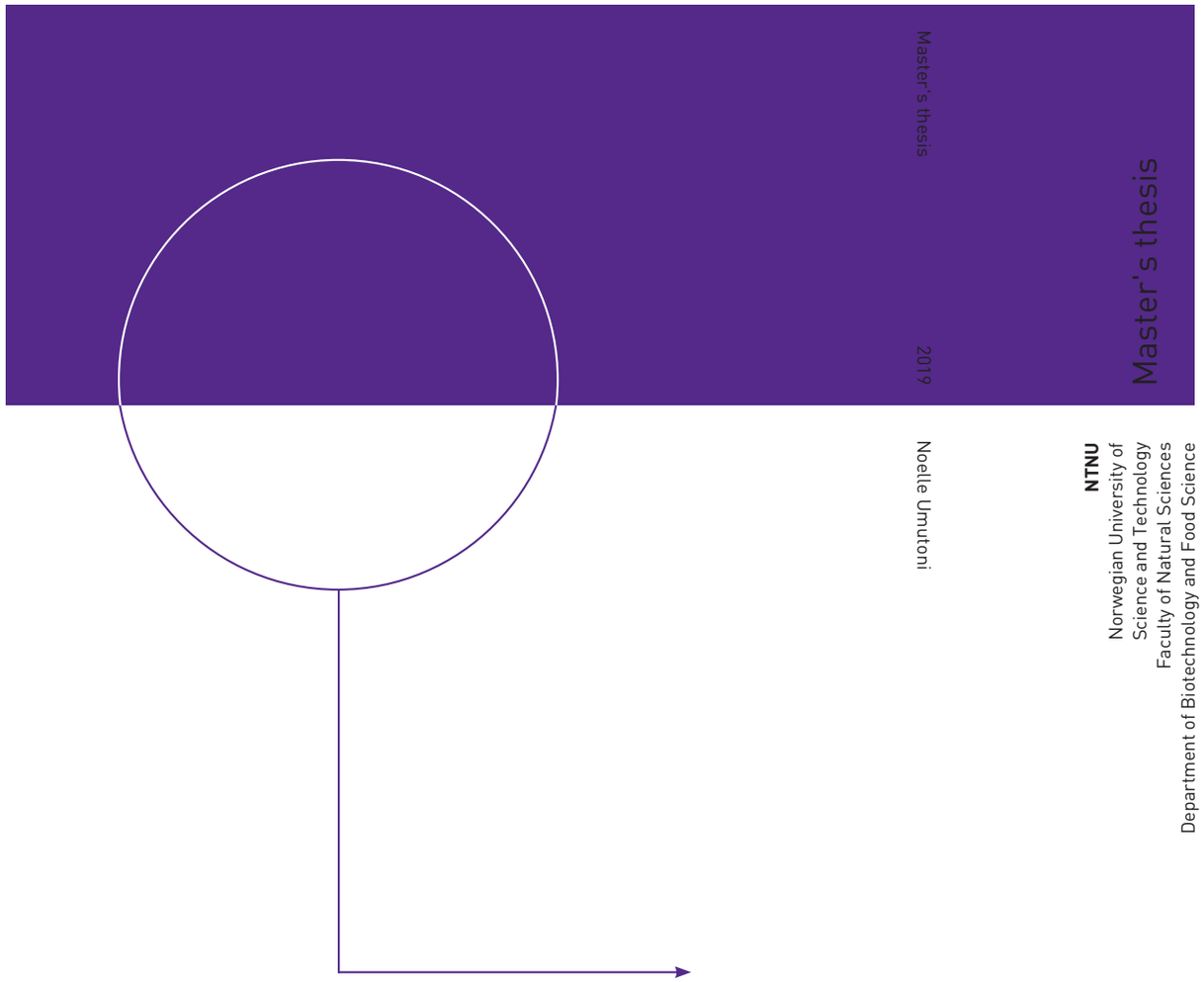

Master's thesis

2019

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Identification and Characterization of Aeromonas species isolated from ready-to-eat lettuce products.

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Food science and Technology
Submission date: May 2019
Supervisor: Lisbeth Mehli

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Preface

This thesis covers 45 ECTS-credits and was carried out as part of the M. Sc. programme for Food and Technology at the institute of Biotechnology and Food Science, faculty of natural sciences at the Norwegian University of Science and Technology in Trondheim in spring 2019.

First, I would like to express my gratitude to my main supervisor Associate professor Lisbeth Mehli. Thank you for the laughs, advice, and continuous encouragement throughout the project. Furthermore, appreciations to PhD Assistant professor Gunn Merethe Bjørge Thomassen for valuable help in the lab. Great thanks to my family and friends for their patience and encouragement these past years. Thank you for listening, despite not always understanding the context of my studies. A huge self-five to myself, for putting in the work.

Finally, a tremendous thank you to Johan – my partner in crime and in life. I could not have done this without you. You kept me fed, you kept sane. I appreciate you from here to eternity.

Mama, we made it!

15th of May 2019

Author
Noelle Umutoni

Abstract

Aeromonas spp. are ubiquitous bacteria in all environments, and can be isolated from aquatic habitats. *Aeromonas* spp. are recognized opportunistic pathogens, with the ability to grow at a temperature range for growth between 4 and 37 °C. *Aeromonas* spp. can cause food spoilage, and represent a potential risk to consumers health due to their pathogenicity and ability to grow at refrigeration temperatures.

Ready-to-eat (RTE) fruits and vegetables are an important and rapidly growing class of foods because of their convenience, and noted health benefits. The production and sale of such products has increased and a further increase is expected in the future. Foodborne pathogens can be present in RTE vegetables, and a number of outbreaks of foodborne disease have been traced to RTE vegetables because they are susceptible to contamination at several stages of production. The risks associated with water employed in the production of leafy vegetables must be identified, as the use of water containing pathogens in edible crops may result in contamination of the crop. Gastroenteritis is the most common infection caused by *Aeromonas* spp., and is associated with the consumption of large numbers of bacteria by both immune-competent and immune-compromised individuals.

The objective of this work was to characterize and identify *Aeromonas* spp. isolated from ready-to-eat lettuce products. The isolates were part of an earlier project «*Lokale spiseklare produkter - mattrygghet langs hele verdikjeden»* at the Norwegian University of Science and Technology (NTNU). The work was divided in four parts. First, growth of *Aeromonas* isolates was analyzed after several years of storage at -80 °C, and the isolates were thereafter identified by biochemical and molecular methods. The identified isolates were further analyzed for prevalence of virulence characteristics described for the *Aeromonas* genus. Lastly, growth characteristics of selected isolates from the four producers we evaluated.

The overall results showed that storage of bacterial samples at below 80 degrees kept the DNA intact. Furthermore, a combination of traditional biochemical and modern molecular methods were powerful tools that identified *Aeromonas* species at genus and genospecies level. All isolates harbored several of the virulence genes analyzed in this work, and a proportion were hemolytic. The growth of *Aeromonas* spp. seems to be highly affected by several factors, including natural microbiota, plant composition content, storage conditions, and pre- and post-harvesting processes. However, further studies that assess the growth of *Aeromonas* species under stimulated storage conditions are necessary. Moreover, further identification and characterization studies are necessary to verify the identification results found in this work.

Sammendrag

Aeromonas spp. er vidt utbredt i naturen, og kan isoleres fra akvatiske miljøer. *Aeromonas* spp. er en anerkjent opportunistisk patogen, som kan formere seg og vokse ved temperaturer mellom 4 and 37 °C. Av den grunn, utgjør denne bakterien en fare for folkehelsen, med tanke på forringelse og mattrygghet av produkter som lagres ved kjøletemperatur, deriblant spiseklare salatblandinger.

Spiseklare produkter blir stadig mer populære, da de er praktiske og sunne alternativer til forbrukeren i en travel hverdag. I de siste årene, har både salg og produksjon av slike produkter økt og en større økning forventes i fremtiden. Matbårne patogener kan forekomme i spiseklare salatblandinger, og en rekke utbrudd av matbåren sykdom har blitt sporet til spiseklare grønnsaker. Kontaminasjon forårsaket av *Aeromonas* spp. kan forekomme ved ulike ledd i produksjonskjeden, hvorav dårlig vannkvalitet anses den viktigste kilden til forringelse forårsaket av *Aeromonas* spp. Gastroenteritt er den vanligste infeksjonen forårsaket av *Aeromonas* spp., og forekommer både hos friske individer, og blant utsatte grupper som har svekket immunforsvar.

Målet med denne oppgaven var å identifisere og karakterisere tidligere isolerte *Aeromonas* spp. fra spiseklare salatblandinger produsert av fire lokale produsenter. Isolatene var en del av et tidligere prosjekt ved Norges teknisk-naturvitenskapelige universitet (NTNU), «*Lokale spiseklare produkter - mattrygghet langs hele verdikjeden»*. Oppgaven var delt i fire deler. Aller først ble vekst av *Aeromonas* isolatene analysert etter flere års lagring ved -80 °C. Videre ble identifisering av isolatene utført ved hjelp av biokjemiske og molekylære metoder. De identifiserte artene ble deretter analysert for tilstedeværelse av virulensgener som har blitt beskrevet for *Aeromonas* arten. Til slutt ble det gjennomført et lagringsforsøk, hvor vekstegenskapene til noen utvalgte isolater ble undersøkt.

Resultatene viste at DNA ikke ble degradert etter flere års lagring ved -80 °C.
En kombinasjon av biokjemiske- og molekylære metoder var viktige verktøy som identifiserte artene på slekt- og artsnivå. Alle isolatene hadde tilstedeværelse av ulike virulensgener som ble analysert i denne oppgaven, og en andel var hemolytiske. Flere faktorer som blant annet, naturlig mikroflora, lagringsforhold, naturlige komponenter i produktene, og andre før- og etter høstings prosesser kan ha påvirket veksten av *Aeromonas* spp. under lagringsforsøket.

Flere studier som analyserer vekst av *Aeromonas* spp. er nødvendige. Tilsvarende behøves det flere studier for å verifisere identifikasjonsresultatene funnet i denne oppgaven.

