Listeria test (BioMérieux, France)

6.3.5 Microbact ™ Listeria 12L (Oxoid, UK)

This is a standardised micro-substrate system designed to simulate conventional biochemical substrates used for the identification of *Listeria* spp. The identification strip consists of 12 tests (11 sugar utilisation and one haemolysis test). The reactions occurring during the incubation period are demonstrated through either a colour change in the sugar utilisation tests or in the lysis of sheep red blood cells in the haemolysis test.

Proceeding steps

For an 18 – 24 hour test a single 24 h old colony is transferred with an inoculation loop into suspending medium and mixed to prepare a homogenous suspension. For a 4 hour test 4 to 5 colonies are used to prepare the bacterial suspension.

- a test strip is prepared and the haemolysis reagent is warmed to room temperature
- the lid of the test strip is removed and using a sterile pipette 4 drops of bacterial suspension are placed into each well
- one drop of haemolysis reagent is added to well number 12 and the lid is replaced
- one drop of the inoculum is streaked on a non-selective medium (TSA) for a purity check
- strip and the agar plate for the purity check are incubated for 18 to 24 h at 35 ± 2°C

The strip is removed from the incubator to read the reaction results. A table of reactions and a colour chart is referred to interpret the results and these are recorded in report form. The identification is performed using the Microbact™ identification software, where the 4-digit octal code is entered manually. The code number is the sum of indices of the positive reaction in groups of three reactions. Figure 35 shows an example of a Microbact ™ result and Figure 36 shows a colour chart of the colour changes during reactions of the Microbact™ test.

	Oxidase	Catalase	Latex Agglut.	Esculin	Mannitol	Xylose	Arabitol	Ribose	Rhamnose	Trehalose	Tagatose	Gluc-1-Phos	M-D-Gluc	M-D-Man	Haemolysis
Result	-	+	+	+	-	+	+	+	-	+	-	+	+	+	+
				4	2	1	4	2	1	4	2	1	4	2	1
Sum				5			6			5			7		

Microbact™ code = 5657

Figure 35: Example result of a Microbact™ test (Oxoid, UK)

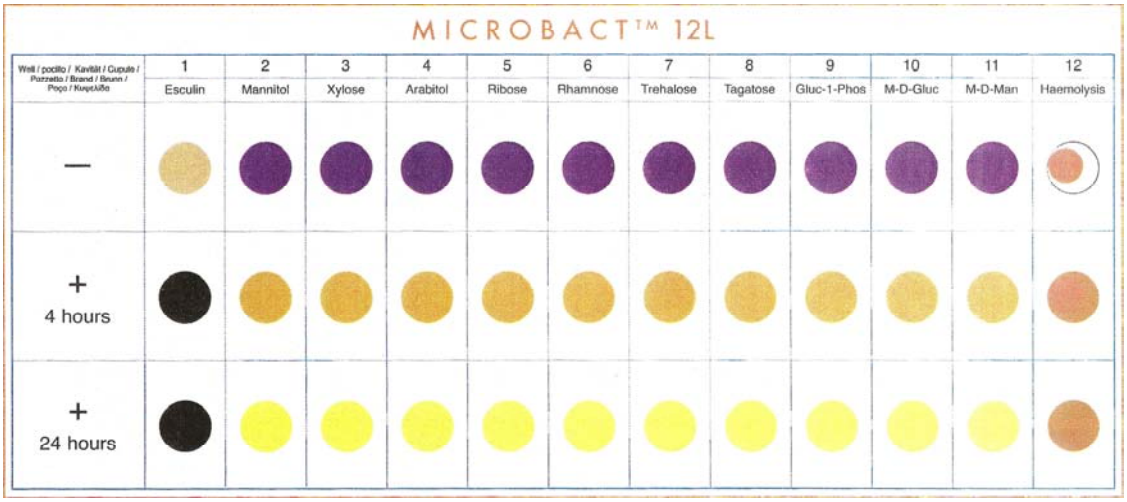


Figure 36: Colour chart showing the colour change of positive reactions of the Microbact™ test (Oxoid, UK)

6.3.6 Comparison of qualitative methods

The alternative method of *Listeria monocytogenes* detection by VIDAS® *L. monocytogenes* Xpress has to be verified to check the effectiveness of the method. The aim is to get results which are comparable to the standard reference method for the detection of *L. monocytogenes*, so that it is a guaranteed that the alternative method (VIDAS®) is able to detect *L. monocytogenes* in the shrimp samples.

The international standard ISO 16140 Microbiology of food and animal feeding stuffs – protocol for the validation of alternative methods offers protocols for the validation of qualitative and quantitative methods (ANONYMUS e, 2003). AOAC International developed "Guidelines for Validation of Qualitative and Quantitative Food Microbiological Official Methods of Analysis. These guidelines describe the unique steps of the validation and are an initiative for the harmonisation of validation methods of ISO 16140 (FELDSINE et al., 2002)

For further theoretical background and descriptions about validation problems and potential for advancement of the validation methods it is relegated to check the diploma thesis of Isolde Ebersdorfer (EBERSDORFER, 2009) where the method comparison is described.

Method comparison study

According following parameters, presented in Table 42, the comparison of VIDAS® *L. monocytogenes* Xpress with the reference method of ISO 11290-1:1996 is performed. The data of the paired results of the reference and alternative method is tabulated as seen in Table 40.

Table 41: Paired results of the reference and alternative method

Responses	Reference method positive (R+)	Reference method negative (R-)
Alternative method positive (A+)	+/+ positive agreement (PA)	-/+ positive deviation (PD) (R-/A+)
Alternative method negative (A-)	+/- negative deviation (ND) (A-/R+)	-/- negative agreement (NA)

Out of the paired results which are arranged according Table 41 the parameters according Table 42 are calculated.

Table 42: Parameter for the comparison of methods (

Parameter	Formula	Description
Relative accuracy (AC)	$AC = \frac{(PA + NA)}{N} \times 100\%$	Degree of the correspondence between the response obtained by the reference method and the alternative method.
Relative sensitivity (SE)	$SE = \frac{PA}{N_+} \times 100\%$	Ability of the alternative method to detect the analyte when it is detected by the reference method.
Relative specificity (SP)	$SP = \frac{NA}{N_-} \times 100\%$	Ability of the alternative method to not detect the analyte when it is not detected by the reference method

False positive rate	pf+ = 100 – SP	Alternative method shows a positive, the reference method a negative result
False negative rate	pf- = 100 – SE	Alternative method shows a negative, the reference method a positive result
Relative detection level	Smallest number of culturable microorganisms that can be detected in the sample in 50% of occasions by the alternative and reference method	

PA = number of positive agreement of the methods
NA = number of negative agreement
PD = number of positive deviation
ND = number of negative deviation
N+ = is the total number of positive results with the reference method (PA + ND)
N- = is the total number of negative results with the reference method (NA + PD)
N = is the total number of samples (NA + PA + PD + ND)

Table 43 shows different tests for the estimation of discordant results. After the results of the comparison parameter are calculated follows the decision which test for the estimation of discordant results will be performed.

Table 43: Tests for the estimation of discordant results

(PD + ND = Y) < 6	**6 ≤ (PD + ND = Y) ≤ 22**	**(PD + ND = Y) > 22**
no test required	Binomial theorem: if m ≤ M (Y), then 2 methods are different at α < 0.05	Mc Nemar test: $X^2 = \frac{\lvert PD - ND \rvert^2}{PD + ND}$ if $X^2 > 3.841$ than methods are different at α < 0.05

6.4 Antibiotic susceptibility testing according to the guideline of the Clinical and Laboratory Standard Institute - CLSI (formerly NCCLS)

The antibiotic susceptibility tests were performed following the guideline of the NCCLS (National Committee for Clinical Laboratory Standards) document M2 – M7 (2000): Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard – Seventh Edition (CLSI a, 2000).

6.4.1 Agar well diffusion method

For the agar well diffusion method preferably fresh prepared Mueller Hinton agar plates (Table 45) were used. The procedure starts by inoculation the agar surface with certain *Listeria* strain suspensions by using a swab. Antibiotic discs which have a definite antibiotic concentration are transferred on the agar surface. During the incubation time the antibiotic diffuses into the agar and a concentration gradient is developed around the antibiotic disc, with the highest antibiotic concentration near by the antibiotic disc.

The bacteria are growing during the incubation time according their sensibility to the antibiotics. Is the strain sensible to certain antibiotic, there is no growth around the antibiotic disc and an inhibition zone is appearing. Another characteristic of antibiotic susceptibility of tested strains is an intermediate behaviour (moderate sensitivity) and a resistant behaviour to the antibiotic (no effect of the antibiotic to the bacterial conditions).

These three characteristic (sensible, intermediate, resistant) have to be analysed by measuring the inhibition zone diameters around the antibiotic discs. Zone diameter breakpoints for these categories of behaviour are available from CLSI standards and from EUCAST standards (European Committee on Antimicrobial Susceptibility Testing).

Zone diameter breakpoints for *Listeria* spp. have been chosen according a study (HANSEN et al., 2005). Until now no *Listeria* specific breakpoints have been defined. In the study of HANSEN et al., 2005 breakpoints of staphylococci were used for the characterisation of antibiotic susceptibility tests of *Listeria* strains.

The breakpoints which were used for this examination were chosen from CLSI standard for *Staphylococcus* spp. Vol. 29, No. 3, Table 2C, M02 and M07 (CLSI b, 2009) and from EUCAST Clinical Breakpoint Table 1.0 2009-12-22 (EUCAST, 2009). Breakpoints for the antibiotic vancomycin were chosen from *Enterococcus* spp. because of no availability of breakpoints from *Staphylococcus* spp. (Table 44).

Table 44: Used antibiotics for the agar well diffusion method and zone diameters

Antibiotic (Oxoid, UK)	Zone diameter			Strain	Reference
	S (sensible)	I (intermediate)	R (resistant)		
Clindamycin 2 µg	>=21	15-20	<=14	Staph. aureus	CLSI Standard Breakpoints
Linezolid 30 µg	>=21	-	-	Staph. aureus	CLSI Standard Breakpoints
Ciprofloxacin 5 µg	>=21	16-20	<=15	Staph. aureus	CLSI Standard Breakpoints
Ampicillin 10 µg	>=29	-	<=28	Staph. aureus	CLSI Standard Breakpoints
Rifampicin 5 µg	>=20	17-19	<=16	Staph. aureus	CLSI Standard Breakpoints
TMP-SMX 1,25 µg-23,75µg	>=16	11-15	<=10	Staph. aureus	CLSI Standard Breakpoints
Vancomycin 30 µg	>=17	15-16	<=14	Enterococcus spp.	CLSI Standard Breakpoints
Tetracycline 30 µg	>=19	15-18	<=14	Staph. aureus	CLSI Standard Breakpoints
Chloramphenicol 30 µg	>=18	13-17	<=12	Staph. aureus	CLSI Standard Breakpoints
Erythromycin 15 µg	>=21	-	<=18	Staph. aureus	EUCAST Breakpoints
Oxacillin 1µg	>=13	11-12	<=10	Staph. aureus	CLSI Standard Breakpoints

6.4.2 Procedure steps of the antibiotic susceptibility test

Preparation of Mueller Hinton agar

The Mueller Hinton agar with the composition shown in Table 45 was prepared according the manufacturer instructions and after the sterilisation plates with a consistent agar depth of 3 – 4 mm were prepared. That was approved by weighing the agar plates during filling it with the agar.

Table 45: Composition of Mueller Hinton agar (

Composition	Conc. g/L
Beef infusion	2.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
pH: 7.3 ± 0.2 at 25°C	

Preparation of the inoculum

Brain heart infusion broth was prepared according the manufacturer instructions (composition is shown in Table 46) and after the sterilisation aseptically filled with a volume of 5 mL into small sterile closable tubes with a volume of about 7 mL. One colony of a *Listeria* strain from TSA, or bacterial suspension from a thawed cryo-culture was transferred using an inoculation loop into sterile small closable tubes with brain heart infusion broth. Afterwards the BHI broths were incubated for 18 – 24 h at 37°C. The incubation should result in a bacterial suspension with a turbidity of 0.5 McFarland which is a bacterial count of about 10^6 - 10^7 cfu per mL. The same procedure was performed with the quality control strains.

Table 46: Composition of Brain Heart Infusion broth (bioMérieux, France)

Composition	Conc. g/L
Nutrient substrate (brain infusion, heart infusion, peptone)	27.5
Glucose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
pH: 7.0 ± 0.2 at 25°C	

Antibiotic susceptibility test performance

The antibiotic discs (Oxoid, UK) are removed from the deep frozen storage and warmed to room temperature for at least 30 min before starting the work. Afterwards the antibiotic discs can be prepared in the disc dispenser (Oxoid, UK).
Mueller Hinton agar plates were prepared and warmed to room temperature. Within 15 min the bacterial suspension was spread over the entire surface of the agar using a sterile swab. After the inoculation of the agar surface antibiotic discs were stamped on the agar surface. The space between the antibiotic discs should be equal and allow a full inhibition zone between the discs. Minimum space should be 24 mm. Therefore just 4 discs were stamped in one Mueller Hinton agar plate.