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1 Introduction

Fresh fruits and vegetables are an essential part of a healthy human diet, because they contain high amounts of vitamins and fibers, as well as minerals and antioxidants. Leafy greens is a term used to describe all vegetables of a leafy nature and of which the leaf (and core) is intended to be consumed raw, such as lettuce, spinach, cabbage, leafy herbs (cilantro, basil, parsley), et cetera. (Food and Agriculture Organization of the United Nations & World Health Organization, 2008a; Pingulkar, 2001) A regular and sufficient consumption of fruits and vegetables can help prevent cardiovascular diseases, and certain cancers. (International Agency for Research on Cancer, 2003; Key, 2010; World Health Organization, 2019) Lettuce, spinach, cabbages, and other leafy vegetables have been identified to make an important contribution against these diseases. (Hung et al., 2004; Link & Potter, 2004)

Ready-to-eat (RTE) fruits and vegetables are an important and rapidly growing class of foods because of their convenience. (Food and Agriculture Organization of the United Nations & World Health Organization, 2008b; Pingulkar, 2001) Many countries have taken initiative to encourage consumers to eat more leafy greens. In recent years, a shift in consumption trends where consumers seek convenient options such as RTE salads has been recognized. This has led to a significant increase in the consumption of leafy greens, and the demand for fresh vegetables has resulted in a growth of the market share for RTE vegetables. (Abadias, Usall, Anguera, Solsona, & Viñas, 2008; De Giusti et al., 2010; J.M. Soriano, 2000; Oliveira et al., 2015) In particular, the fresh market for leafy vegetables and herbs has increased significantly, and it is expected that both the consumption and production of leafy green vegetables will continue to increase in the future.

Between 1990 and 2004, the worldwide consumption of fruits and vegetables increased by an average of 4.5 %, which led to a progressive increase in the world's production and harvest area of leafy vegetables such as lettuce, chicory, cabbages and other brassicas. By 2006, the harvest area for lettuce and chicory had increased by 218 %, whereas spinach had increased by 300 %. The major producers of lettuce and chicory, by 2006, were China (50 %) and the United States of America (20 %). (Food and Agriculture Organization of the United Nations & World Health Organization, 2008b)

As the demand for fresh, preservative-free vegetables increases, so does the importance of good hygiene practices, especially in countries where hygiene standards might be compromised. According to the Codex Alimentarius Commission "*Food safety is assurance that food will not cause harm to the consumer when it is prepared and/or eaten according its intended use.*" This means that everyone involved in the food chain, from farm to fork, shares a joint responsibility to ensure that food is safe for consumption. (Codex Alimentarius Commission, 2009)

The risks associated with water employed in the production of leafy vegetables must be identified, as the use of water containing pathogens in edible crops may result in contamination of the crop. (Food and Agriculture Organization of the United Nations & World Health Organization, 2008b; Thurston-Enriquez et al., 2002) For pathogenic microorganisms, this applies especially to watering of vegetable plants that are meant to be consumed without further treatment that can inactivate the infectious agent. (Norwegian Scientific Committee for Food Safety, 2014)

Pathogens and undesirable chemical substances that are present in such water can cause human disease if the water is used for irrigation of vegetable crops. Several risk assessments have quantified the risks associated with the contamination of leafy vegetables and herbs with enteric pathogens in irrigation water (Hamilton, Stagnitti, Premier, Boland, & Hale, 2006; Petterson, Ashbolt, & Sharma, 2001; Stine, Song, Choi, Gerba, & Stine, 2005). Experimental evidence has also confirmed that water used for irrigation can transfer human pathogens to several growing leafy vegetables. (Amoah et al., 2005; Melloul, Hassani, & Rafouk, 2001; Solomon, Yaron, & Matthews, 2002; Song, Stine, Choi, & Gerba, 2006)

Notably, these collectively provide strong evidence that water quality is an important risk factor that must be taken in consideration while producing these foods. (Food and Agriculture Organization of the United Nations & World Health Organization, 2008b) The presence and prevalence of pathogenic organisms in leafy green vegetables can be a potential problem for consumer health and is a public health issue. (De Giusti et al., 2010; J.M. Soriano, 2000; McMahon & Wilson, 2001)

Ready-to-eat products can serve as vehicles for foodborne pathogens and toxins. Okafo, Umoh, and Galadima (2003) reported the detection of *Salmonella* and *Vibrio* on lettuce and amaranthus when irrigation water was contaminated. (Okafo et al., 2003) *Campylobacter* and *Listeria monocytogenes* have also been detected in watercress grown in and harvested from contaminated water (Edmonds & Hawke, 2004; Prazak, Murano, Mercado, & Acuff, 2002). Söderström, Lindberg, and Andersson (2005) reported a large outbreak of *Escherichia coli* O157 contamination in iceberg lettuce in Sweden, which was linked to the use of contaminated irrigation water drawn from a small stream. (Söderström et al., 2005)

Several factors including indigenous microbiota, inherent food characteristics, processing technology, packaging, storage temperature, production hygiene, slicing equipment and utensils, and hygienic design, can affect the product's safety. (Hoel, Vadstein, & Jakobsen, 2019) Among the greatest concerns with human pathogens on fresh fruits and vegetables are enteric pathogens such as *E. coli* O157:H7 and *Salmonella*. This is due to their potential for growth prior to consumption, or low infectious doses. (L. R. Beuchat, 2002; Buck, Walcott, & Beuchat, 2003)

Opportunistic and emerging pathogens are thought to be involved in a large number of foodborne illnesses each year. In the United States of America, the Centre for Disease Control and Prevention (CDC) estimates that 38.6 million foodborne illnesses each year are caused by unknown pathogens. (Bhunia, 2018; Hausdorf, 2012)

Bacterial pathogens that can cause human disease, such as *Campylobacter jejuni*, *Shigella*, *Staphylococcus*, *Citrobacter freundii*, *Enterobacter cloacae*, *Vibrio cholerae*, *Klebsiella* spp. and *Aeromonas* spp. have previously been isolated from lettuce and salad samples (L. Beuchat, 1996; L. R. Beuchat, 2002; National Advisory Committee on Microbiological Criteria for, 1999; National Advisory Committee on Microbiological Criteria for Foods, 1999b). For this reason, the hygienic quality of such products must be controlled to reduce microbial populations and the risk for foodborne disease. (Francis, Thomas, & O' Beirne, 1999)

Bacteria belonging to the genus *Aeromonas* are ubiquitous in the environment, and can be present in ready-to-eat vegetables due to potential sources of contamination such as fresh water, seawater, chlorinated drinking water, animal feces and sewage. (Latif-Eugenín, Beaz-Hidalgo, & Figueras, 2016) *Aeromonas* spp. are recognized as increasingly important human pathogens because of their frequent prevalence in all types of food, including vegetables. (Hoel et al., 2019) They also represent a potential risk to consumers health due to their possible pathogenicity and ability to grow at refrigeration temperatures. (Cahill, 1990; J. Michael Janda & Abbott, 2010; J. Michael Janda & Duffey, 1988)

In the project «*Lokale spiseklare produkter - mattrygghet langs hele verdikjeden»* proceeding this master's thesis, the microbial quality of ready-to-eat leafy salads was assessed through a period of three years. In total, 307 different lettuce products were sampled from retail stores and analysed in triplicates. The lettuce products were mainly from regional producers. The microbial quality between product types (whole lettuce vs pre-cut and washed lettuce) is significant (results not published; Mehli pers.com). Pre-cut and washed lettuce is more heavily contaminated compared to whole lettuce. The levels of *Aeromonas* in the lettuce samples varied between 0 and 4,5 log CFU. *Aeromonas* contamination in samples from the different producers varied between 41 to 66 %, producer A (59 %), B (50 %), C (41 %) and D (66 %), respectively (results not published).

The objective of this work was to identify and characterize presumptive *Aeromonas* spp. isolated from regional ready-to-eat lettuce products by biochemical and molecular methods. The work was divided into four parts:

1. Growth of *Aeromonas* isolates after storage at -80°C.
2. Identification of *Aeromonas* isolates from 4 producers in Mid Norway.
3. Prevalence of virulence characteristics of the identified isolates.
4. Growth characteristics of selected isolates from the four producers.

2 *Aeromonas*

2.1 Taxonomy, identification and classification

The genus *Aeromonas* belongs to the Aeromonadaceae family and comprises a collection of gram-negative, facultatively anaerobic, oxidase positive, catalase positive, glucose fermentative, chemoorganotrophic rod-shaped bacteria that are generally motile by means of polar flagella. (Daskalov, 2006; Hazen, Fliermans, Hirsch, & Esch, 1978; Khardori & Fainstein, 1988) Aeromonads that belong to the *Aeromonas* genus have traditionally been classified in two main groups; the motile mesophilic species versus the non-motile psychrophilic species. The classification is based upon growth characteristics and other biochemical features. (Christopher J Grim, 2013; Hoel, 2018; J. Michael Janda & Abbott, 2010; Martin-Carnahan & Joseph, 2005; Yáñez, Catalán, Apráiz, Figueras, & Martínez-Murcia, 2003)

Previously, *Aeromonas* spp. were recognized based on the analysis of the 16S rRNA gene and DNA-hybridization groups. 16S rDNA sequence analysis is routinely used to distinguish and establish relationships between bacterial genera. However, in the case of *Aeromonas*, 16S rRNA gene sequencing has proven to be inefficient due to low taxonomic resolution. (Morandi, Zhaxybayeva, Gogarten, & Graf, 2005; Nagar, Shashidhar, & Bandekar, 2013) The *Aeromonas* 16S rRNA gene sequences are extremely conserved when compared to other bacteria genera, and lacks enough specificity for differentiating closely related species. (Küpfer, Kuhnert, Korczak, Peduzzi, & Demarta, 2006) Thus, it has become necessary to sequence for alternative genes that are selective to Aeromonads. Studies have shown that sequencing for different housekeeping genes that include an RNA polymerase B subunit (*rpoB*), an RNA polymerase D subunit (*rpoD*), and a DNA gyrase B subunit (*gyrB*) give a more precise identification of *Aeromonas* strains to genospecies level. (R. Beaz-Hidalgo et al., 2015; Percival & Williams, 2014; Persson, Al.Shuweli, Yapici, Jensen, & Olsen, 2015)

After sequencing of protein-coding housekeeping genes was implemented, several new species have been discovered and the genus has gained a more clear and robust taxonomy. Currently, 36 species are now recognized as part of the *Aeromonas* genus (table 1). (M. J. Figueras et al., 2017)