Afterwards all agar plates were incubated for 18 - 24 h at 37°C. For a sterility control an empty agar plate was incubated too. During the incubation the agar plates should be arranged in a pile of a maximum of five to six plates.

Evaluation of the incubated agar plates

A constant bacterial growth over the agar surface allowed the examination of the agar plates. Without any or weak growth on the plates an examination was not possible. The inhibition zones were visible as clear zones around the antibiotics discs (Figure 42). Using a calliper rule the entire inhibition zone diameters were measured including the diameter of the discs and of the inhibition zones.

Flow chart of the antibiotic susceptibility test procedure steps

Figure 37: Inoculation tubes in the incubation chamber

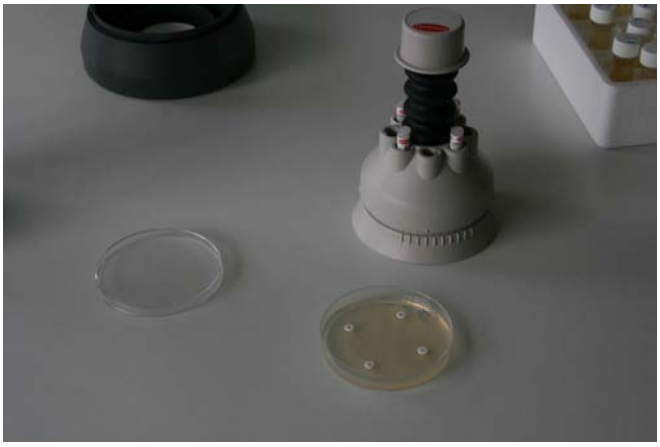
Listeria isolates and reference strains are inoculated in BHI and incubated for 18 - 24 h at 37°C.

Inoculated and incubated Brain Heart Infusion broth, a disc dispenser (Oxoid, UK), Mueller Hinton agar plate and a sterile swab is prepared.

Figure 38: Preparation before starting the inoculation

The inoculum is spread over the entire agar surface by using a sterile swab.

Figure 39: Inoculation of the agar surface by swabbing



Antibiotic discs are stamped on the agar surface. On one Mueller Hinton agar plate a maximum number of four different antibiotics discs were stamped on the agar.

Figure 40: Antibiotic discs stamped on the agar surface

After incubation for 18 - 24 h at 37°C the inhibition zone diameters are measured by using a calliper rule.

Figure 41: Measuring of the inhibition zone diameters

Quality control of *St. aureus* ATCC 25923.

Figure 42: Inhibition zones of *St. aureus* ATCC 25923

7 Results

Overall 58 different shrimp samples which are sold in Austria in retail markets were examined. In the microbial screening the presence of different bacteria families and groups (e.g. total viable count, lactic acid bacteria, *Enterobacteriaceae*, yeasts, molds, *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas sp.*, *Aeromonas sp.*, *Clostridium perfringens*, *Brochothrix thermosphacta*) were examined additionally *Listeria* species were detected and isolated and used for antimicrobial disc susceptibility tests, which followed the microbiological screening.

In following tables the bacterial counts of the shrimp samples are categorized into the three different convenience degree stages blanched, cooked and raw frozen. The bacterial counts are divided in these categories because of the different processing procedures with or without heat treatment and therefore significant different bacterial counts are expected. The bacterial counts are specified as colony forming unit per gram.

Resulted bacterial counts are compared with the recommended limits of cfu/g according to different recommendations for the microbial quality of fish and seafood products. The microbiological limits are specified by two limit designations. The letter "**m**" specifies the maximum recommended bacterial counts for good quality products and the letter "**M**" presents the value of the maximum recommended bacterial counts for marginally acceptable quality products (ICMSF, 1986).

Plate counts below "m" are considered good quality. Plate counts between "m" and "M" are considered marginally acceptable quality, but can be accepted if the number of samples does not exceed "**c**" The letter "c" specifies the maximum number of acceptable sample units with bacterial counts between "m" and "M" within "**n**" which is the number of representative sample units (ICMSF, 1986). Plate counts at or above "M" are considered unacceptable quality (ICMSF, 1986).

7.1 Results of the microbiological examinations

7.1.1 Examination of the total viable count (TVC)

The bacterial counts of TVC varied in high factors (10^1 to 10^5) between the different convenience degrees of the shrimp samples (Table 47). These differences in the counts of TVC may depend on the different procedure and storage conditions. The lowest TVC was examined in a cooked shrimp sample with the identification K-01 with a bacterial count of 1.3 x 10^2 cfu/g the highest count was detected in a raw frozen sample with a value of 1.2 x 10^7 cfu/g (sample CN-07 – appendix Table 63). In Table 47 the TVC of the shrimp samples according their convenience degrees are listed. Within the convenience degrees the factors of the different bacterial counts ranged between 10^3 and 10^4 cfu/g.

Table 47: Results of the total viable count (TVC)

Shrimp samples (n = 58)	Number of colony forming units on plate count agar
Blanched	1.9 x 10^2 – 8.4 x 10^5
Cooked	1.3 x 10^2 – 2.7 x 10^5
Raw frozen	2.4 x 10^3 – 1.2 x 10^7

7.1.2 Examination of lactic acid bacteria (LAB)

Table 48 shows the bacterial counts of LAB detected in the shrimp samples. The bacterial counts of LAB show high differences within the convenience degrees. The counts are ranging from less than 10 cfu/g to 6.1 x 10^3 cfu/g (sample M-09 – appendix Table 63) in blanched shrimp samples. Contrary to the expectation the bacterial count in blanched and cooked samples resulted in relatively high numbers. The highest count of cooked shrimps reached sample QF-03 with a value of 2.2 x 10^4 cfu/g. In raw frozen shrimp samples the highest bacterial count was expected and the results ranged from a value of 1.0 x 10^1 (sample KP-01 – appendix Table 63) to 1.1 x 10^7 cfu/g (sample CN-07 – appendix Table 63).

Table 48: Bacterial counts of the examination of lactic acid bacteria (LAB)

Shrimp samples (n = 58)	Number of colony forming units on MRS agar
Blanched	< 1.0 x 10^1 – 6.1 x 10^3
Cooked	< 1.0 x 10^1 – 2.2 x 10^4
Raw frozen	< 1.0 x 10^1 – 1.1 x 10^7

7.1.3 Examination of yeasts and moulds

The count of colony forming units of yeasts and moulds divided in the different convenience degrees of the samples are presented in Table 49. Summarised, the counts of yeasts and moulds determined have a relatively low value. As expected the counts of heat treated products are lower compared to raw frozen shrimp products. The cfu count of blanched samples does not reach a higher value of 10^2 cfu/g for yeasts and moulds. In cooked shrimp samples the highest amount of yeasts was examined in sample RA-02 (appendix - Table 63). The colony count of moulds in cooked samples does not reach 10 cfu/g. In raw frozen products the colony count of moulds keeps the low value. The count just reaches a number less than 10^2 cfu/g. The colony count of yeast in raw frozen products is higher,

whether not significantly. It reaches a value of 9.1 x 10^2 cfu/g in sample CN-23 (appendix - Table 63).

Table 49: Microbial counts of the examination of yeasts and moulds

Shrimp samples (n = 58)	Number of colony forming units on YGC agar	
	Yeasts	Moulds
Blanched	< 1.0 x 10^1 – < 1.0 x 10^2	< 1.0 x 10^1 – < 1.0 x 10^2
Cooked	< 1.0 x 10^1 – 4.9 x 10^2	< 1.0 x 10^1
Raw frozen	< 1.0 x 10^2 – 9.1 x 10^2	< 1.0 x 10^1 – < 1.0 x 10^2

7.1.4 Examination of *Staphylococcus aureus*

In two different shrimp samples with the identification codes CN-14 (cooked) and CN-24 (raw) (appendix - Table 63) growth of presumptive Staphylococcus aureus was observed. In total three presumptive colonies were isolated from these two samples.

For the maximum recommended bacterial counts of *St. aureus* for marginally acceptable quality products (M) of frozen cooked crustaceans no value is defined according to ICMSF (1986) but certainly a maximum microbial count for good quality products (m) of frozen cooked crustaceans for *St. aureus* is defined at a value of 1 x 10^3 cfu/g. The cooked shrimp sample CN-14 has a microbial count of < 10^3 cfu/g and do not exceed a critical value. Sample CN-24 which is a raw frozen product resulted in the same microbial count of *St. aureus* of < 10^3 cfu/g. The "M" value of *St. aureus* of frozen raw products is defined according ICMSF (1986) at a value of 1 x 10^4 cfu/g. Consequently this microbial count does not exceed the critical value.

7.1.5 Examination of *Bacillus cereus*

Bacillus cereus was examined using two different selective media – PEMBA and MYP. The confirmation of *Bacillus cereus* using these two media is done by the colony appearance, a lecithin precipitation and a negative mannitol reaction. The mannitol content allows the differentiation of mannitol-positive microbial flora easily from mannitol-positive Bacillus cereus. In total in two samples with the identifications CN-02 (sample of cooked shrimp) (appendix Table 63) and M-11 (raw shrimp) (appendix Table 63) presumptive *Bacillus cereus* shows growth. The isolates were determined by gram-staining and a microscopic examination for the presence of lipid globules in the vegetative cells by a rapid confirmatory staining procedure according to HOLBROOK and ANDERSON, 1980.

The guideline levels of *B. cereus* in ready-to-eat food of the Food Standard of Australia and New Zealand (FSANZ, 2001) recommend a marginal quality of ready-to-eat foods with a bacterial count of *B. cereus* of 10^2-10^3 cfu/g. The unsatisfactory level is between 10^3 and 10^4 cfu/g. The bacterial counts of *B. cereus* of the positive shrimp samples show a value of < 1 x 10^3 cfu/g, so the unsatisfactory level is not exceeded in the case of these two samples.

7.1.6 Examination of *Brochothrix thermosphacta*

Brochothrix thermosphacta was examined using the selective STAA medium. The medium shows basically growth of *Brochothrix*. Pseudomonas species which are able to grow on STAA agar were differentiated by a positive oxidase-reaction, compared to *Brochothrix thermosphacta* which is oxidase negative, and by gram staining (*Brochothrix thermosphacta* is gram-positive). In samples with a higher count of Pseudomonas species the accompanied growth caused problems on plates with high colony counts, so these differentiation procedures were performed. The geometric mean of the cfu counts of the different convenience degrees of the samples are shown in Table 50. The different bacterial counts show a significant difference in their values between the convenience degrees of the shrimp products. In cooked samples the bacterial count reached a level of 4.5×10^3 cfu/g in sample QF-02 (appendix Table 63). Blanched shrimp samples had a general higher bacterial count by factor 10. A value of 5.2×10^4 cfu/g was reached by sample M-09 (appendix Table 63). The bacterial counts of raw frozen products reached the highest values in the level of 10^5 cfu/g (sample M-10 reached 5.4×10^5 cfu/g – Table 63) but also 10 raw frozen samples had a colony less than 10^2 cfu/g.

Table 50: Bacterial counts of the examination of *Brochothrix thermosphacta*

Shrimp samples (n = 58)	Number of colony forming units on STAA agar
Blanched	$< 1.0 \times 10^2 – 5.2 \times 10^4$
Cooked	$< 1.0 \times 10^2 – 4.5 \times 10^3$
Raw frozen	$< 1.0 \times 10^2 – 5.4 \times 10^5$

7.1.7 Examination of *Clostridium perfringens*

Presumptive colonies of *Clostridium perfringens* could be examined in four samples. The highest count was 4.9×10^2 cfu/g of a cooked shrimp sample (QF-02). One sample was blanched (M-05) and two were raw frozen products (sample M-04 and M-08). The value of the bacterial counts in these three samples was $< 1.0 \times 10^2$ cfu/g. This value is in the satisfactory level ($< 10^2$ cfu/g) of the bacterial counts according to the guidelines for microbiological examination of ready-to-eat foods in Australia and New Zealand (FSANZ, 2001). Furthermore the highest count of *Cl. perfringens* with 4.9×10^2 cfu/g (QF-02) is in the marginal range of 10^2-10^3 cfu/g according these guidelines (FSANZ, 2001).

7.1.8 Examination of *Enterobacteriaceae*

The examination was performed using VRBD medium with overlay. In total 58 samples were analysed. 41 samples showed no growth after 48 hours of incubation at 37°C. Summarised 5 blanched, 14 cooked and 22 raw frozen samples no growth of *Enterobacteriaceae* occurred (appendix Table 63). In Table 51 the different bacterial count ranges of the shrimp samples according their convenience degrees are listed.

The highest colony count of *Enterobacteriaceae* was found in one raw frozen sample with a count of 9.7×10^2 cfu/g (sample . This colony count is lower than the critical /guidance value of 1.0×10^4 cfu/g in sea fish (DGHM, 2010).

Table 51: Bacterial counts of the examination of *Enterobacteriaceae*

Shrimp samples (n = 58)	Number of colony forming units on VRBD agar
Blanched	$< 1.0 \times 10^1 – 2.4 \times 10^2$
Cooked	$< 1.0 \times 10^1 – < 1.0 \times 10^2$
Raw frozen	$< 1.0 \times 10^1 – 9.7 \times 10^2$

Confirmation of coliform bacteria and *E. coli*

Isolates (n = 95) from VRBD agar medium were streaked out on unselective tryptic-soy-agar and incubated at 37°C for 24 - 48 hours. Isolates which showed a positive KOH reaction (gram-negative bacteria) and additionally a negative oxidase reaction were further inoculated in Fluorocult® Lauryl Sulfate Broth. The selective broths were incubated for 16 - 24 hours at 35-37°C.

After incubation of the Lauryl Sulfate broth, 33 isolates showed growth in the test tubes and gas formation because of a degradation of lactose. Three isolates have shown fluorescence and a positive indole reaction indicating the presence of E. coli.
In total three presumptive *E. coli* and 30 coliform bacteria species were identified. A further identification by the biochemical test system Api® 20 E (BioMérieux) was planned. Unfortunately the isolates could not be reanimated after storage which was unavoidable during the work, so it was not possible to perform the identification by Api® 20 E.

7.1.9 Examination of *Pseudomonas* spp. and *Aeromonas* spp.

The colony counts of presumptive *Pseudomonas* spp. of the 58 shrimp samples in total have not reached the critical value of 1.0×10^6 cfu/g for sea fish (DGHM, 2010). For *Aeromonas* spp. no critical value of the microbial load was available. In Table 52 the geometric mean of the bacterial counts of the shrimp samples in their different convenience degree is presented.

Table 52: Bacterial counts *Pseudomonas* spp. and *Aeromonas* spp.

Shrimp samples (n = 58)	Number of colony forming units on GSP agar	
	***Pseudomonas* spp.**	***Aeromonas* spp.**
Blanched	$< 1.0 \times 10^2 – 5.2 \times 10^3$	$< 1.0 \times 10^2$
Cooked	$< 1.0 \times 10^2 – 1.7 \times 10^3$	$< 1.0 \times 10^2$
Raw frozen	$< 1.0 \times 10^2 – 1.0 \times 10^4$	$< 1.0 \times 10^2$

Summarised the examination of *Pseudomonas* spp. and *Aeromonas* spp. was not an ordinary procedure. First it was a problem to handle accompanying flora, e.g. yeasts showed a high growth on the GSP selective medium (acc. to KIELWEIN, 1969; 1971). The reason for that unrequested growth was that the first examinations were performed with GSP media without the supplement pimaricin, which inhibits accompanied microbial growth. The usage of pimaricin maintained better results and the differentiation and counting of the colonies was much easier. The second problem of the examination was that the general colony appearance was not sufficient to handle the differentiation from other colonies. To get countable results of presumptive *Pseudomonas* spp. each colony had to be examined by gram staining. During this examination all types of bacteria forms except of gram-negative rods (*Pseudomonas* spp.) like gram-positive rods and coccoid species and yeasts were found.

Performing the examination resulted in the isolation of different colonies. In total four presumptive *Aeromonas* spp. and 250 presumptive *Pseudomonas* spp. colonies were isolated.

Confirmation of *Pseudomonas* spp. and *Aeromonas* spp.

Isolates from GSP medium were streaked and inoculated on unselective tryptic-soy-agar. This procedure had to be repeated for some isolates up to four times until pure cultures were growing.

During the storage of the isolates it was not possible to reanimate different *Pseudomonas* sp. isolates. Also the enrichment in brain-heart-infusion or nutrient broth showed no positive results. Consequently 78 isolates could not be used for further confirmation.

The biochemical confirmation was performed using an oxidation-fermentation medium according to (HUGH and LEIFSON, 1953). The results of the oxidation-fermentation tests are summarised in Table 53.

Table 53: Results of the confirmation procedure

Species	oxidative glucose degradation	fermentative glucose degradation	no reaction
Pseudomonas spp.	109	36	27
Aeromonas spp.	1	2	1

Figure 43: OF-test example

In total the result of the oxidation-fermentation test is that 63.4 percent of the presumptive *Pseudomonas* spp. isolates degraded glucose in oxidative form, by changing the indicator colour of the medium to yellow on the surface of the medium (aerobic conditions). These isolates are bacteria of the Pseudomonas species. Just 50 percent of the presumptive *Aeromonas* spp. degraded glucose fermentative, so two isolates are bacteria of the *Aeromonas* species. Figure 43 shows an example of a positive oxidation reaction.

For further identification of the *Pseudomonas* species it is recommended to use biochemical identification, e.g. Api® 20 NE or other capable systems to determine the exact species.

7.1.10 Examination of *Photobacterium* spp.

The counting and isolation of *Photobacterium* spp. was achieved by the application of Differential Photobacterium broth with an additional amount of 1.5 percent of agar, so that the spread plate method was possible to perform.

In total on 50 samples showed bacterial growth. The medium has shown very different forms of growth. On many agar plates four to six colony forms (shape, colour, general form) appeared and hence a serious examination of the microbial count of *Photobacterium* spp. was not possible. Gram staining of different colonies showed the result of high diverse bacterial growth of all forms and gram-characters. Due to this problem of a high diversity of bacterial growth a counting of the colony forming units of *Photobacterium* spp. was not performed.

7.2 Summary of the microbiological screening

7.2.1 Graphical interpretation of the microbial counts

The interpretation the microbial counts was performed by the comparison of the results from the samples with a different convenience degree as seen in Figure 44. The three convenience degrees of the shrimp samples were raw frozen, blanched and cooked and shipped and stored at freezing temperature of -18°C. The graphical interpretation of the microbial counts is carried out through the geometric mean of the different bacterial count results. The calculations were performed in the spreadsheet application Microsoft Excel.

The geometric mean of a data set $\{a_1, a_2, ..., a_n\}$ is given by:

$$\left(\prod_{i=1}^{n} a_i\right)^{1/n} = \sqrt[n]{a_1 a_2 \cdots a_n}.$$

In Figure 44 the geometric mean of the bacteria counts of the examined bacterial families are presented. As shown the geometric mean of the different bacteria counts of the sample is separated in their convenience degrees.

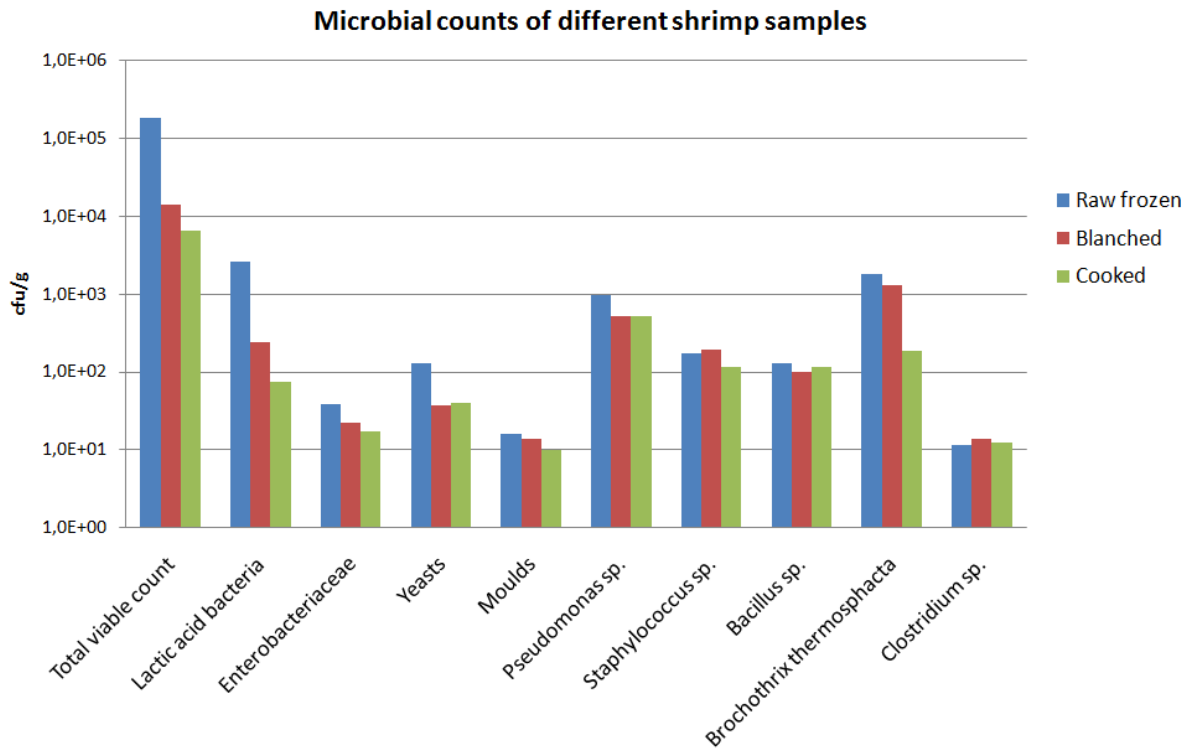


Figure 44: Microbial counts of the different convenience degrees of the shrimp samples

In general, the overall microbial quality of raw, blanched and cooked shrimp samples sold in Austria is satisfactory. As expected the lowest bacterial load could be observed in heat treated products, such as cooked shrimps similar to blanched products (Figure 44). Shrimp samples without any heat treatment during the processing (raw frozen) show more or less the

same bacterial profile, compared to heat treated products, with the exception that the bacterial count of lactic acid bacteria (LAB) is significant higher (about the factor 10). Except the total viable count and the value of lactic acid bacteria, the bacterial counts of all bacteria families show more or less the same level. The highest values of TVC are reached for raw frozen shrimp samples. In total 7 raw frozen shrimp samples have a total viable count between 10^6 and 10^7 cfu/g. One sample (CN-07) exceeds the value of 10^7 cfu/g with a bacterial count of 1.2 x 10^7 cfu/g (appendix Table 63) which is over the maximum recommended count for marginally acceptable quality products according ICMSF (1986). The largest part of all shrimp samples has a TVC within the range of 10^4 and 10^5 cfu/g.

The highest bacterial count of lactic acid bacteria was also reached by the raw frozen sample CN-07 with a value of 1.1 x 10^7 cfu/g which gives the information, that the main species of bacteria causing the high value of TVC of sample CN-07 is dominated by lactic acid bacteria. The count of lactic acid bacteria of sample CN-07 is also significantly higher (factor 10^2) compared to the count of LAB of other shrimp samples. The bacterial count of LAB of three samples (CN-21, M-10 and R-03 – Table 63) exceeds the value of 10^5 cfu/g, 10 samples are exceeding the value of 104 cfu/g and the remaining 44 shrimp samples have bacterial counts between lower than 10 to 10^4 cfu/g. As presented in Figure 44 the LAB counts in blanched and cooked shrimp samples are lower which can be explained by the reduction of the bacterial load from heat treatment during the processing of these shrimp products.

The overall good quality of the examined shrimp samples can be seen by the bacterial load of *Enterobacteriaceae*. The geometric means of the different convenience degrees do not exceed the value of 10^2 cfu/g. In particular two samples exceeded the limit of 5.0 x 10^2 cfu/g according ICMSF, 1986 which were the raw frozen samples CN-07 with a value of 9.7 x 10^2 cfu/g (Table 63) and CN-23 with a value of 9.3 x 10^2 cfu/g (Table 63). Within 100 and 500 cfu/g 4 samples were determined. These samples were three raw frozen (CN-20, M-04 and CN-13) and one blanched (M-05) samples. From the other samples 11 had a value of < 100 cfu/g and 41 samples a bacterial load of less than 10 cfu/g.

Specific spoilage organisms such as the *Pseudomonas* species have shown very similar geometric means of the bacterial counts between the convenience degrees (Figure 44). No significant difference between raw frozen and heat treated products was measureable. The bacterial count of raw frozen shrimp is marginal higher than to blanched and cooked samples which have the same value of bacterial counts. The bacterial counts in particular were on the value of 10^3 cfu/g. Bacterial counts below 10^3 cfu/g were examined in 26 samples (Table 63), counts below 10^2 cfu/g had 18 shrimp samples (Table 63). Just two raw frozen samples (CN-07 and CN-01) were examined with a bacterial count of 10^4 cfu/g. Samples with bacterial counts under 10^4 ranged between 1.1 to 8.6 x 10^3 cfu/g.