Table 1. Recognized species that belong to the *Aeromonas* genus. The species in bold represent the most prevalent clinical species. (M.J Figueras & Beaz-Hidalgo, 2014) The lowercase superscript letters show where the species were isolated; clinical species (a), water(b), and fish/seafood (c). The table is adapted from Hoel, Vadstein, and Jakobsen (2017)

Species	Source of Isolation (year) of type strain	Reference
A. allosaccharophila [a,c]	Eel (1992)	(Martinez-Murcia, Esteve, Garay, & Collins, 1992)
A. aquatica [b]	Lake water (2015)	(R. Beaz-Hidalgo et al., 2015)
A. aquatilis [b]	Lake water (2017)	(M. J. Figueras et al., 2017)
A. australiensis [b]	Water (2013)	(Aravena-Román et al., 2013)
A. bestiarum [a–c]	Diseased fish (1996)	(Ali, Carnahar, Altwegg, & Lüthy-Hottensterin, 1996)
A. bivalvium [c]	Bivalve mollusks (2007)	(Miñana-Galbis, Farfán, Lorén, & Fusté, 2002)
A. cavernicola [b]	Fresh water (2013)	(A. Martínez-Murcia et al., 2013)
A. caviae [a–c,†]	Guinea pig (1984)	(Popoff, 1984)
A. crassostreae [c]	Oyster (2017)	(M. J. Figueras et al., 2017)
A. dhakensis [a–c,*]	Aquarium water (2008)/Human (diarrheic stool) (2002)	(A. J. Martínez-Murcia et al., 2008), (G. Huys et al., 2002), (Roxana Beaz-Hidalgo, Martinez-Murcia, & Figueras, 2013)
A. diversa [a]	Human (wound infection) (2010)	(Miñana-Galbis, Farfán, Gaspar Lorén, & Carmen Fusté, 2010)
A. encheleia [a,c]	Eel (1995)	(Esteve, 1995)
A. enterica [a]	Human (diarrheic stool) (2017)	(M. J. Figueras et al., 2017)
A. eurenophila [a–c]	Fresh water fish (1998)	(SIngh & Sanyal, 1997)
A. finlandiensis [b]	Lake water (2015)	(R. Beaz-Hidalgo et al., 2015)
A. fluvialis [b]	River water (2010)	(Alperi, Martinez-Murcia, Monera, Saavedra, & Figueras, 2010)
A. hydrophila [a–c]	Milk (1943)	(Stanier, 1943)
A. intestinalis [a]	Human (diarrheic stool) (2017)	(M. J. Figueras et al., 2017)
A. jandaei [a–c]	Human (feces) (1991)	(Carnahan, Fanning, & Joseph, 1991)
A. lacus [b]	Lake water (2015)	(R. Beaz-Hidalgo et al., 2015)
A. lusitana [b]	Water (2012)	(A. Martínez-Murcia et al., 2016)
A. media [a–c]	Water (1983)	(Allen, Austin, & Colwell, 1983)
A. molluscorum [c]	Bivalve mollusks (2004)	(Miñana-Galbis, Farfán, Fusté, & Lorén, 2004)
A. piscicola [c]	Diseased fish (salmon) (2009)	(R. Beaz-Hidalgo, Alperi, Figueras, & Romalde, 2009)
A. popoffii [a,b]	Drinking water (1997)	(Geert Huys et al., 1997)
A. rivipollensis [b]	River sediments (2016)	(Marti & Balcázar, 2015)
A. rivuli [b]	Water rivulet (2011)	(M. J. Figueras et al., 2011)
A. salmonicida [a–c,**]	Fish (salmon) (1953)	(Griffin, Snieszko, & Friddle, 1953)
A. sanarellii [a]	Human (wound infection) (2010)	(Anabel Alperi et al., 2010)
A. schubertii [a,c]	Human (1988)	(Hickman-Brenner, Fanning, Arduino, Brenner, & Farmer, 1988)
A. simiae	Monkey feces (2004)	(Harf-Monteil et al., 2004)
A. sobria [a–c]	Fish (1976)	(Popoff & Véron, 1976)
A. taiwanesis [a]	Human (wound infection) (2010)	(Anabel Alperi et al., 2010)
A. tecta [a,c]	Human feces (2008)	(Demarta et al., 2008)
A. trota [a,††]	Human feces (1991)	(A. M. Carnahan et al., 1991)
A. veronii [a–c]	Human (sputum) (1987)	(Hickman-Brenner et al., 1987)

†synonymous with *A. punctata* †† Synonymous with *A. enteropelogenes*

**A. aquariorum* and *A. hydrophila subsp. dhakensis* were synonymized under the name *A. dhakensis*

***The subset of mesophilic, motile stains that can grow at 37 °C

The nutritional requirements of aeromonads are simple because they ferment many monosaccharides, disaccharides, glycosides and some alcoholic sugars. They can, therefore, be cultured on many non-selective, differential and selective agars. (Percival & Williams, 2014, p. 49) Aeromonads can also reduce nitrate, and produce several extracellular enzymes such as cytolytic toxins, proteases, nucleases, lipases, sulphatases, lecithinase, chitinase, amylase and stapholysin. (McCoy et al., 2010; Meng, Liu, & Lu, 2009; Palumbo, Bencivengo, Del Corral, Williams, & Buchanan, 1989; Pemberton, Kidd, & Schmidt, 1997) Blood agar can be used to screen aeromonads for cytochrome oxidase and tryptophanase deamination, which are both positive for most aeromonads. (Kelly, Stroh, & Jessop, 1988) Several *Aeromonas* strains exhibit β-hemolysis on sheep blood agar. Some produce double zone hemolysis, which is when there is an outer zone of incomplete clearing and inner zone of complete β-hemolysis. (Percival & Williams, 2014, p. 58)

Aeromonads are as noted above identified by colony morphology, oxidase tests and hemolytic reaction on blood agar. Several selective media have been used to isolate aeromonads from sewage, water, sludge and polluted waters. (Seidler et al., 1980) Identification of phenospecies depends on biochemical testing. (Percival & Williams, 2014, p. 57) *Aeromonas* colonies are buff-colored, smooth, convex, and about 3-5 mm in diameter after 24h incubation at 37 °C. It has previously been documented that most aeromonads are capable of growth in media containing up to 4 % NaCl and a pH range of 5-9, which is similar to their natural aquatic environments. (D. A. Austin, McIntosh, & Austin, 1989)

2.2 Aeromonas spp. in human disease

The mesophilic aeromonads are the main group associated with human disease, and includes human pathogens such as *Aeromonas hydrophila*, *Aeromonas dhakensis* and *Aeromonas media*. The main species within the group that are responsible for a majority of human infections are *Aeromonas caviae*, *A. hydrophila*, *A. dhakensis* and *A. veronii* biovar *sobria*. (J. Michael Janda & Abbott, 2010) These species can cause a variety of extraintestinal and systemic infections, as well as gastrointestinal infections. It is clear that water is the source of most cases of gastroenteritis caused by *Aeromonas* spp. worldwide, as they can be isolated from virtually all fresh and brackish water. (P. E. Granum, 2015, p. 59)

Gastroenteritis is the most common infection caused by *Aeromonas* spp., and is associated with the consumption of large numbers of bacteria by both immune-competent and immune-compromised individuals. (Nagar & Bandekar, 2011) 85 % of *Aeromonas* gastrointestinal infections are caused by *A. hydrophila*, *A. caviae* and *A. veronii* biovar *sobria*. (J. M. Janda, 1991)

Mesophilic strains are most often motile and grow well between 35 to 37 °C, but they can also grow at temperatures as low as 4 °C, thereby posing as a health threat in foods stored at refrigerator temperatures. (Christopher J Grim, 2013; J. Michael Janda & Abbott, 2010; Ørmen, Granum, Lassen, & Figueras, 2005) The second group, psychrophilic *Aeromonas*, consists of non-motile strains where *A. salmonicida* is the primary bacteria that is pathogenic to fish and cold-blooded animals. Strains in the latter group have optimal growth temperatures of 22 to 25 °C. An overview of environmental sources for aeromonads, that might lead to human disease is shown in figure 1. (J. Michael Janda & Abbott, 2010)