Another spoilage causing organisms is the species *Brochothrix thermosphacta*. The geometric means of the bacterial counts of raw frozen and blanched samples have a marginal higher value compared to *Pseudomonas* spp. (Table 50). Cooked shrimp samples show a value of bacterial count of *Brochothrix thermosphacta*, which is lower than raw frozen samples by a factor of 10. Maximum bacterial counts were examined in 4 raw frozen shrimp samples with bacterial counts between 1.6 x 10^5 and 5.4 x 10^5 cfu/g (M-10, CN-24, CN-07 and M-02). Bacterial counts below 10^4 cfu/g were examined in 15 samples, 9 samples showed counts below 10^3 cfu/g and in 26 samples examined, the bacterial load was < 10^2 cfu/g.

The critical level of the microbial count of *Staphylococcus aureus* is 1.0 x 10^3 cfu/g for frozen cooked crustaceans acc. ICMSF (1986). This level was not reached by any sample of all 58 examined samples (raw frozen and heat treated). Summarised in two samples *B. cereus* was detected and in three samples *Clostridium* sp. were examined. In both cases the bacterial load were not exceeding any critical microbiological limits of microbiological guidelines according FSANZ, 2001.

Yeasts and moulds were examined to get some knowledge of any contamination by environmental influences, e.g. through air pollution by air condition systems. The geometric means of the microbial counts between the different convenience degrees gave no significant level of difference. The geometric mean of the microbial load of yeasts in raw frozen products reached a value over 10^2 cfu/g. The maximum count of yeasts was reached by sample CN-23 with a count of 9.1 x 10^2 cfu/g. An average microbial count for yeasts has a value fewer than 100 cfu/g. Moulds with an microbial load at a value below 100 cfu/g were examined in 8 samples in total. In the majority of shrimp samples examined no growth of moulds was detectable.

In Figure 45 the geometric means of the bacterial counts of the different bacteria families from shrimp samples according their different production technologies are presented. The comparison of the microbial counts of the shrimp samples separated to the different origins of the production gives no significant differences. The geometric means of the bacterial counts of all bacterial families show the same results. Therefore the results give no definite conclusion of a significant difference of the microbial quality from different production sources.

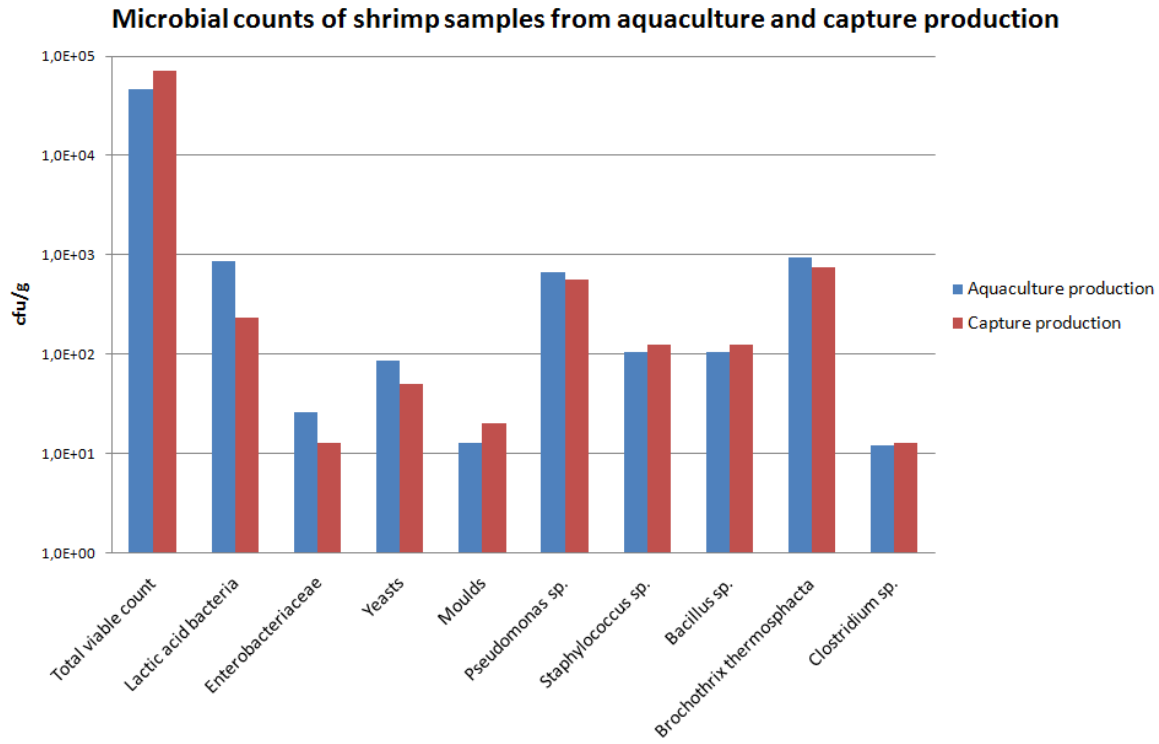


Figure 45: Microbial counts of shrimp samples from different production sites

7.3 Detection of Listeria species

The detection with VIDAS® *Listeria monocytogenes* Xpress yielded in one positive result of detected *L. monocytogenes*. The sample with the identification M-09 showed this positive *L. monocytogenes* result of the VIDAS® test. In the Figures 46 to 49 the set test data of the mini VIDAS® test system and the confirmation of the positive result are given. In Figure 46 the entered master lot data is given. Figure 47 shows the report of the VIDAS® test update.

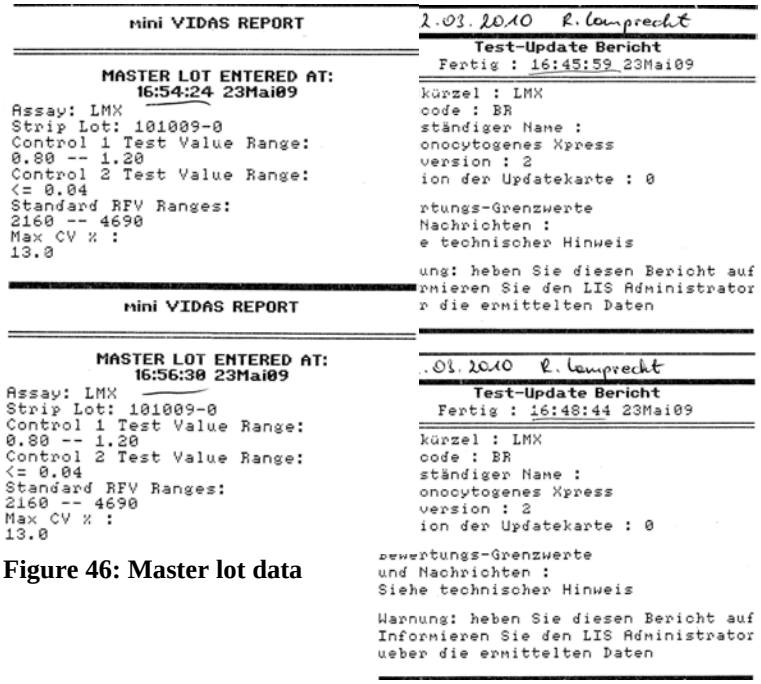


Figure 46: Master lot data

Figure 47: Report of the test update

Figure 48 present the print out of the positive Mini VIDAS® test result of sample M-09.1 (Position A5). The print out of the successful calibration procedure and the results of positive and negative controls are given in Figure 49.

Figure 49: Positive result of sample M-09.1

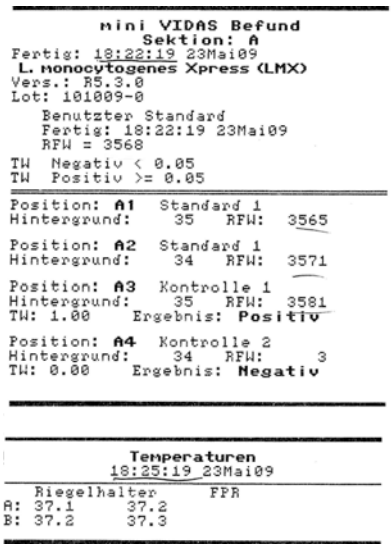


Figure 48: Results of the VIDAS control standards

Inoculation on OCLA and PALCAM agar plates

After the finished VIDAS® *L. monocytogenes* Xpress test one inoculum from each enrichment broth was streaked on OCLA and PALCAM agar plates and incubated at 37°C for 24 – 48 hours. During this incubation other samples showed positive growth of *Listeria* species. To ensure the method performance of the VIDAS® *L. monocytogenes* Xpress test the reference method according to DIN EN ISO 11290-1, 2005 was performed. From all enrichment steps of Half-Fraser and Fraser inoculums were also streaked out on OCLA and PALCAM agar plates.

In total 34 *Listeria* species were isolated from 58 shrimp samples. Table 54 shows the number of isolates from the different samples according their convenience degrees. In total twelve *L. monocytogenes* isolates in five different shrimp samples were examined (Table 54), hence the prevalence of *L. monocytogenes* of all shrimp samples accounts 8.6%. Out of these five samples three were blanched, one was a cooked and one was a raw frozen product without any prior treatment.

The number of samples were different *Listeria* species were examined are given in Table 55. *L. innocua* was found in two (1 raw, 1 cooked) samples, *L. welshimeri* was present in 2 raw frozen and 1 blanched sample. Furthermore *L. ivanovii* was present in 4 raw and 1 cooked, *L. seeligeri* in 1 raw and 1 cooked and finally *L. grayi* was found in 1 raw frozen shrimp sample. In total in 14 different shrimp samples *Listeria* species were examined.

Table 54: Number of isolated *Listeria* species of 58 shrimp samples according their convenience degrees

Convenience degree	Number of Isolates					
	L. monocytogenes	***L. innocua***	***L. welshimeri***	***L. ivanovii***	***L. seeligeri***	***L. grayi***
	(n=12)	(n=5)	(n=6)	(n=8)	(n=2)	(n=1)
Raw	3	2	4	6	1	1
Blanched	5		2			
Cooked	4	3		2	1	

Table 55: Number of samples with positive *Listeria* species out of 58 shrimp samples

Convenience degree	Number of positive samples					
	L. monocytogenes	***L. innocua***	***L. welshimeri***	***L. ivanovii***	***L. seeligeri***	***L. grayi***
	(n=5)	(n=2)	(n=3)	(n=5)	(n=2)	(n=1)
Raw	1	1	2	4	1	1
Blanched	3		1			
Cooked	1	1		1	1	

Confirmation of the *Listeria* isolates

Presumptive colonies from OCLA and PALCAM agar plates were used for further confirmation procedures. To perform these procedures all presumptive colonies were inoculated on tryptic-soy-agar and incubated at 37°C for 24 hours. All isolates were testet for the catalase and oxidase reaction were tested. catalase-positive, oxidase-negative isolates were further used for gram staining. Gram-positive rods with katalase-positive and oxidase-negative reactions, as presumptive Listeria species, were used for biochemical identification using API® Listeria and Microbact™ Listeria 12L. In Table 56 all isolated *Listeria* species with their source of isolation (enrichment steps) and their sample identification are listed.

Furthermore the results of the β-haemolysis tests and the plausibility of the biochemical identification are also shown in Table 56.

Table 56: Isolates of *Listeria* species detected in different shrimp samples

Sample character	Identification and source of the isolate		Species	β-Haemolysis	Plausibility (%)		Comments
				(-, +, ++)	API®	Microbact™	
raw	ISO-0,5F-M-10-1	0.5 F	*L. grayi*	-	99.9		
cooked	V-RA-02-1	VIDAS	*L. innocua*	-	99.6		
cooked	ISO-0,5F-RA-02-2	0.5 F	*L. innocua*	-	99.6		
cooked	ISO-F-RA-02-2	F	*L. innocua*	-	99.6		
raw	ISO-F-CN-23-1	F	*L. innocua*	-		99.3	
raw	ISO-F-CN-23-2	F	*L. innocua*	-			
cooked	V-CN-16-1	VIDAS	*L. ivanovii*	-		99.9	
cooked	V-CN-16-2	VIDAS	*L. ivanovii*	-			
raw	V-CN-07-1	VIDAS	*L. ivanovii*	-		99.9	
raw	V-M-04-1	VIDAS	*L. ivanovii*	+		98.9	
raw	V-M-04-2	VIDAS	*L. ivanovii*	++			
raw	V-M-10-1	VIDAS	*L. ivanovii*	++		98.9	
raw	ISO-F-M-02-1	F	*L. ivanovii*	++		99.3	
raw	ISO-F-M-02-2	F	*L. ivanovii*	++			
blanched	V-M-09-1 No1	VIDAS	*L. monocytogenes*	-			positive on plate
blanched	ISO-0,5F-M-06-2	0.5 F	*L. monocytogenes*	-			positive on plate
blanched	ISO-F-M-06-1	F	*L. monocytogenes*	-		99.9	
blanched	ISO-F-RA-03-1	F	*L. monocytogenes*	-			positive on plate
blanched	V-M-09-1 No2	VIDAS	*L. monocytogenes*	-			positive on plate
cooked	ISO-0,5F-CN-19-1	0.5 F	*L. monocytogenes Lec-*	-		99.3	
cooked	ISO-0,5F-CN-19-2	0.5 F	*L. monocytogenes Lec-*	-		99.3	
cooked	ISO-F-CN-19-1	F	*L. monocytogenes Lec-*	-		99.3	
cooked	ISO-F-CN-19-2	F	*L. monocytogenes Lec-*	-			
raw	V-CN-23-1	VIDAS	*L. monocytogenes Lec-*	-		99.9	
raw	V-CN-23-2	VIDAS	*L. monocytogenes Lec-*	-			
raw	ISO-0,5F-CN-23-2	0.5 F	*L. monocytogenes Lec-*	-		99.9	
cooked	V-CN-10-2	VIDAS	*L. seeligeri*	-	94.2		
raw	V-M-08-1	VIDAS	*L. seeligeri*	+		98.2	
blanched	ISO-F-M-09-2	F	*L. welshimeri*	+	99.9		
blanched	ISO-0,5F-M-09-2	0.5 F	*L. welshimeri*	-	99.9		
raw	V-RA-01-1	VIDAS	*L. welshimeri*	++	99.9		
raw	ISO-0,5F-RA-01-1	0.5 F	*L. welshimeri*	++	99.9		
raw	ISO-0,5F-RA-01-2	0.5 F	*L. welshimeri*	++	99.9		
raw	ISO-F-M-10-1	F	*L. welshimeri*	++	99.9		

Beta-haemolysis test of Listeria isolates

All identified *Listeria* species were tested on their beta haemolysis characters. *L. monocytogenes* should show a significant haemolysis zone close to a beta lysine disc on Columbia blood agar plate. The results of the haemolysis tests are shown in Table 56. Positive tests show a crescent-shaped zone of haemolysis, negative tests show no haemolytic zone or a

small shaped haemolytic zone (Figure 50). The description was done using – for negative tests, + for weak positive tests and ++ for clear positive beta haemolysis tests. All isolated *L. monocytogenes* species showed no positive haemolysis reaction.

Figure 50: Examples of β haemolysis tests of isolated Listeria species

7.3.1 Comparison of the method VIDAS® Listeria monocytogenes Xpress and the reference method according to EN ISO 11290-1

In Table 57 the results of the VIDAS L. monocytogenes Xpress detection method and the results of the reference method according ISO 1290-1 are presented. The table shows the sample identification and gives the information if *Listeria monocytogenes* was detected in this shrimp sample.

Table 57: Overview of the *Listeria monocytogenes* detection results of VIDAS® method and the reference method according EN ISO 11290-1

Sample	Appendages per sample	VIDAS® LMX	Appendages per sample	Reference method EN ISO 11290-1
		L. monocytogenes		*L. monocytogenes*
K-01	K 01.1	-	K 01.1	-
	K01.2	-	K01.2	-
B-02	B-02.1	-	B-02.1	-
	B-02.2	-	B-02.2	-
R-03	R-03.1	-	R-03.1	-
	R-03.2	-	R-03.2	-
C-04	C-04.1	-	C-04.1	-
	C-04.2	-	C-04.2	-
G-05	G-05.1	-	G-05.1	-
	G-05.2	-	G-05.2	-
L-06	L-06.1	-	L-06.1	-
	L-06.2	-	L-06.2	-
RG-01	RG-01.1	-	RG-01.1	-
	RG-01.2	-	RG-01.2	-
KP-01	KP-01.1	-	KP-01.1	-
	KP-01.2	-	KP-01.2	-
QF-01	QF-01.1	-	QF-01.1	-
	QF-01.2	-	QF-01.2	-
QF-02	QF-02.1	-	QF-02.1	-
	QF-02.2	-	QF-02.2	-
QF-03	QF-03.1	-	QF-03.1	-
	QF-03.2	-	QF-03.2	-
Y-01	Y-01.1	-	Y-01.1	-
	Y-01.2	-	Y-01.2	-

Y-02	Y-02.1	**-**	Y-02.1	**-**
	Y-02.2	**-**	Y-02.2	**-**
Y-03	Y-03.1	**-**	Y-03.1	**-**
	Y-03.2	**-**	Y-03.2	**-**
CN-01	CN-01.1	**-**	CN-01.1	**-**
	CN-01.2	**-**	CN-01.2	**-**
CN-02	CN-02.1	**-**	CN-02.1	**-**
	CN-02.2	**-**	CN-02.2	**-**
CN-03	CN-03.1	**-**	CN-03.1	**-**
	CN-03.2	**-**	CN-03.2	**-**
CN-04	CN-04.1	**-**	CN-04.1	**-**
	CN-04.2	**-**	CN-04.2	**-**
CN-05	CN-05.1	**-**	CN-05.1	**-**
	CN-05.2	**-**	CN-05.2	**-**
CN-06	CN-06.1	**-**	CN-06.1	**-**
	CN-06.2	**-**	CN-06.2	**-**
CN-07	CN-07.1	**-**	CN-07.1	**-**
	CN-07.2	**-**	CN-07.2	**-**
CN-08	CN-08.1	**-**	CN-08.1	**-**
	CN-08.2	**-**	CN-08.2	**-**
CN-09	CN-09.1	**-**	CN-09.1	**-**
	CN-09.2	**-**	CN-09.2	**-**
CN-10	CN-10.1	**-**	CN-10.1	**-**
	CN-10.2	**-**	CN-10.2	**-**
CN-11	CN-11.1	**-**	CN-11.1	**-**
	CN-11.2	**-**	CN-11.2	**-**
CN-12	CN-12.1	**-**	CN-12.1	**-**
	CN-12.2	**-**	CN-12.2	**-**
CN-13	CN-13.1	**-**	CN-13.1	**-**
	CN-13.2	**-**	CN-13.2	**-**
CN-14	CN-14.1	**-**	CN-14.1	**-**
	CN-14.2	**-**	CN-14.2	**-**
CN-15	CN-15.1	**-**	CN-15.1	**-**
	CN-15.2	**-**	CN-15.2	**-**
CN-16	CN-16.1	**-**	CN-16.1	**-**
	CN-16.2	**-**	CN-16.2	**-**
CN-17	CN-17.1	**-**	CN-17.1	**-**
	CN-17.2	**-**	CN-17.2	**-**
CN-18	CN-18.1	**-**	CN-18.1	**-**
	CN-18.2	**-**	CN-18.2	**-**
CN-19	CN-19.1	**-**	CN-19.1	**+**
	CN-19.2	**-**	CN-19.2	**+**
CN-20	CN-20.1	**-**	CN-20.1	**-**
	CN-20.2	**-**	CN-20.2	**-**
CN-21	CN-21.1	**-**	CN-21.1	**-**
	CN-21.2	**-**	CN-21.2	**-**
CN-22	CN-22.1		CN-22.1	**-**
	CN-22.2	**-**	CN-22.2	**-**
CN-23	CN-23.1	**-**	CN-23.1	**-**
	CN-23.2	**-**	CN-23.2	**+**
CN-24	CN-24.1	**-**	CN-24.1	**-**
	CN-24.2	**-**	CN-24.2	**-**
CN-25	CN-25.1	**-**	CN-25.1	**-**
	CN-25.2	**-**	CN-25.2	**-**
CN-26	CN-26.1	**-**	CN-26.1	**-**
	CN-26.2	**-**	CN-26.2	**-**
CN-27	CN-27.1	**-**	CN-27.1	**-**
	CN-27.2	**-**	CN-27.2	**-**
CN-28	CN-28.1	**-**	CN-28.1	**-**

	CN-28.2	-	CN-28.2	-
CN-29	CN-29.1	-	CN-29.1	-
	CN-29.2	-	CN-29.2	-
CN-30	CN-30.1	-	CN-30.1	-
	CN-30.2	-	CN-30.2	-
M-01	M-01.1	-	M-01.1	-
	M-01.2	-	M-01.2	-
M-02	M-02.1	-	M-02.1	-
	M-02.2	-	M-02.2	-
M-03	M-03.1	-	M-03.1	-
	M-03.2	-	M-03.2	-
M-04	M-04.1	-	M-04.1	-
	M-04.2	-	M-04.2	-
M-05	M-05.1	-	M-05.1	-
	M-05.2	-	M-05.2	-
M-06	M-06.1	-	M-06.1	+
	M-06.2	-	M-06.2	+
M-07	M-07.1	-	M-07.1	-
	M-07.2	-	M-07.2	-
M-08	M-08.1	-	M-08.1	-
	M-08.2	-	M-08.2	-
M-09	M-09.1	+	M-09.1	-
	M-09.2	-	M-09.2	-
M-10	M-10.1	-	M-10.1	-
	M-10.2	-	M-10.2	-
M-11	M-11.1	-	M-11.1	-
	M-11.2	-	M-11.2	-
RA-01	RA-01.1	-	RA-01.1	-
	RA-01.2	-	RA-01.2	-
RA-02	RA-02.1	-	RA-02.1	-
	RA-02.2	-	RA-02.2	-
RA-03	RA-03.1	-	RA-03.1	+
	RA-03.2	-	RA-03.2	-

Comparison of the *L. monocytogenes* detection methods VIDAS® LMX and EN ISO 11290-1

Table 58 shows the comparison of the detection results of the alternative method to the reference method. In Table 58 the number of positive and negative agreements as well as the number of positive and negative deviations is presented. Summarised *L. monocytogenes* was detected in 5 samples from 58 samples in total by performing both methods.

Table 58: Comparison of the detection of L. monocytogenes of VIDAS® LMX and EN ISO 11290-1(ANONYMUS e, 2003)

		EN ISO 11290-1 results		Total
		positive	negative	
VIDAS® LMX results	positive	0 (PA)	1 (PD)	1
	negative	4 (ND)	53 (NA)	57
Total		4 (N_+)	54 (N_-)	**58 (N)**

As presented in Table 58 the detection of *L. monocytogenes* resulted in one false positive VIDAS® outcome (sample M-09). The sample is mentioned as false positive, because the sample was not detected as *L. monocytogenes* positive by performing the reference method ISO 11290-1. The *Listeria* species detected by VIDAS® LMX in sample M-09 was determined and definitely confirmed as *Listeria monocytogenes* species, so consequently this

sample detection is correct positive. Further the reference method detected *L. monocytogenes* in 4 samples (CN-19, CN-23, M-06 and RA-03), which were not detected by the VIDAS method. Consequently these 4 samples are signified as wrong negative results.

Out of the results from Table 58 the parameters for the method performance of VIDAS® LMX are calculated. Formulas for the calculation of relative accuracy, relative sensitivity and relative specificity are presented and described in chapter "Comparison of qualitative methods" in Table 42.

The calculations results for the VIDAS® *L. monocytogenes* Xpress method are shown in Table 59. The relative accuracy (AC) resulted to a value of 91.37%. This result is relatively correct because the false positive result of the VIDAS® detection was confirmed later as correct positive result. As the accuracy stands for "the closeness of agreement between a test result and the accepted reference value "(ANONYMUS e, 2003), the result shows that the VIDAS® detection method and reference method have similar outcomes.

The calculated relative specificity (SP) result was 98.15 % which can be explained that the negative agreement of VIDAS method and reference method is high. Hence 98.15 % of all negative samples were detected negative from VIDAS® and from the reference method (HÜBNER et al., 2002). Because of the inhomogeneous dispersion of bacteria in naturally contaminated samples, generally the determination of the relative specificity is difficult (HÜBNER et al., 2002). Based on the relative specificity the false positive rate is low with a value of 1.85 % (Table 59).

The term relative sensitivity (SE) in use with qualitative methods is explained as the efficiency of the VIDAS® method to detect *Listeria* positive samples, when they are detected by the reference method (HÜBNER et al., 2002). The result of relative sensitivity is 0 %, which can be explained that none of the samples which were detected *Listeria* positive by the reference method was detected by the alternative VIDAS® method (Table 57). Again the inhomogeneous dispersion of the *Listeria monocytogenes* contamination in the shrimp samples may explain this outcome. Hence the calculation of the false negative rate resulted to 100%. This parameter says that 100% of positive samples detected by the reference method were not (respectively negative) detected by VIDAS® LMX.