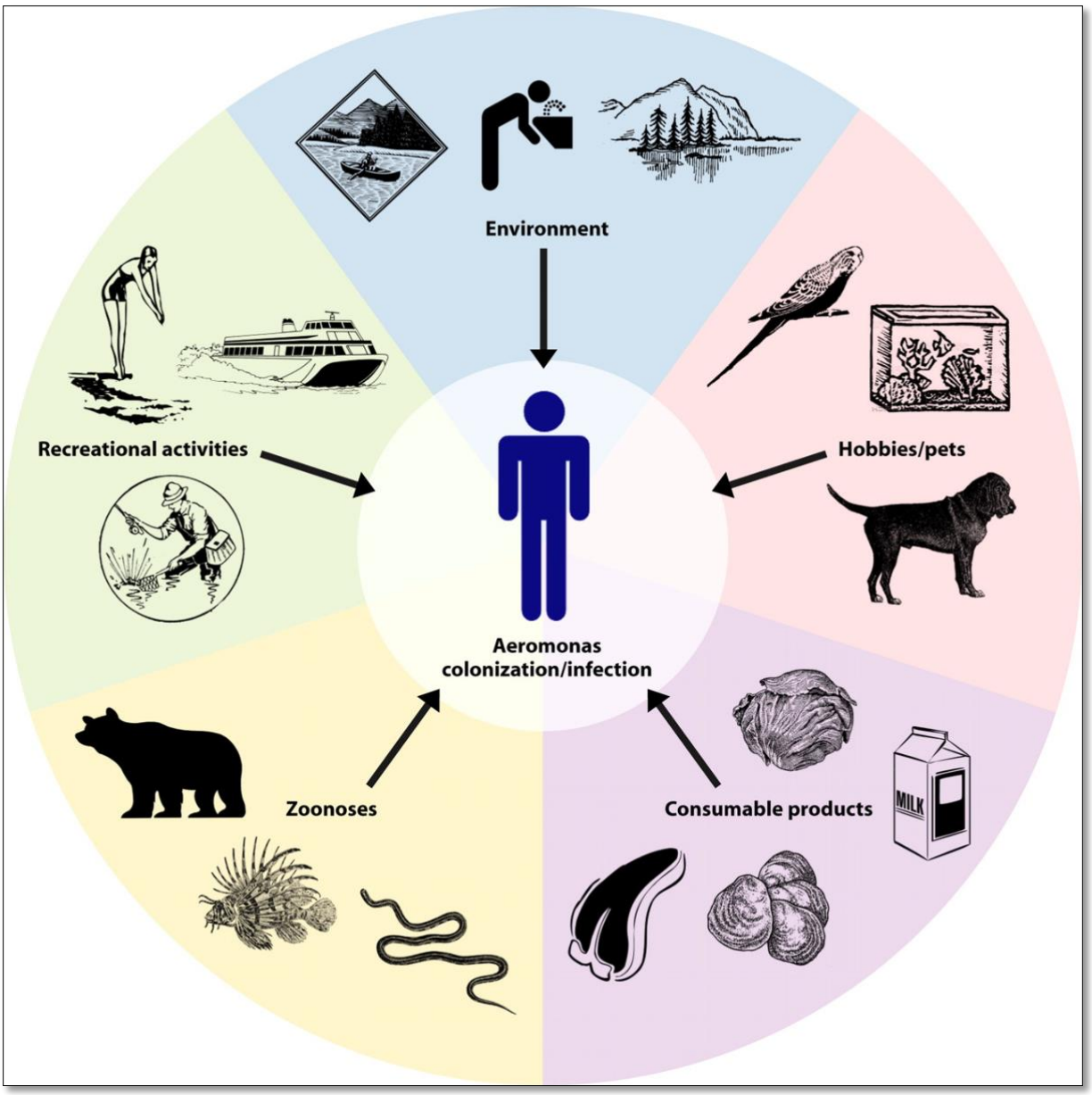

Figure 1. Environmental sources of Aeromonas species potentially leading to infection or colonization in humans. (J. Michael Janda & Abbott, 2010)

A. media has been described in river freshwater, and found in sewage water, drinking water, activated sludge, animals and humans. It has been isolated from clinical material on more than one occasion or unequivocally linked to human disease. (J. Michael Janda & Abbott, 2010) *A. media* is an opportunistic emerging pathogen that causes a wide spectrum of diseases in humans and animals. When present in fish, *A. media* causes skin ulcers, and diarrhea in humans.

Compared to other *Aeromonas* species, *A. media* has distinctive genetic features: (i) members of this species have a genetic polymorphism showed by 7 gene multilocus sequence typing (MLST) higher than that of other species within the genus (5.8 % versus 2.5 % for other species), (ii) a high heterogeneity in the tRNA genes intergenic spacers, (iii) one

of the highest rate of intragenomic heterogeneity among ribosomal RNA (rrn) operons, and (iv) a strikingly high diversity of rrn chromosomal distribution. (Talagrand-Reboul et al., 2017)

The infective dose for *Aeromonas* spp. is estimated to be between 10^6 to 10^8 cells with an incubation period between 6 and 48 hours. Although in some cases, the infective dose has been reported to be as low as 10^3 to 10^4 cells. (Ørmen, 2000) However, in a study where human volunteers were exposed to *A. hydrophila* strains up to 10^{10} CFU/g, only 2 of 57 individuals developed diarrhea and the bacterial colonization in the gut was low. (Bhunia, 2018; P. E. Granum, 2015, p. 62) The severity of the disease varies from simple diarrhea to massive dysentery, with bloody, mucus-containing, frequent stools, abdominal pain and fever. Although gastroenteritis symptoms in humans are still controversial, epidemiological data and clinical symptoms (diarrhea), and stool analysis have established *Aeromonas* as an enteropathogen. (Bhunia, 2018; Bin Kingombe et al., 2010; Dallal, Fard, Talkhabi, Aghaiyan, & Salehipour, 2016; P. Granum, 2014; Christopher J Grim, 2013; Tomás, 2012)

A. veronii biovar *sobria* has frequently been registered in cases of dysenteric-like gastroenteritis, while *A. caviae* is commonly associated with pediatric diarrhea. (Christopher J Grim, 2013) A list of recognized *Aeromonas* species that are associated with human disease is presented in table 2. (M. J. Figueras et al., 2017)

Table 2. *Aeromonas* species associated with human disease. (J. Michael Janda & Abbott, 2010; Martin-Carnahan & Joseph, 2005)

Species	Occurrence in clinical specimens	Disease association
A. hydrophila	Common	Wounds, septicemia, gastroenteritis, leech therapy, peritonitis
A. dhakensis	Common	Wounds, septicemia, gastroenteritis, leech therapy, peritonitis
A. veronii biovar sobria	Common	Wounds, septicemia (including "primary septicemia"), gastroenteritis, leech therapy, peritonitis
A. caviae	Common	Gastroenteritis (esp. pediatric), septicemia
A. veronii biovar veronii	Rare	Gastroenteritis, septicemia
A. jandaei	Rare	Wounds, septicemia, gastroenteritis
A. schubertii	Rare	Traumatic aquatic wounds, septicemia
A. trota	Rare	Gastroenteritis

Aeromonads can cause severe forms of infections in immunocompromised individuals, e.g necrotizing fasciitis and hemolytic-uremic syndrome (HUS), but further studies are necessary to establish such associations. (Khajanchi et al., 2010) Aeromonads also play a role in the development of myonecrosis in individuals with lever disease or malignancy, leaving a mortality rate of 60-77 % in patients with this disease. Other clinical diseases where *Aeromonas* spp. can play a role are pneumonia, urinary tract infection, eye infection, and peritonitis. (Bhunia, 2018)

In recent years, the number of documented outbreaks of human infections associated with the consumption of raw fruits and vegetables has increased. (Buck et al., 2003) Such increase in produce-related human infections can be attributed to several factors. Some reasons that have been proposed include notable changes in dietary habits that rose over a decade ago. A higher per capita consumption of fresh or minimally processed fruits and vegetables, as well as an increase in the use of salad bars and meals consumed outside of the home arose. (Altekruse & Swerdlow, 1996)

In addition, changes in production and processing methods, sources of produce, and the emergence of pathogens that previously have not been associated with fresh produce have also enhanced the potential for foodborne illness outbreaks associated with minimally processed fruits and vegetables. (Hedberg & Osterholm, 1994) As a consensus, these changes contribute to the increased exposure of consumers to products which exacerbate potential problems with contamination by human pathogens. (Buck et al., 2003)

2.3 The multifactorial virulence of *Aeromonas*

The pathogenesis of *Aeromonas* infection is multifactorial and depends on the bacterial strain, the infection route and the animal used as a model organism. Several virulence factors have been associated with pathogenicity of aeromonads, including production of hemolysins, cytotonic and cytotoxic enterotoxins, proteases, lipases, adhesins, and surface layer (S-layer) proteins that act in concert to cause disease in the host. (Ghenghesh, Ahmed, Cappuccinelli, & Klena, 2014; Khajanchi et al., 2010; Martins, Marquez, & Yano, 2002)

The bacteria's virulence factors that are considered to play a role in invasive disease include the lipopolysaccharides, the S-layer, outer-membrane proteins, pili and flagella, resistance to complement mediated lysis and several secretion systems. (Percival & Williams, 2014, pp. 52-53) Aeromonads have a pH tolerance range of between 5 and 10, which highlights their resilience to the demands inflicted on them in aquatic environments, particularly in natural waters. Several factors such as pH (<4.5), NaCl (>4%) and temperature (<3 °C) can significantly slow their growth. (Bhunia, 2018)

A. caviae is the most prevalent mesophilic Aeromonas species in raw sewage and other effluents. This has led to the proposal of using this species as an indicator for fecal pollution. (Ramteke, Pathat, Gutam, & Bhattacherjee, 1993) However, it seems *A. hydrophila* and *A. veronii* biovar *sobria* are more toxigenic when compared to *A. caviae*. Therefore, the species composition of aeromonads in sewage effluents may be of a particular interest when effluents are used for irrigation of crops or are discharged into recreational waters. (Percival & Williams, 2014, p. 54)

2.3.1 Cytotoxins and Hemolysins

Exotoxins are major virulence factors of *Aeromonas* spp. that include a cytotoxic heat-labile enterotoxin (*Act*), a cytotonic heat-labile enterotoxin (*Alt*), and a cytotonic heat-stable enterotoxin (*Ast*). (Bin Kingombe et al., 2010; Dallal et al., 2016; Christopher J Grim, 2013; J. Michael Janda & Abbott, 2010; Tomás, 2012) *Act* is a toxin that plays an important role in the pathogenesis of *A. hydrophila*, it creates pores in the cell membrane, resulting in cell death. It is secreted by a type II secretion system (T2SS) that is found in many *A. hydrophila* and *A. veronii* strains. *Act* has the ability to cause both diarrhea and severe tissue damage, and can lead to apoptosis or necrosis of host cells, depending on the dosage. (Christopher J. Grim et al., 2014) *Alt* exhibits exciting homology with lipases and phospholipase C. (Ghenghesh et al., 2014) Genomic comparisons have shown that *alt* and *ast* are core elements of *A. hydrophila*. (Li, Ni, Liu, & Lu, 2011; Rasmussen-Ivey, Figueras, McGarey, & Liles, 2016; Sha, Kozlova, & Chopra, 2002)

Shiga-like toxins (stx) represent a group of bacterial toxins that have been described in human and animal disease. Some bacteria genera, including *Aeromonas* spp. produce these toxins. Alperi and Figueras (2010) described the presence of stx-1 and stx-2 in clinical isolates of *Aeromonas* spp. associated with gastroenteritis, hemorrhagic colitis, and HUS. Genes that encode these toxins are located in different lambdoid bacteriophages that lysogenize this strain. The *Aeromonas* genus also has a zero-secretion system named Outer Membrane Vesicle (OMVs), that could be a path for horizontal transfer of some proteins, RNA, periplasmatic space components and other components associated with virulence to other genera. OMVs are therefore believed to play an important role in pathogenicity of *Aeromonas* spp. (Guerrero, Lopez, Longa, & Castro, 2015; Guerrero-Mandujano, Palma-Martinez, & Castro-Escarpulli, 2015)

Another powerful virulence factor is aerolysin, which has been associated with Aeromonas-mediated gastrointestinal illness. (Martins et al., 2002) Over 75 % of *A. hydrophila* strains produce aerolysin, which has been well-characterized as a hemolytic toxin. Hemolysins are a broad group of multifunctional enzymes that play a role in the bacterium's pathogenesis. The gene encoding this pore-forming toxin (*aerA*) has been isolated from foodborne isolates including *A. hydrophila*, *A. dhakensis*, *A. salmonicida*, *A. media*, *A. piscicola*, and *A. caviae*. (Bhunia, 2018; Hoel et al., 2017) The production of hemolysin, and differences in the presence of/and type of hemolysis that is present, varies between and within the species depending on the type of cells that are used. (Martin-Carnahan & Joseph, 2005)