Table 59: Performance parameters for VIDAS® *L. monocytogenes* Xpress

Parameter	L. monocytogenes
Relative accuracy (AC)	91.37 %
Relative sensitivity (SE)	0 %
Relative specificity (SP)	98.15 %
False positive rate (pf+)	1.85 %
False negative rate (pf-)	100 %

Estimation of discordant results

According the formula in Table 42 (PD + ND = Y) the result for the variable Y is 5. Therefore no test for the estimation is recommended because the value of Y is lower 6 by following the formula (PD + ND = Y) < 6 and so no test is required.

The values for PD and ND are low and almost equal and so no statistical difference between the method VIDAS® *L. monocytogenes* Xpress and the reference method acc. EN ISO 11290-1 could be determined.

7.4 Results of the antibiotic susceptibility testing

All isolated *Listeria* species were used for antibiotic susceptibility tests. By performing an agar diffusion test all 34 *Listeria* isolates were tested on their susceptibility respectively resistance to 11 different antibiotics.

All antibiotics used for the susceptibility testing were chosen according different publications, were the use of antibiotics in aquacultures is described (HOLMSTRÖM et al., 2003), and antibiotic susceptibility testing on *Listeria* species was performed (PESAVENTO et al., 2010; WALSH et al., 2001; CONTER et al., 2009; CHEN et al., 2010; HANSEN et al., 2005). After the tests of the shrimp isolates were performed, the same test procedure of susceptibility testing was performed on *Listeria* reference strains, which were available from the reference stock of the institute for food sciences.

In Table 60 the results of the antibiotic susceptibility tests of the different isolated *Listeria* species are presented. Of all tested strains 30 strains were resistant to clindamycin (88.2 %). Just 4 strains (2 *L. ivanovii* and 2 *L. seeligeri*) showed a sensitive reaction to this antibiotic. Almost all strains were resistant to the beta lactam antibiotic oxacillin (33 strains – 97.0 %). One strain of *L. seeligeri* was sensitive to this antibiotic.

In total 29 strains (85.3 %) of all isolated *Listeria* species were resistant to ampicillin. Most strains of the isolated *Listeria* species were sensitive to rifampicin, co-trimoxazole (trimethoprim/sulfomethoxazole), tetracycline and erythromycin. Resistance to these antibiotics were detected altogether from 14 strains – 2 strains *L. ivanovii* and respectively one strain of *L. seeligeri* and *L. grayi* were resistant to rifampicin. To co-trimoxazole 2 strains of *L. welshimeri* and 2 strains of L. ivanovii showed resistance. *L. grayi* as unique strain was resistant to tetracycline and 4 strains (2 *L. welshimeri* and 2 *L. ivanovii*) were resistant to erythromycin. To ciprofloxacin one strain of *L. ivanovii* (2.9 %) showed resistance. No resistance could be observed for the antibiotics linezolid, vancomycin and chloramphenicol. All strains were sensitive to these antibiotics.

The highest number of resistance was detected from the unique *L. grayi* species. This strain showed resistance in total to 6 different antibiotics. The species *L. ivanovii* different strains were resistant to 7 antibiotics.

Table 60: Number and percentage of isolated *Listeria* strains resistant to antibiotics

Antibiotic	*L. monocytogenes* No. (%)	*L. innocua* No. (%)	*L. welshimeri* No. (%)	*L. ivanovii* No. (%)	*L. seeligeri* No. (%)	*L. grayi* No. (%)
	(n=12)	(n=5)	(n=6)	(n=8)	(n=2)	(n=1)
Clindamycin	12 (100.0)	5 (100.0)	6 (100.0)	6 (75.0)	0 (-)	1 (100.0)
Linezolid	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Ciprofloxacin	0 (-)	0 (-)	0 (-)	1 (12.5)	0 (-)	0 (-)
Ampicillin	9 (75.0)	5 (100.0)	6 (100.0)	7 (87.5)	1 (50.0)	1 (100.0)
Rifampicin	0 (-)	0 (-)	0 (-)	2 (25.0)	1 (50.0)	1 (100.0)
Co-trimoxazole	0(-)	0 (-)	2 (33.3)	2 (25.0)	0 (-)	1 (100.0)
Vancomycin	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Tetracycline	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	1 (100.0)
Chloramphenicol	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Erythromycin	0 (-)	0 (-)	2 (33.3)	2 (25.0)	0 (-)	0 (-)
Oxacillin	12 (100.0)	5 (100.0)	6 (100.0)	8 (100.0)	1 (50.0)	1 (100.0)

For *L. welshimeri* all tested strains (100 %) were resistant to clindamycin, ampicillin and oxacillin. To co-trimoxazole and erythromycin two strains (33.3 %) showed resistance. Nearly the same resistance profile was detected for the *L. innocua* strains. As the *L. welshimeri* strains 100% showed resistance to the antibiotics clindamycin, ampicillin and oxacillin (Table 59). From the *L. monocytogenes* strains 100 % were resistant to clindamycin and oxacillin, 9 out of 12 strains (75 %) showed resistance to ampicillin (Table 60).

In Table 61 the results of the antibiotic susceptibility tests of different *Listeria* reference strains are presented. Compared to the isolated strains from the shrimp samples the number of reference strains resistant to antibiotics was lower. Additionally the antibiotic gentamicin was tested in that series of the susceptibility test. In total 11 strains (61.1 %) were resistant to clindamycin. From the *L. monocytogenes* strains 7 showed resistance to clindamycin. To oxacillin all *L. monocytogenes* strains (100.0 %) were resistant. No resistance was detected on the antibiotics linezolid, ciprofloxacin, co-trimoxazole, vancomycin, tetracycline, chloramphenicol and gentamicin. In contrast to the shrimp isolates, the reference strains showed not the same resistance profile to the beta lactam antibiotic ampicillin. Just the unique strains of *L. innocua* and *L. welshimeri* showed a resistant reaction to ampicillin. One of the *L. grayi* reference strains was additionally resistant to rifampicin and the one tested L. welshimeri strain showed resistance to erythromycin. The *L. seeligeri* strain tested, showed no resistance pattern, *L. monocytogenes*, *L. welshimeri* and *L. ivanovii* showed resistances to two antibiotics and *L. innocua* and *L. grayi* resulted in resistances to three antibiotics.

Table 61: Number and percentage of resistant *Listeria* reference strains

Antibiotic	*L. monocytogenes* No.(%)	*L. innocua* No.(%)	*L. welshimeri* No.(%)	*L. ivanovii* No.(%)	*L. seeligeri* No.(%)	*L. grayi* No.(%)
	(n=12)	(n=1)	(n=1)	(n=2)	(n=1)	(n=2)
Clindamycin	7 (58.3)	1 (100.0)	0 (-)	1 (50.0)	0 (-)	2 (100.0)
Linezolid	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Ciprofloxacin	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Ampicillin	0 (-)	1 (100.0)	1 (100.0)	0 (-)	0 (-)	0 (-)
Rifampicin	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	1 (50.0)
Co-trimoxazole	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Vancomycin	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Tetracycline	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Chloramphenicol	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Erythromycin	0 (-)	0 (-)	1 (100.0)	0 (-)	0 (-)	0 (-)
Oxacillin	12 (100.0)	1 (100.0)	0 (-)	2 (100.0)	0 (-)	2 (100.0)
Gentamicin	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)

Furthermore it was possible to test human *L. monocytogenes* isolates on their antibiotic susceptibility. In total 11 isolates were used for the tests. The results are presented in Table 62. The resistance pattern is similar to *L. monocytogenes* isolates from the shrimp samples. The isolates were resistant to clindamycin (81.8 %), ampicillin (45.5 %) and oxacillin (90.9 %). Additionally one strain showed resistance to linezolid, which was not determined in *L. monocytogenes* strains isolated from the shrimp samples and neither detected in the *L. monocytogenes* reference strains.

Table 62: Antibiotic susceptibility of human isolate s of *L. monocytogenes*

Antibiotic	Human isolates of *L. monocytogenes* No. (%) (n = 11)
Clindamycin	9 (81.8)
Linezolid	1 (9.1)
Ciprofloxacin	0 (-)
Ampicillin	5 (45.5)
Rifampicin	0 (-)
Co-trimoxazole	0 (-)
Vancomycin	0 (-)
Tetracyclin	0 (-)
Chloramphenicol	0 (-)
Erythromycin	0 (-)
Oxacillin	10 (90.9)

8 Discussion

Before summarising the results of the microbiological screening first the description of different problems which occurred during the work have to be discussed. One problem during the screening was that all bought products were available deep frozen. During the transport of the products it was important to keep the temperature stable at temperatures which have no influence on microbial growth in the product. This risk of increased temperature during transportation of the products which may occur in bacterial growth was prevented by using cooler bags. So more or less the temperature of the samples were kept stable, even though the temperature increased and the samples could not be kept on a minimum temperature of -18°C. Nevertheless the results of the microbiological screening determined showed that it is doubtful that an increased bacterial growth during transportation proceeded.

Over the period of the examinations another problem was that the different product types had to be thawed before it was possible to take samples for the microbiological examination. It was not possible to define a general time of thawing the products because of different product variations (product size, thickness of the glazing layer, marketing form). The thawing times ranged between 3 and 12 hours at 4°C. The problem is that through these different thawing times it was assumed that there may be a possible growth of psychrophilic bacteria, which cannot be controlled. However, the evaluation of the microbial count results has shown no significant differences and so it is supposed that the thawing intervals had no significant influences on the bacterial counts.

Another possible influence on resulting bacterial counts is the sample preparation procedure. It was necessary to remove the shrimp flesh from the whole shrimp body and transfer it aseptically into a stomacher bag for further examination. The problem was that it was not possible to remove the shell from all kinds of shrimp samples and so there is the possibility that the amount of shell in the analysed shrimp samples inhibited the bacterial growth because of the antimicrobial properties of chitin (LIMAM et al., 2011; DYAHNINGTYAS, 2010) which is the dominant material in the shell of crustaceans. Hence, the possible inhibition of microbiological growth, influenced by parts of the crust of shrimps may occurred, which could not be confirmed in this study.

In general the microbial quality of all examined shrimp products sold in Austria is satisfactory. An overview of the geometric means of the bacterial counts presented in Figure 44 shows a good microbiological status of all products. As it was expected, the highest bacterial loads were examined in raw frozen products followed by blanched and cooked products. The highest bacterial count of the total viable count (TVC) could be observed in sample CN-07 with a bacterial count of 1.2×10^7 cfu/g. The critical level for good quality products of frozen raw crustaceans is limited up to 10^6 cfu/g according to recommended microbiological limits for seafood (ICMSF, 1986). The recommended microbial count for marginally acceptable quality products is 10^7 cfu/g (ICMSF, 1986). Only one sample out of 58 reached a bacterial load over the maximum marginal level. The bacterial count of lactic acid bacteria (LAB) with a level of 1.1×10^7 cfu/g of sample CN-07 mainly represents the high bacterial load in the sample. Hence, the product cannot be estimated as unacceptable for consumption. Additionally, the product is raw frozen, therefore a heat treatment has to be performed before consumption, reducing the bacterial load significantly.

Further, eight out of 34 samples of raw frozen shrimps exceeded the maximum level for good quality products of 10^6 cfu/g (ICMSF, 1986). The geometric mean of TVC of blanched and cooked samples is lower by the factor 10^1. In blanched shrimp samples the geometric mean of TVC accounts 1.4×10^4 cfu/g and the count of cooked samples accounts

6.6 x 10^3 cfu/g. A TVC level higher than 5 x 10^5 cfu/g for good quality products (ICMSF, 1986) of cooked crustaceans was reached by one blanched sample (M-09) (8.3 x 10^5 cfu/g) out of 7 samples with the same convenience degree. As blanching is a low pressure heat treatment compared to cooking and the TVC level does not reached the maximum level of good quality of raw frozen products the microbiological status can be estimated as satisfactory. Beyond that, an additional heat treatment before consumption is recommended for blanched products. Blanched products have this recommendation for consumers labelled on the package. All cooked samples have a lower total viable count than 5.0 x 10^5 cfu/g, which is the recommended maximum level of good quality products of cooked crustaceans. This low bacterial load in the cooked shrimp samples was reached, because these products undergo a crucial heat treatment during processing, which significantly reduces the bacterial load of these products. Summarised, the microbial quality of all examined shrimp products focused on the total viable count is acceptable and comparable to other studies (MOHAMED HATHA et al., 2003). The maximum recommended bacterial counts for good quality products acc. ICMSF, 1986 were not reached by 84.5 % of all samples. 13.8 % of the samples have shown TVC levels between m and M and 1.7 % were higher than the M level of the recommended total viable count.