2.3.2 Adhesion and biofilm formation

Aeromonas spp. have evolved multiple regulatory mechanisms for biofilm formation that are intimately linked with the production of virulence factors. Biofilm formation helps the bacterium to adhere and colonize, it also provides with resistance to antimicrobial agents and host defenses. (Lynch et al., 2002) In order to form biofilm, aeromonads are dependent on the presence of type IV pili and cell surface hydrophobicity, as well as flagella in promoting coaggregation and adherence. (Béchet & Blondeau, 2003) Adherence to enterocytes can be mediated by two types of pili consisting of short and rigid pili. (Bhunia, 2018; Kirov, Barnett, Pepe, Strom, & Albert, 2000)

Aeromonads have quorum sensing systems that modulate bacterial virulence genes, in addition to swim and swarm mobility that contributes to the overall virulence of genus. (Christopher J. Grim et al., 2014) However, there are notable differences within the *Aeromonas* species. For example, *A. piscicola* AH-3 has a single lateral flagellin, and both the polar and lateral flagella are glycosylated. In contrast, *A. hydrophila* has two lateral flagellins and only the polar flagellum is glycosylated. S-layer proteins and bacterial capsules promote

adhesion to the host's cells and employ resistance to complement activation and anti-phagocytic activity. (Bhunia, 2018; Rasmussen-Ivey et al., 2016)

2.3.3 Secretion systems

Aeromonads express several secretion systems. The type-three secretion system (T3SS) is considered a major virulence attribute to *A. salmonicida* and *A. hydrophila.* T3SS is mainly found in clinical isolates. It is thought that bacterium uses the T3SS to secrete and translocate a large number of toxins and effector proteins into the host cells, thereby killing them. (Frey & Origgi, 2016) A schematic overview of the T3SS system and its genetic arrangement in *A. salmonicida subsp. salmonicida* is shown in figure 2.

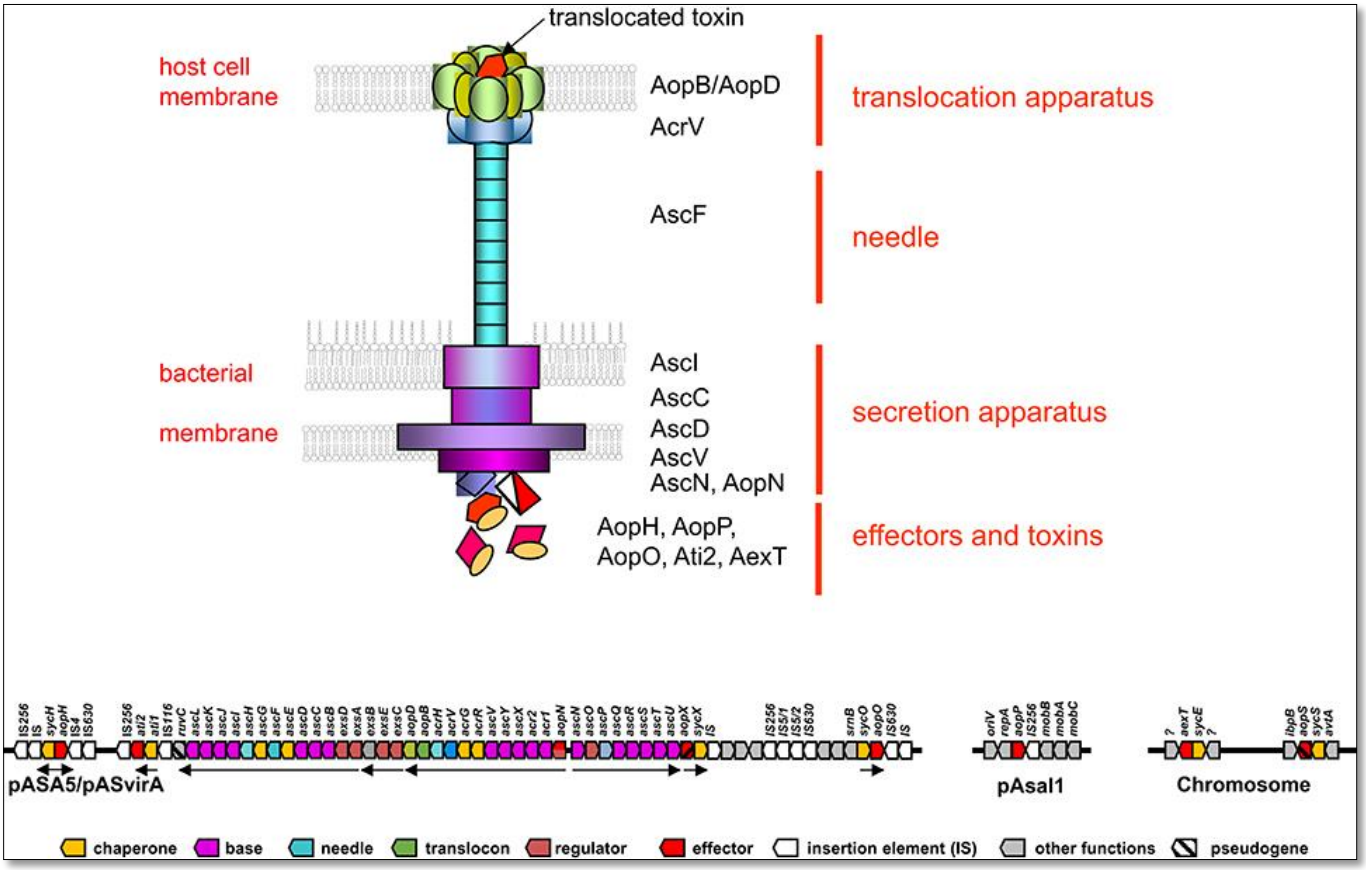

Figure 2. Schematic overview of a T3SS apparatus (top) and its genetic arrangement in *A. salmonicida* subsp. *salmonicida* (bottom)**.** The colored arrows represent the individual genes and are labeled with analogous colors of the constituents of the T3SS apparatus drawn on top. Black arrows indicate the major operons. (Burr, Stuber, & Frey, 2003; Cornelis, 2010; Galán, Lara-Tejero, Marlovits, & Wagner, 2014; Reith et al., 2008)

Two other potent and diverged T3SS effectors (*AexT* and *AexU*) have been identified in aeromonads. Khajanchi et al. (2010) characterized one of the T3SS effectors, which designated *AexU*, and found it to be associated with ADP ribosylation of host cell proteins, a rounded phenotype in HeLa cells, inhibition of phagocytosis, induction of apoptosis, and mouse mortality. (Khajanchi et al., 2010) *AexT* and *AexU* have a GTPase-activating protein (GAP) and ADP-ribosyltransferase (ADP-RT) activities that lead to host cell death. (Christopher J. Grim et al., 2014)

Furthermore, the role of two type VI (T6SS) effectors, the valine-glycine repeat G (VgrG) family of proteins (VgrG1,-2,-3), and the hemolysin-coregulated protein (Hcp) has been characterized in the virulence of *A. hydrophila*. Studies show that VgrG1 of *A. hydrophila* has actin-ADP ribosylation activity that induces host cell cytotoxicity. Based on the T6SS model, the VgrG1 protein must assemble with the highly homologous VgrG2 and VgrG3 proteins to form a cell puncturing device in order to deliver effector proteins into the host cells. (Christopher J. Grim et al., 2014; Khajanchi et al., 2010)

2.3.4 Antimicrobial susceptibility

A majority of motile Aeromonas spp. are generally resistant to penicillin, ampicillin, carbenicillin, and ticarcillin. Regardless, they continue to be susceptible to second and third generation cephalosporins, aminoglycosides, carbapenems, chloramphenicol, tetracyclines, trimethoprim-sulfamethoxazole and quinolones. Resistance to β-lactam antibiotics, such as ampicillin, in *Aeromonas* is often the result of chromosomally-mediated and inducible β-lactamases. (Martin-Carnahan & Joseph, 2005; Ye, Xu, & Li, 2010) *Aeromonas*' resistance to quinolones is primarily due to chromosomal mutations that change the structure of DNA gyrase and topoisomerase IV, which are target enzymes for quinolone antibiotics. (Alcaide, Blasco, & Esteve, 2010)

3 Ready-to-eat (RTE) vegetables

RTE leafy vegetables are the fastest growing sector of the UK and Europe markets. (Ariffin, Gkatzionis, & Bakalis, 2017) As shown in figure 3, there is a broad variety in RTE vegetable products on the market, and such products have become healthy and convenient food options for consumers. RTE vegetables are products that retain much of their indigenous microflora after minimal processing. A general definition of "minimal processed products" is that the product contains live tissue, or that the product has slightly been modified from fresh condition but still maintains fresh-like character and quality. The tissues do not have the same physiological responses as raw, untreated intact live plant tissues, and the cutting process or minimal heating of the tissues can cause different responses in various environmental and packaging situations. (Uhlig et al., 2017; Yildiz & Wiley, 2017)

The aim of minimal food processing is to preserve the sensory and nutritional properties of the raw material to the greatest extent possible. (Hoel et al., 2019) During the production process from raw material to bagged precut lettuce, they are picked, washed, dried, trimmed, cored, cut and packed before being marketed as ready-to-eat without further handling. (Caldera & Franzetti, 2013; Yildiz & Wiley, 2017)

Figure 3. A display of different RTE salad options that are available in a local grocery store. (Source: Rema 1000, Heimdal)

RTE vegetables differ from dehydrated vegetables in term of texture and water activity (a_w). RTE vegetables usually have a_w between 0.97-0.99 and are sensitive to a_w reduction. In terms of microbiological hazards, such products present a great concern as they are mostly grown in soil and processed in diverse and complex ways. Post-harvesting processes such as harvesting, transportation, cutting, packaging and storage can also damage the vegetables. (Francis et al., 1999; Hausdorf, 2012) Manual handling, in particular, can damage fresh produce and render it more suitable for growth and/or survival of spoilage and pathogenic microorganisms. (Brackett, 1994)

3.1 Production of RTE vegetables

There are several production stages involved in the production of RTE vegetables. A schematic overview of the different stages involved in the production of RTE lettuce from a national producer is shown in figure 4.

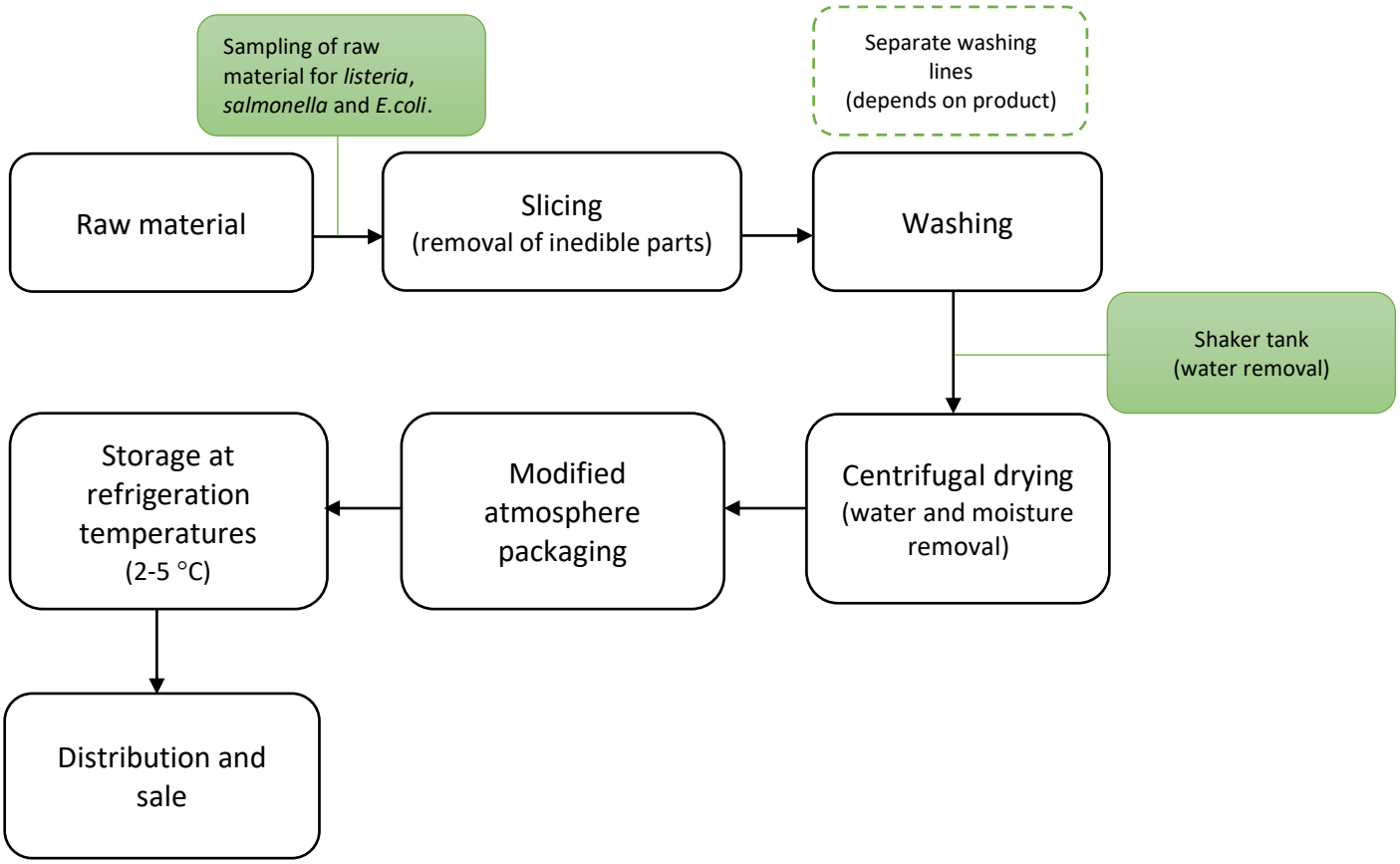

Figure 4. A flow chart of the production process of minimally processed vegetables. (Umutoni, 2019)

The initial step, before further handling of raw material, is random sampling of the raw product for analysis of the most frequent enteric pathogens that are found in RTE products (*Listeria*, *Salmonella*, and *E. coli*) (Dutta; Pers comm.)

Once each batch is cleared for further handling, whole heads of lettuce are sent to the slicing station, whereas precut lettuce is sent directly to the washing tanks. Depending on what type of lettuce is being processed, there are separate washing lines at the factory. Lettuce that can handle rough treatment (i.e iceberg lettuce) is sent to a washing tank that operates at a high pace. All lettuce is strictly washed in water, without further antimicrobial dipping. The washing water is replaced continuously, for as long as production is ongoing. Microbiological analysis of the washing water are performed minimum twice a day; the first samples are collected before production starts, and thereafter continuously throughout the production period.

Before the product is packed in a modified atmosphere, the excess water that has been added during washing is removed in a shaker tank and furthermore by centrifuging. The produce is thereafter stored at refrigeration temperature before it is distributed and sold. The overall goal of RTE vegetable production is to remove as much water as possible, thus reducing a_w and minimizing optimal conditions for growth of spoilage organisms and opportunistic pathogens. Moreover, it is in the producer's best interest to deliver a fresh-like product to the consumer, with an extended shelf-life, while at the same time ensuring food safety and maintaining a nutritional and sensory quality. (Yildiz & Wiley, 2017)

3.1.1 Production of RTE-vegetables in Norway

Production of Norwegian lettuce has shifted from greenhouse production in the early 1990s to increased field production (Nordskog, Nærstad, Herrero, Sundheim, & Hermansen, 2008). The Norwegian farming season lasts from May to October, depending on spring and autumn temperatures. Norwegian lettuce production consists of iceberg, carrots, spinach, tomatoes, celery, reddish, and several other vegetables. The production of field grown iceberg lettuce was initiated by the growers in the 1990s to give alternatives to imported lettuce. Since then, the sale of Norwegian iceberg lettuce has increased from 2.3 million heads in 1993, to 8 million heads in 2003. (Nordskog et al., 2008)

Due to geographical reasons and climatic limits, the production of Norwegian vegetables is not sufficient to supply the country's inhabitants with an adequate amount of vegetables. Therefore, to maintain variety among products, import is necessary. In recent years, the value of lettuce imported to Norway has increased, as shown in figure 5. This statistic shows the value of lettuce imported to Norway over the past eight years, displayed in Norwegian Kroner (NOK). By 2012, the import value of lettuce amounted to approximately 114 million NOK. In 2017, the total import value of lettuce had increased to roughly 134 million NOK. However, available data for the past year shows that value decreased to about 123 million NOK. (Statista, 2019)

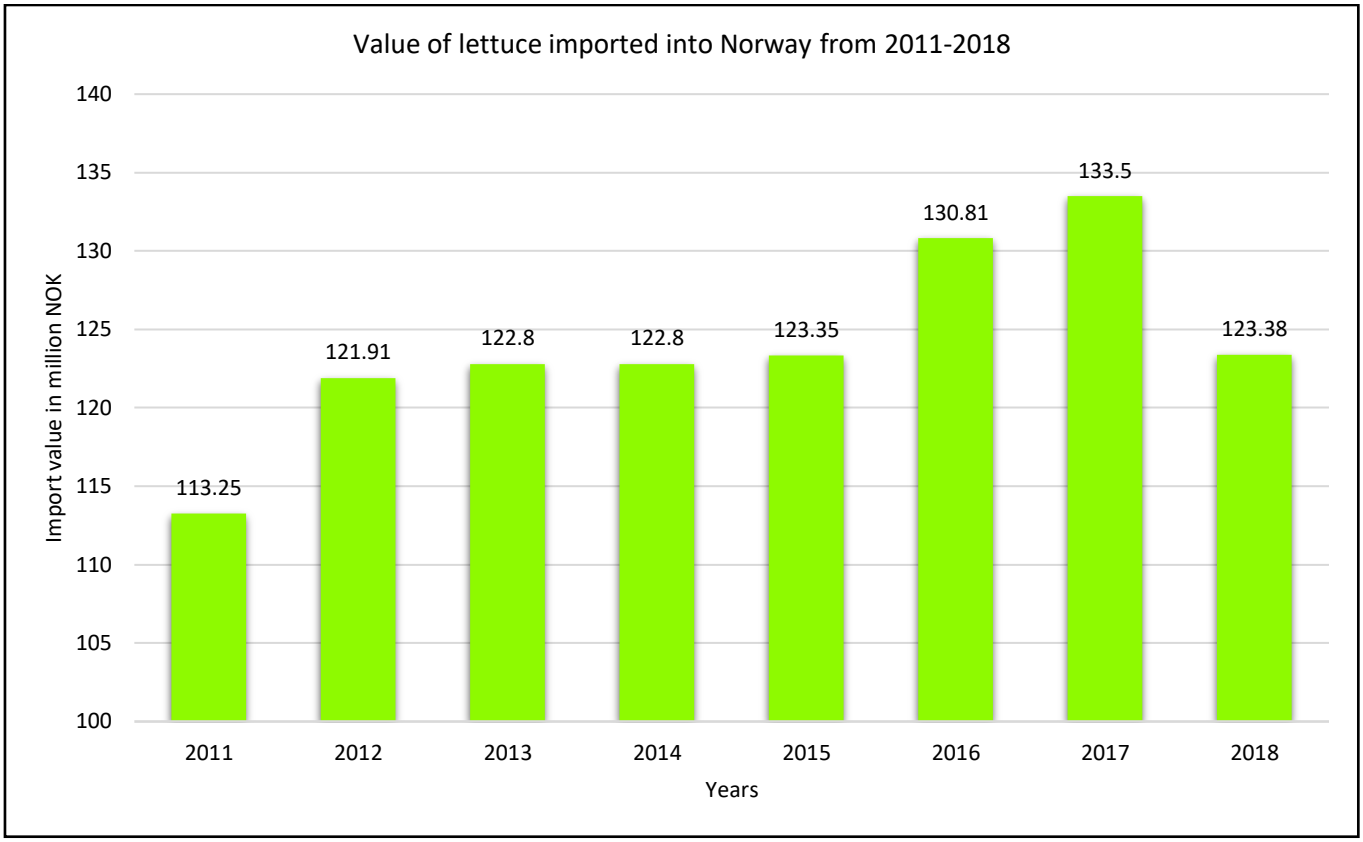

Figure 5. The value of lettuce imported to Norway from 2011 to 2018, displayed in million Norwegian Kroner (NOK) (Source: Statista, 2019)