According to the critical values of colony forming units for *E. coli* (ICMSF, 1986) and the results of the examination, it can be presumed that the hygienic conditions during production are appropriate. The overall good quality of the examined shrimp samples can be seen in the bacterial load of *Enterobacteriaceae*. Out of 58 shrimp samples two raw frozen samples showed a bacterial load of *Enterobacteriaceae* of 9.7 x 10^2 cfu/g (CN-07) and 9.3 x 10^2 cfu/g (CN-23). A range between 100 and 500 cfu/g was reached in 4 samples. For crustaceans no recommended levels of bacterial counts for *Enterobacteriaceae* could be found. Therefore, the bacterial counts have been compared with levels according DGHM (2010) for marine finfish. The maximum recommended level for *Enterobacteriaceae* of 1 x 10^4 cfu/g could not be reached by all shrimp samples. Hence, according to this level (DGHM, 2010) the quality of the shrimp samples is satisfactory. In total, out of 95 isolated *Enterobacteriaceae*, 3 isolates were identified as *E. coli* and 30 isolates as coliforms. According this result the presumptive number of *E. coli* in all shrimp samples has a low level which does not reach maximum bacterial counts of 11 cfu/g (ICMSF, 1986) for good quality crustacean products.

Also the examination of *Staphylococcus aureus* resulted in good microbial quality and the processing hygiene seems to be on a high level, even though most shrimp processing facilities are found in developing countries. Summarised, two samples showed growth of presumptive *Staphylococcus aureus*, which were CN-14 (cooked) and CN-24 (raw frozen) with a count of < 10^3 cfu/g. Both bacterial counts did not exceed the criteria recommended for crustaceans acc. ICMSF, 1986. Hence, these products are of a satisfactory quality.

Presumptive *Bacillus cereus* was examined in two samples with counts under the critical limit for ready-to-eat food so with a satisfactory level, furthermore *Clostridium perfringens* was also detected in four samples. One cooked shrimp sample (QF-02) reached the marginal range of a count between 10^2 and10^3 cfu following the recommendation for ready-to-eat foods acc. FSANZ (2001). Other positive samples (M-05, M-04 and M-08) had counts below 10^2 cfu/g, which is within the satisfactory level for ready-to-eat products (FSANZ, 2001). Hence, no critical hazard of intoxication by both *Bacillus* and *Clostridium* is given.

Specific spoilage organisms such as *Pseudomonas* species and *Brochothrix thermosphacta* showed very similar bacterial counts between the different convenience degrees. The bacterial counts of *Pseudomonas* species showed no significant differences between heat treated and raw products. The geometric mean of *Pseudomonas* counts ranged at a level of 10^3 cfu/g which is under the critical value of 10^6 cfu/g acc. DGHM (2010) for marine finfish. The small differences of the bacterial counts between raw and heat treated products can be explained by the character of the psychrophilic growth potential of *Pseudomonas* species. After heat treatment potential growth of spoilage causing *Pseudomonas* spp. may be possible (REHBEIN & OEHLENSCHLÄGER, 2009). The bacterial counts have not reached a higher level than 10^5 cfu/g, which approves the good microbial status of the examined shrimp products. The bacterial counts of the spoilage causing *Brochothrix thermosphacta* in general were higher than the bacterial counts of *Pseudomonas* species. Significant in the results of the bacterial counts of *Brochothrix thermosphacta* was, that the count in cooked shrimp samples was about the factor 10 lower as in raw shrimp samples, which shows the limiting effect of heat treatment during the processing of shrimp. Blanched shrimp samples showed no significant lower value of the bacterial count of *Brochothrix thermosphacta*, which may occur that the intensity of the heat treatment during blanching is not high enough to eliminate a significant amount of the bacterial load. *Brochothrix thermosphacta* does not survive heating at 63°C for 5 min (BERGEY, 2009). Blanching is defined as a heat treatment reaching a minimum core temperature of 60°C. If during the blanching process no higher temperature than 63°C is reached, a lower amount of the bacterial load is eliminated during the procedure.

The low counts of yeasts and moulds in the examined products indicate that this parameter is not relevant for the spoilage of shrimp products.

Informative results were determined by the detection of *L. monocytogenes* and *Listeria* spp. in the shrimp products. Summarised, all species of the genus *Listeria* could be detected by using the reference method and the VIDAS® LMX method (Table 56). Out of all 34 isolated *Listeria* species 50 % of the isolates were detected in raw frozen products. *L. monocytogenes* could be identified in 35.3 % out of all isolated *Listeria* species. In 18 different shrimp samples *Listeria* spp. could be detected, 5 samples were *L. monocytogenes* positive which is a percentage of 27.8%. In nearly one third of *Listeria* positive samples *L. monocytogenes* occurred. In total of all examined shrimp samples the presence of *L. monocytogenes* was 8.6% and the presence of *Listeria* spp. was 22.4 %. Compared to other studies which determined the number of *Listeria* species in shrimp, these levels are within the normal discovery range (WAN NORHANA et al., 2009). In other studies (STONSAOVAPAK and BOONYARATANAKORNKIT, 2010; KARUNASAGAR and KARUNASAGAR, 2000) the prevalence of the *Listeria* species and *L. monocytogenes* had a lower percentage or no prevalence was determined. The prevalence results of this study were similar to these of WAN NORHANA et al. (2009). The higher number of *L. monocytogenes* positive heat treated samples (3 blanched, 1 cooked and 1 raw frozen) presumes that the main contamination of shrimp products took place during the processing steps after the heat treatment. As a ubiquitous bacterium *L. monocytogenes*, the contaminations may take place during peeling, cooling, glazing or packing of the shrimp products and *L. monocytogenes* may grow during the cooling storage or during thawing processes which may occur if the cooling chain is not correctly performed.

Further different problems occurred during the detection of *Listeria* species in the shrimp products. To control the method performance of the VIDAS® LMX detection system, the reference method acc. EN ISO 11290-1 was performed. The detection resulted that the VIDAS® method detected one sample as *L. monocytogenes* positive, which could not be confirmed by the reference method. Also 4 *L. monocytogenes* positive samples could not be

detected by the fast detecting method. Thus the method performance showed the significant results of relative sensitivity of 0 % by the VIDAS® method. Otherwise the values of relative accuracy and relative specificity were significantly close to 100 % which shows that the method performance is alright. The problem is that the dispersion of the *L. monocytogenes* contamination of the shrimp is very high and a full homogenisation of the samples was not performed. Consequently without a complete homogenisation of examined samples the method performance may result to these different parameters.

Another problem occurred in the results of the beta-haemolysis tests. All confirmed *L. monocytogenes* species showed no positive result of haemolysis. Furthermore *L. welshimeri* strains, which are not-haemolytic (BERGEY, 2009) strains appeared positive results for beta-haemolysis. This effect of different haemolysis characteristic cannot be explained. *L. monocytogenes* strains may have lost the character during storage, although fresh inoculated strains were used performing this test. This effect leads to the question if the identification results of *L. welshimeri* strains were totally correct.

The determinations of the antibiotic susceptibility tests have shown no significant new results of antibiotic resistance patterns of *Listeria* species. As expected, nearly all tested *Listeria* strains (isolated from shrimp samples and tested reference strains) were resistant to the antibiotic oxacillin. According to TROXLER et al. (2000) *Listeria* spp. are naturally sensitive to resistant to oxacillin. The examination showed that all *Listeria* strains were resistant.

Listeriosis usually has been treated with penicillin or ampicillin (WALSH et al, 2001) additionally in combination with an aminoglycoside such as gentamicin (tested on the reference strains) (PESAVENTO et al., 2010). Further tetracycline, erythromycin or chloramphenicol alone or in combination were also used for treatment (WALSH et al., 2001; PESAVENTO et al., 2010). Results of the determination have shown that 75% of the isolated *L. monocytogenes* strains were resistant to ampicillin, but no resistance was determined for tetracycline, erythromycin or chloramphenicol, hence resistance to ampicillin has developed in *L. monocytogenes* strains which occur in shrimp products. Also the results of determined *Listeria* spp. were resistant to ampicillin, which shows a similarity to the study acc. PESAVENTO et al. (2010) were around 20 % of determined strains of *Listeria* spp. were resistant. Other studies have not determined resistance (CHEN et al., 2010; CONTER et al., 2009). According to TROXLER et al. (2000) *Listeria* spp. are naturally sensitive to ampicillin, hence a resistance has developed in the determined strains. The examination showed no resistance of *L. monocytogenes* to tetracycline, erythromycin or chloramphenicol, hence no resistance has developed. In the study acc. to WALSH et al. (2001) resistance to tetracycline of *L. monocytogenes* was already determined. Rifampicin and vancomycin were determined because these antibiotics are also used for listeriosis treatment. Both antibiotics showed no resistance pattern of *L. monocytogenes*, but two *L. ivanovii*, one *L. seeligeri* and one *L. grayi* strain were resistant to rifampicin. Co-trimoxazole is used for listeriosis treatment for people allergic to other antibiotics or in the presence of multiresistant bacteria. All *Listeria* species are naturally sensitive against this antibiotic (TROXLER et al., 2000) nevertheless 33.3 % of *L. welshimeri* strains and 25.0 % of *L. ivanovii* strains were resistant. This pattern was also determined in the study acc. PESAVENTO et al. (2010). Significant resistance patterns were also examined for the antibiotic clindamycin. According to TROXLER et al. (2000) *Listeria* spp. are naturally sensitive to this antibiotic, hence resistance developed. These results are similar to other studies (CONTER et al., 2009; PESAVENTO et al., 2010) which have also examined resistance patterns, except the results of this study show significantly higher percentages of resistance strains. All *Listeria* strains were sensitive to linezolid and ciprofloxacin. Only one strain of *L. ivanovii* showed resistance to ciprofloxacin, a pattern which is according to TROXLER et al. (2000) possible because *L. ivanovii* may be

naturally intermediate or resistant to the antibiotic ciprofloxacin. Summarised, all antibiotic susceptibility test results show no new resistance patterns, which have not been published before. The exceptions are that the percentage of resistance to the antibiotics clindamycin and ampicillin are significantly higher compared to other studies, which leads to expect that the resistance development is increasing hence the therapeutic use of these antibiotics may not show positive results.

This study on the microbiological quality of commercial shrimp and prawns gives a general overview over the microbial status of products sold on the Austrian retail market. Summarised the products show a very satisfactory quality. It is recommended to focus on the more precise detection of pathogens and also especially focus on aquatic pathogens and their antibiotic resistance profiles. Further, antibiotic susceptibility testing should be performed on specific spoilage organisms as they may be vectors of antibiotic resistance genes to pathogen organisms (WARD & HACKNEY, 1991). Thus, it is recommended to focus on the resistance development of pathogens caused by the usage of antibiotics against non pathogenic bacteria. For the future it is recommended to start a sustainable production of shrimp, so that problems occurred, do not further increase and the pollution of the environmental is avoided.

9 Conclusion

This study was performed to evaluate the microbiological quality of shrimp products sold in Austria differing in the types of convenience degrees (raw frozen, blanched and cooked shrimp). Between the processing types of the shrimp samples no significant differences based on the results regarding the bacterial counts could be determined. In general, the microbiological quality of all examined shrimp products (raw, blanched and cooked) is satisfactory. *L. monocytogenes* resulted in higher prevalence in heat-treated shrimp products, which indicates an insufficient heating technology, recontamination during further processing steps by production facilities or temperature variations during storage and transportation. As shrimp products are mainly imported from developing countries, globally spreading pathogens may pose a risk to the public health. Therefore consistent testing of the overall microbiological diversity is essential in order to estimate perspectives of changing bacterial populations and the effect of processing and treatment with antibiotics. Performed antibiotic susceptibility tests to investigate presumed resistances of *L. monocytogenes* to antibiotics have not given any significant results. Nevertheless, such investigations might be important in future.