Commercial vegetable cultivation is strongly limited to specific districts such as outer settlements in Østfold, Lier, villages at Arendal and Grimstad, the best agricultural villages around Mjøsa, Jæren, Frosta, the coastal municipalities from Borre to Larvik, and the Levanger area. (Ellestad & Bratberg, 2019)

Spain is the world's largest exporter of lettuce. 56 % of vegetables on the Norwegian market are mainly imported from Spain and Italy. A total of 14 % of world trade for these types of products are Spanish. Import is not limited to shredded lettuce, producers also import whole lettuce heads that are shredded when they arrive at the factory. In 2007, Spanish export of fruits and vegetables to Norway was 18.2 %. By 2016, this had increased to 26.7 %. It is estimated that Spain exports fruits and vegetables equal to 12 billion euro per year. By 2017, this had increased by approximately 7 %. (Henriksen, 2017)

Factors such as warm climate, location, quality and price are essential to why Spanish lettuce is imported into Norway. When a producer imports Spanish products, they are certain they will receive robust products of good quality. All foodstuffs imported into Norway must comply with Norwegian food law, and the importer must be registered as an importer in the Norwegian food safety authority's (NFSA) form services. He/she is responsible for ensuring that the food they import is safe for human health and that content and labeling are in accordance with Norwegian rules. If this is not the case, food products might be denied entry to Norway and consequently not be sold. (Norwegian Food Safety Authority, 2012)

However, the rules are regulated depending on the country of export. Foodstuffs imported from the European Union (EU) are not obligated to be reported. In contrast, all imports from countries outside the EU must be notified in advance to the Norwegian Food Safety Authority. (Norwegian Food Safety Authority, 2012) In addition to these guidelines, importers also set their own guidelines and rules, which their business partners must comply with. These include, but are not limited to billing systems, labor conditions (no child labor), documents of certification, et cetera. (Dutta, Pers comm.)

3.2 The growth and prevalence of *Aeromonas* in RTE vegetables

Foodborne pathogens can be present in RTE vegetables, and a number of outbreaks of foodborne disease have been traced to RTE vegetables. The US Food and Drug Administration only considers *A. hydrophila* as a food safety issue in RTE vegetables, as it is a psychrotrophic, facultative anaerobe bacteria that can survive at 0 °C, but also be capable of growth at 4-5 °C. The literature contains numerous reports and articles of the presence of *A. hydrophila* on vegetables, premade salads, and commercial RTE mixed vegetable salads.

RTE vegetables, including leafy vegetables, are often contaminated with large and diverse populations of microorganisms, where counts of 10^5–10^7 CFU/g have been reported to be frequently present. A majority of the present bacteria (80-90 %) are gram-negative rods. (Francis et al., 1999; Johnston et al., 2005)

High counts of *A. hydrophila* (10^3–10^6 CFU/g) have previously been isolated from retail RTE vegetable salads. (C.R: Fricker, 1989; Marchetti, Casadei, & Guerzoni, 1992; R. M. Garcia-Gimeno, 1996) Callister and Agger (1987) performed a study that involved conventionally farmed vegetables, where *A. hydrophila* and *A. caviae* were isolated from lettuce, spinach, celery, parsley and broccoli. 92 % of the studied vegetable samples harbored cytotoxic aeromonads. (Callister & Agger, 1987)

Monge, Arias-Echandi, and Utzinger (1998) detected *Aeromonas* spp. in 30 %, 52 %, and 46 % of coriander, lettuce, and celery samples, respectively. Forty-five percent of the isolated aeromonads were cytotoxic. (Monge et al., 1998) Palú et al. (2006) isolated 28 strains of *A. hydrophila* and *A. caviae* from lettuce, and the strains were tested for their antibiotic

susceptibility. Data showed that *A. hydrophila* was resistant to ampicillin and tetracycline in 96.2 % and 7.7 % of the strains, respectively. In the case of *A. caviae*, the species was resistant to ampicillin, tetracycline, and cefoxitin in 55.1 %, 10.3 %, and 31 % of the strains, respectively. (Callister & Agger, 1987; Laird, 2015; Palú et al., 2006)

McMahon and Wilson (2001) isolated aeromonads from organic vegetables and raw, minimally processed RTE vegetables. In a study that compared microbial growth in organic and minimally processed vegetables, *Aeromonas* spp. were isolated from 34 % of the organic vegetables, and 41 % of the vegetables that had undergone minimal processing.

Xanthopoulos, Tzanetakis, and Litopoulou-Tzanetaki (2010) performed another study where they examined 26 types of minimally processed salads from five producers. In this particular study, 33 isolates of *A. hydrophila* and 12 isolates of *A. caviae* were present. (McMahon & Wilson, 2001; Xanthopoulos et al., 2010)

It is important to note that many other species may be more relevant as well. For instance, *A. dhakensis* is widely distributed in the environment and in clinical samples. It has, among others, been previously mistaken for *A. hydrophila*, which has probably led to an overestimation of the clinical significance of *A. hydrophila*. Thus, there is reason to believe that an inaccurate prevalence of *A. hydrophila* in foods, water and clinicals cases has been reported. Because of these reasons, it is important to have an accurate taxonomy to understand the distribution and virulence potential of species belonging to the genus *Aeromonas*. (Francis et al., 1999; Hoel et al., 2019)

The growth and survival of microorganisms on fresh-cut fruit and vegetables is influenced by inner properties of the product, such as pH, nutrient composition, water activity, but exterior factors such as transport and storage, can also play a role and affect the growth and survival of the organisms. (Oliveira et al., 2015) The plants natural composition content of antibiotics can also play a role in the growth an survival of microorganisms.

Antibiotics like oxytetracline, sulfadimethoxine/ormetoprim and florfenicol have been shown to be effective against *A. hydrophila*. (Ramena, Ramena, & Challa, 2018) Natural antibiotics inhibit bacterial growth by multiple mechanisms, the most common are bactericidal and bacteriostatic effects. Ramena et al. (2018) described that a selection of herb and spice extracts showed antimicrobial activity against *A. hydrophila*. (figure 6) Sharma, Pandey, A.K, and Y.P (2014) and Tagboto and Townson (2001) have also shown that that the bioactive compounds produced by plants have antifungal and antibacterial properties that are effective against *A. hydrophila*. (Sharma et al., 2014) and (Tagboto & Townson, 2001)

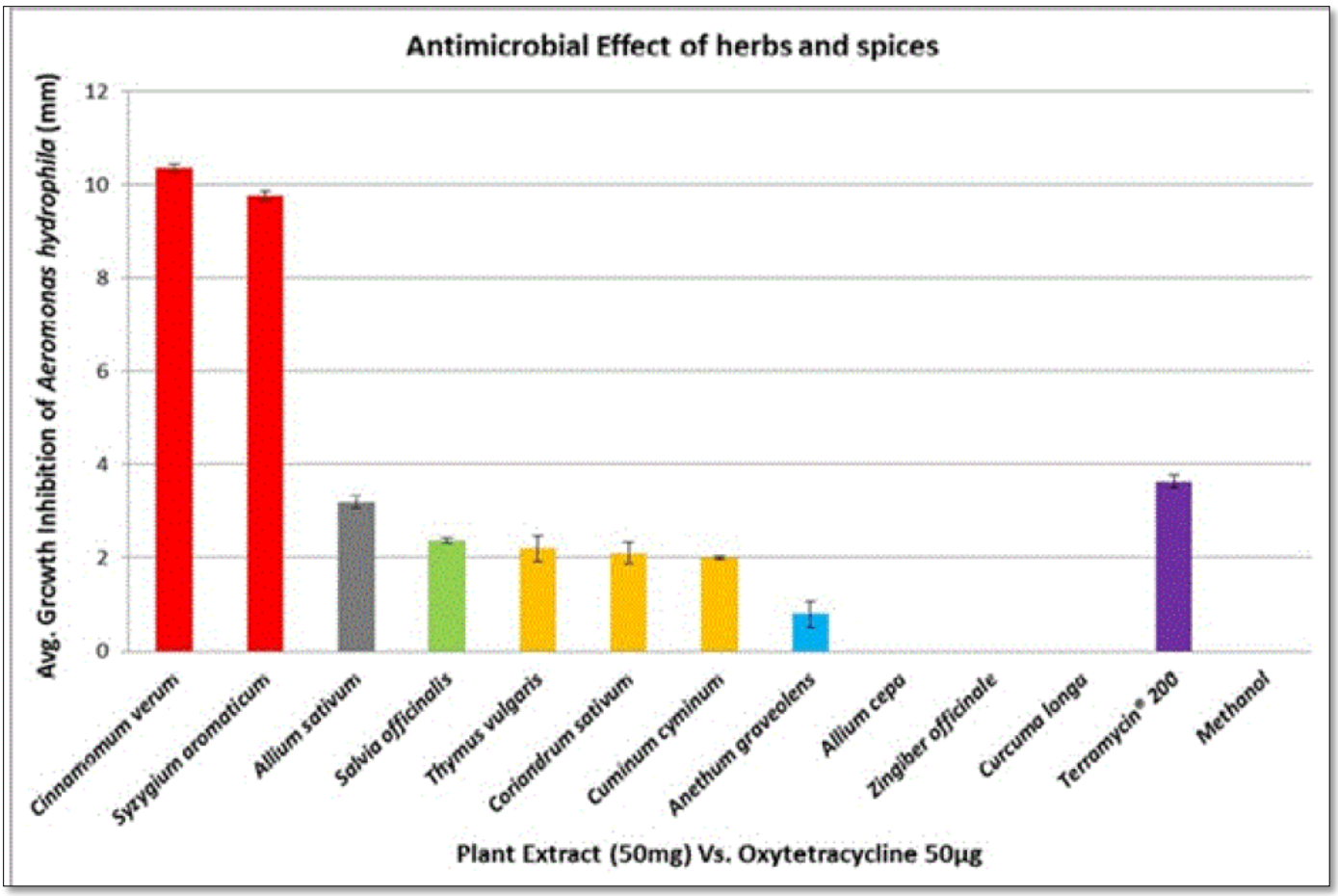

Figure 6: Screening of herb and spice extracts compared to Oxytetracycline for antimicrobial activity against *A. hydrophila*. (Ramena et al., 2018) *Tetramycin=Oxytetracyline