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11 Appendix

11.1 Bacterial counts of the microbial screening

Table 63: Bacterial counts of the microbial screening

Serial number	Identification	Sample characteristic	Total viable count	Lactic acid bacteria	Enterobacteriaceae	Yeasts	Moulds	Pseudomonas sp.	Staphylococcus sp.	Bacillus sp.	Brochothrix thermosphacta	Clostridium sp.
2	B-02	blanched	1916,7	270,0	10,0	100,0	10,0	1000,0	100,0	100,0	100,0	10,0
12	Y-01	blanched	2941,7	10,0	10,0	100,0	10,0	2550,0	100,0	100,0	100,0	10,0
13	Y-02	blanched	5132,2	1514,3	100,0	100,0	10,0	5200,0	100,0	100,0	1100,0	10,0
49	M-05	blanched	281982,0	1770,0	240,0	100,0	10,0	1100,0	100,0	100,0	4851,5	100,0
50	M-06	blanched	83636,4	100,0	10,0	10,0	100,0	100,0	100,0	100,0	21181,8	10,0
53	M-09	blanched	838095,2	6100,0	10,0	10,0	10,0	100,0	100,0	100,0	52000,0	10,0
58	RA-03	blanched	185,0	10,0	10,0	10,0	10,0	100,0	100,0	100,0	100,0	10,0
1	K-01	cooked	130,0	100,0	10,0	10,0	10,0	100,0	100,0	100,0	100,0	10,0
7	RG-01	cooked	20000,0	10,0	10,0	10,0	10,0	100,0	100,0	100,0	100,0	10,0
9	QF-01	cooked	20772,7	100,0	10,0	100,0	10,0	100,0	100,0	100,0	100,0	10,0
10	QF-02	cooked	36750,0	325,0	10,0	100,0	10,0	100,0	100,0	100,0	4500,0	490,0
11	QF-03	cooked	162381,0	22416,7	10,0	100,0	10,0	1000,0	100,0	100,0	1000,0	10,0
14	Y-03	cooked	12909,1	100,0	10,0	100,0	10,0	100,0	100,0	100,0	100,0	10,0
16	CN-02	cooked	940,0	10,0	10,0	100,0	10,0	100,0	100,0	1000,0	100,0	10,0
17	CN-03	cooked	5400,0	10,0	100,0	275,0	10,0	1000,0	100,0	100,0	100,0	10,0
23	CN-09	cooked	1800,0	10,0	100,0	10,0	10,0	1000,0	100,0	100,0	100,0	10,0
24	CN-10	cooked	267272,7	10000,0	10,0	100,0	10,0	100,0	100,0	100,0	100,0	10,0
25	CN-11	cooked	4800,0	10,0	10,0	10,0	10,0	1000,0	100,0	100,0	100,0	10,0
28	CN-14	cooked	7300,0	10,0	100,0	10,0	10,0	1600,0	1000,0	100,0	100,0	10,0
29	CN-15	cooked	1300,0	10,0	10,0	10,0	10,0	1000,0	100,0	100,0	100,0	10,0
30	CN-16	cooked	2800,0	100,0	10,0	10,0	10,0	100,0	100,0	100,0	1000,0	10,0
33	CN-19	cooked	3500,0	120,0	10,0	100,0	10,0	100,0	100,0	100,0	100,0	10,0
51	M-07	cooked	355,0	10,0	10,0	10,0	10,0	100,0	100,0	100,0	100,0	10,0
57	RA-02	cooked	51904,8	909,5	10,0	485,7	10,0	1700,0	100,0	100,0	1000,0	10,0

3	R-03	raw	2000000,0	134545,5	100,0	135,0	10,0	1900,0	100,0	100,0	8401,1	10,0
4	C-04	raw	26409,1	2131,8	10,0	100,0	10,0	100,0	100,0	100,0	1000,0	10,0
5	G-05	raw	47500,0	120,0	10,0	100,0	10,0	1000,0	100,0	100,0	1700,0	10,0
6	L-06	raw	18318,2	2350,0	10,0	100,0	10,0	1000,0	100,0	100,0	100,0	10,0
8	KP-01	raw	4750,0	10,0	100,0	100,0	10,0	1000,0	100,0	100,0	100,0	10,0
15	CN-01	raw	120900,0	2014,0	100,0	260,0	10,0	10000,0	100,0	100,0	1000,0	10,0
18	CN-04	raw	17640,0	100,0	10,0	100,0	10,0	1000,0	100,0	100,0	100,0	10,0
19	CN-05	raw	57000,0	180,0	10,0	180,0	10,0	100,0	100,0	100,0	1000,0	10,0
20	CN-06	raw	50000,0	390,0	100,0	100,0	10,0	1000,0	100,0	100,0	1600,0	10,0
21	CN-07	raw	12290000,0	11190000,0	970,0	100,0	10,0	10000,0	100,0	100,0	179090,9	10,0
22	CN-08	raw	84000,0	250,0	10,0	100,0	10,0	100,0	100,0	100,0	1000,0	10,0
26	CN-12	raw	284600,0	100,0	10,0	130,0	10,0	1000,0	100,0	100,0	100,0	10,0
27	CN-13	raw	164500,0	2000,0	170,0	150,0	10,0	1000,0	100,0	100,0	100,0	10,0
31	CN-17	raw	325800,0	15670,0	10,0	100,0	100,0	1000,0	100,0	100,0	5900,0	10,0
32	CN-18	raw	31833,3	100,0	10,0	100,0	100,0	1000,0	100,0	100,0	100,0	10,0
34	CN-20	raw	4697000,0	97000,0	450,0	660,0	100,0	8619,0	100,0	100,0	49500,0	10,0
35	CN-21	raw	417641,8	217606,3	100,0	240,0	100,0	5100,0	100,0	100,0	9095,2	10,0
36	CN-22	raw	69000,0	790,0	10,0	100,0	100,0	1000,0	100,0	100,0	8272,7	10,0
37	CN-23	raw	1600000,0	13000,0	930,0	910,0	100,0	3800,0	100,0	100,0	1700,0	10,0
38	CN-24	raw	1062000,0	13050,0	100,0	200,0	10,0	4809,5	1000,0	100,0	235000,0	10,0
39	CN-25	raw	156700,0	1959,0	10,0	100,0	10,0	1000,0	100,0	100,0	2300,0	10,0
40	CN-26	raw	35000,0	100,0	10,0	100,0	10,0	100,0	100,0	100,0	100,0	10,0
41	CN-27	raw	82500,0	17250,0	10,0	100,0	10,0	1000,0	100,0	100,0	1000,0	10,0
42	CN-28	raw	655000,0	34500,0	100,0	100,0	10,0	1000,0	100,0	100,0	2600,0	10,0
43	CN-29	raw	450000,0	7700,0	10,0	110,0	10,0	1000,0	100,0	100,0	100,0	10,0
44	CN-30	raw	1283333,3	310,0	10,0	100,0	10,0	1000,0	100,0	100,0	7904,8	10,0
45	M-01	raw	149520,0	4200,0	10,0	100,0	10,0	1000,0	100,0	100,0	18590,9	10,0
46	M-02	raw	367768,6	24360,0	10,0	130,0	10,0	1000,0	100,0	100,0	167142,9	10,0
47	M-03	raw	2375,0	100,0	10,0	100,0	10,0	100,0	100,0	100,0	100,0	10,0
48	M-04	raw	465000,0	1986,4	370,0	155,0	10,0	1600,0	100,0	100,0	1400,0	100,0
52	M-08	raw	1859090,9	260,0	10,0	100,0	100,0	1000,0	100,0	100,0	1000,0	100,0
54	M-10	raw	1872727,3	176818,2	10,0	100,0	10,0	1000,0	100,0	100,0	540000,0	10,0
55	M-11	raw	119047,6	1036,4	10,0	100,0	10,0	1900,0	100,0	1000,0	100,0	10,0
56	RA-01	raw	126363,6	13190,5	10,0	100,0	10,0	1000,0	100,0	100,0	2650,0	10,0

11.2 List of examined shrimp samples

Table 64: List of shrimp samples examined in this study

Serial number	Identification	Convenience degree	Market form		Date of expiry	Batch No. / Lot No.	Storage temperature
1	K-01	cooked	headless	peeled, deveined	29.04.2010	L 8309B3	-20°C
2	B-02	blanched	headless	peeled, deveined	30.11.2010	L 8309B0N17	-20°C
3	R-03	raw	headless	peeled	16.12.2009	8304-9050	-20°C
4	C-04	raw	headless	peeled	27.12.2009	8331-9061	-20°C
5	G-05	raw	headless	peeled	14.05.2010	201108M	-20°C
6	L-06	raw	headless	peeled	30.07.2010	29345 07:25 330	-20°C
7	RG-01	cooked	headless	peeled, deveined	20.04.2009	476492.092.11	-20°C
8	KP-01	raw	headless	peeled	31.10.2010	31777 12:35 058	-20°C
9	QF-01	cooked	headless	peeled, deveined, tail on	30.09.2009	8254-8329	-20°C
10	QF-02	cooked	headless	deveined	31.12.2009	8304-9047	-20°C
11	QF-03	cooked	headless	deveined	31.08.2009	8127-8285	-20°C
12	Y-01	blanched	head on	shell on	15.02.2011	L9 047	-20°C
13	Y-02	blanched	headless	deveined	25.09.2010	L8 269	-20°C
14	Y-03	cooked	headless	peeled	25.11.2010	L8 312	-20°C
15	CN-01	raw	headless	shell on	12.09.2010	UTA 08/35 VN/322/V/073	-20°C
16	CN-02	cooked	headless	peeled, deveined, tail on	31.07.2010	L8283 KK 36	-20°C
17	CN-03	cooked	headless	peeled, deveined	31.09.2010	L8341 LS51	-20°C
18	CN-04	raw	headless	peeled, deveined, tail on	03.09.2010	L7 VN/025/V/246038	-20°C
19	CN-05	raw	headless	peeled, deveined	30.09.2010	L8 345 VN/181/V062	-20°C
20	CN-06	raw	headless	peeled, deveined	31.12.2009	HV 87178 VN/391/V069	-20°C
21	CN-07	raw	headless	shell on	30.06.2010	DL 2 VOG 11074	-20°C
22	CN-08	raw	headless	shell on	31.07.2009	DL 2 VOGG 9915 05:05	-20°C
23	CN-09	cooked	headless	peeled	03.04.2010		-20°C
24	CN-10	cooked	headless	peeled	31.05.2010	DL VOGG 2275	-20°C
25	CN-11	cooked	headless	peeled, deveined, tail on	01.10.2009	Job. 9519	-20°C

26	CN-12	raw	head on	shell on	01.06.2010		-20°C
27	CN-13	raw	head on	shell on	27.05.2009		-20°C
28	CN-14	cooked	headless	peeled	20.11.2010		-20°C
29	CN-15	cooked	headless	peeled	31.12.2010	VN 322 V096	-20°C
30	CN-16	cooked	headless	peeled, deveined	09.04.2010	0904 D1	-20°C
31	CN-17	raw	headless	shell on	01.11.2009	Job. 9637	-20°C
32	CN-18	raw	head on	shell on	01.10.2009		-20°C
33	CN-19	cooked	headless	peeled, deveined	30.04.2010	L8282 E1	-20°C
34	CN-20	raw	headless	shell on	01.10.2010	D-6508	-20°C
35	CN-21	raw	headless	shell on	30.06.2009	Job. 9825	-20°C
36	CN-22	raw	headless	shell on	31.07.2009	DL VOGG 9914	-20°C
37	CN-23	raw	headless	shell on	15.10.2010	11226	-20°C
38	CN-24	raw	headless	shell on	20.10.2009		-20°C
39	CN-25	raw	headless	peeled, deveined	31.07.2009	DL VOG 9919	-20°C
40	CN-26	raw	headless	peeled, deveined	18.05.2010	L.8 138	-20°C
41	CN-27	raw	headless	peeled, deveined	31.05.2009		-20°C
42	CN-28	raw	headless	peeled	30.04.2009	Job. 8867	-20°C
43	CN-29	raw	headless	peeled, deveined	13.01.2011	11226	-20°C
44	CN-30	raw	headless	deveined, shell on	30.06.2009	L.7 165	-20°C
45	M-01	raw	headless	peeled	01.03.2011	1001000498 102201 9338	-20°C
46	M-02	raw	headless	peeled, deveined	01.08.2011	VN 458 V042	-20°C
47	M-03	raw	head on	shell on	31.03.2011	P092455	-20°C
48	M-04	raw	head on	peeled, deveined	06.07.2011		-20°C
49	M-05	blanched	headless	deveined, shell on	21.01.2011	L 092134	-20°C
50	M-06	blanched	headless	peeled	15.03.2011	L 092534	-20°C
51	M-07	cooked	head on	shell on	04.05.2011	L 10.11.2009 2 18:17	-20°C
52	M-08	raw	head on	shell on	30.03.2011	0481P	-20°C
53	M-09	blanched	headless	peeled	07.04.2011	L 092573	-20°C
54	M-10	raw	headless	shell on	31.08.2011	12803	-20°C

55	M-11	raw	headless	deveined, shell on	25.09.2011	L9 294	-20°C
56	RA-01	raw	headless	shell on	25.09.2011	110430	-20°C
57	RA-02	cooked	headless	peeled, deveined, tail on	31.07.2011	DL 2 SEA 12685	-20°C
58	RA-03	blanched	headless	peeled	31.07.2011	110731	-20°C