3.4 Food spoilage caused by *Aeromonas* spp.

Several species of the *Aeromonas* genus have been identified as potential food spoilers, and differences might occur in the spoilage potential at species and strain level. (Roxana Beaz-Hidalgo et al., 2015) The predominant species in clinical samples and water isolates have been found to be *A. caviae*. For decades, *A. salmonicida* has been known to be a fish pathogen. This species officially has five subspecies: *salmonicida*, *smithia*, *achromogenes*, *masoucida* and *pectinolytica*. Strains in the latter subspecies are considered motile, because they can grow at 37 °C. The first case of human infection by *A. salmonicida* with a clear clinical background was reported in 2008. (Laird, 2015; Vincent et al., 2019)

Daskalov (2006) isolated *A. hydrophila* from 19 %, 28 %, 90 %, and 22 % of fish samples from the United Kingdom, New Zealand, Switzerland and Taiwan, respectively. Similarly, Davies, Pottage, Bennett, and Walker (2011) isolated 40 % *A. hydrophila* from fresh fish samples from retail outlets in the United Kingdom, France, Greece, and Portugal. (Daskalov, 2006; Davies et al., 2011)

Fontes, Saavedra, Martins, and Martínez-Murcia (2011) showed that *Aeromonas* spp. is widely spread in pig carcasses. In their study, two-third of samples taken from pig carcasses, water, and dehairing equipment in slaughterhouses, contained eight different *Aeromonas* species: *A. hydrophila*, *A. salmonicida*, *A. bestiarum*, *A. caviae*, *A. media*, *A. veronii*, *A. allosaccharophila*, *A. simiae*, and *A. aquariorum*. (Fontes et al., 2011) In 2010, the first identification of human disease caused by *A. smiae* found in pig carcasses was described in Portugal. (Laird, 2015) Previous studies by Hoel, Mehli, Bruheim, Vadstein, and Jakobsen (2015) detected mesophilic *Aeromonas* spp. in a substantial proportion of fresh retail sushi. In a follow up study, mesophilic *A. salmonicida* was most present in salmon (Hoel et al., 2015; Hoel et al., 2017)

Foods where *A. hydrophila* has been isolated have been contaminated by water, animal feces or manual food handling. In most cases, contaminated water and/or use of chlorinated water was proven to be the main source of contamination. As previously mentioned, *Aeromonas* spp. can be isolated from a diverse range of foods, and has, in recent years, emerged as a spoilage organism in meat products as well. (Laird, 2015; R. M. Garcia-Gimeno, 1996)

3.4.1 Factors influencing the prevalence of *Aeromonas* spp.

RTE vegetables are susceptible to contamination at several stages of production. Factors that determine the amount of pathogens that contaminate the product are, among others, the product itself, its source, and the type of microorganisms that are initially present. Nonetheless, contaminations with pathogens can occur during processing and distribution. Thus, exquisite hygiene must be implemented at all stages of processing. Many vegetable cells are broken during processing, and the intracellular products they release may enhance bacterial growth. (Oliveira et al., 2015) Slicing and shredding of products destroys the surface cells, bruises underlying layers and allows juices from inner tissue to leak on both the equipment used, as well as the fresh cut product. Therefore, equipment becomes a source of contamination because it often has inaccessible areas that can harbor bacteria, and be difficult to clean. (Garg, Churey, & Splittstoesser, 1990)

The presence of moisture on surfaces of used equipment provides excellent media for rapid growth of microorganisms, and allows microbial infiltration, thereby providing an increased surface area for contamination and growth. (Brackett, 1994) Pre-cut product has the disadvantage of reduced shelf life because the tissue is disrupted during the cutting process, thereby leading to rapid deterioration. In addition, cutting increases the area of injured tissue available for microbial degradation. (Barriga, Trachy, Willemot, & Simard, 1991)

The more operations a product undergoes, the more its flora will reflect that of the environment in which it is produced. (C.R: Fricker, 1989; J.M. Soriano, 2000; R. M. Garcia-Gimeno, 1996) Insufficient sanitization of vegetables can lead to contamination. In addition, increased antibiotic resistance in food isolates of *Aeromonas* spp. and the ability to produce toxins is of importance when determining the genus' role in food contamination. (Callister & Agger, 1987; Laird, 2015; Palú et al., 2006)

4 Materials and Methods

4.1 Bacterial strains

In 2012, a variety of RTE lettuce products from four local manufacturers were purchased for the "«*Lokale spiseklare produkter - mattrygghet langs hele verdikjeden*»" project. 307 strains were isolated according to Nordic Committee on Food Analysis [NMKL] (2004) method no. 150 as described previously in Hoel et al. (2015).

From each *Aeromonas* positive sample, four presumptive *Aeromonas* colonies were picked from an optimal dilution (up to 100 colonies /plate), propagated to purity, and frozen at – 80 °C. The isolates were stored at the Norwegian University of Science and Technology (NTNU) Campus Kalvskinnet until subsequent analysis.

One isolate from each lettuce sample was used in this study.

4.1.1 Selection of isolates

Bacterial samples were recovered from frozen stocks at – 80 °C, and re-cultivated on tryptone soy agar (TSA)(Oxoid, Oslo, Norway). A small amount of each frozen isolate was streaked onto TSA and incubated overnight at 37 °C under aerobic conditions. The procedure was repeated two to three times for each isolate. Bacterial growth was recorded after 24 hours. *A. caviae* (CCUG 25939), *A. hydrophila* (CCUG 14551), *A. veronii* biovar *sobria* (CCUG 30360) and *A. veronii* biovar *veronii* (CCUG 27821) were included as reference strains. *Staphylococcus aureus* (from an earlier project) was included as a negative control.

4.1.2 Visualization of hemolysis

Aeromonas isolates, including the reference strains, that successfully grew on plates were analyzed for hemolytic activity. One colony of each isolate was streaked onto bovine blood agar (Analysis center, Trondheim Municipalty) and incubated at 37 °C. Six to seven different isolates were distributed per agar plate, and hemolysis was recorded after 24 and 48 hours. The presence and degree of hemolysis varied from no hemolysis (0) to extreme hemolysis (5).

4.2 Identification of *Aeromonas* species

4.2.1 DNA isolation

Total genomic DNA was prepared from cells harvested from TSA and resuspended in 1 ml TE-buffer (10mM Tris/HCl, 1 mM EDTA, pH 8). Further DNA isolation was performed as described in the protocol for Gram-negative bacteria in the DNeasy Blood and Tissue kit (69506; Qiagen 2006), without modifications. The genomic DNA was stored at -20 °C until further analysis.

All samples, including the reference strains, were quantified using the Gen5 2.0 program in the BioTek PowerWave XS Microplate spectrophotometer. The DNA concentration and purity of the samples was determined by evaluating the A_{260nm}/A_{280nm} ratio of each sample. Samples that contained levels higher than 20ng/µl were diluted 1:10 and re-quantified before PCR reactions.

4.2.2 PCR protocols

PCR reactions were performed using primers that target the 16S rRNA, *rpoB* and *gyrB* genes. The presence of five virulence-associated genes (*act*, *ast*, *alt*, *stx-1* and *stx-2*) was also included. *E. coli* (CCUG 29197) was added as a positive control[1] for stx-1 and stx-2. The primer pairs used for amplification and sequencing of each target gene were listed in table 3.

Table 3. PCR primers, annealing temperatures and expected amplicon length for specific housekeeping- and virulence genes associated with the Aeromonas genus. The table is adapted and modified from (Dahllof, Baillie, & Kjelleberg, 2000; Hoel et al., 2017).

Target gene	Primer	Primer sequence (5'-3')	Annealing temperature (°C)	Amplicon length (bp)	Reference
16S rRNA	338F 1492R	CCTACGGGAGGCAGCAG GGTTACCTTGTTACGACTT	58	1154	Muyzer, de Waal, and Uitterlinden (1993)
gyrB	gyrB3F gyrB14R	GAAGGCCAAGTCGGCCGCCAG ATCTTGGCATCGCCCGGGTTTTC	52	1100	Tacão et al. (2005)
rpoB	1698F 2041R	AACATCGGTTTGATCAAC CGTTGCATGTTGGTACCCAT	50	343	Dahllof et al. (2000)
act	actF actR	CCGGGCTCGGCGTCCAATAC CCAGTTCGGGCGGTTGTCCG	58	361	Kingombe et al. (1999)
alt	altF altR	TGCTGGGCCTGCGTCTGGGCGGT AGGAACTCGTTGACGAAGCAGG	58	361	Bin Kingombe et al. (2010)
ast	astF astR	CGCCATCAACAGCTCGCCCA CGGGCCTCGTTGAGGAAGCG	58	536	Bin Kingombe et al. (2010)
stx-1	stx1F stx1R	ATAAATTGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	58	180	Paton and Paton (1998)
stx-2	stx2F stx2R	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	58	255	Paton and Paton (1998)

[1] Personal communication, Hoel 04.04.19.

All PCR reactions were performed in a total volume of 25 µl containing the following reagents: 2,5 µl 10x PCR buffer (QIAGEN: Lot no. 160022527), 0,5 µl $MgCl_2$ (25mM; QIAGEN: Lot no. 160028153), 0,5 µl dNTP (10 µM; QIAGEN: Lot no. 154035644), 1,0 µl of each primer, 0,13 µl Hot Star Taq Polymerase (5 units/µl; QIAGEN: Lot no. 160051558), 16,87 µl nuclease free water (AMBION: Lot no. 1803046) and 2,5 µl (20-50 ng/µl) template DNA. PCR reactions for detection of Aeromonas based on the different target genes were as listed in table 4.

Table 4. PCR-reaction programs for all primer pairs. (Hoel et al., 2017)

Step	Temperature (°C)	Time	Repetition
Initial denaturation	95	15 min	1
Denaturation	95	30 s	
Annealing	52*	30 s	30
	58ˆ	30 s	30
	50+	30s	30
Extension	72	60 s	
Final extension	72	7	1

*Annealing temperature for the *gyrB* primers. +Annealing temperature for the *rpoB* primers.

ˆAnnealing temperature for the 16S rRNA, *act*, *ast*, *alt*, stx-1 and stx-2 primers.

PCR-products were visualized by electrophoresis in a 1 % agarose gel (Lonza: Lot no. 0000477460) in 1x TAE (40 mM Tris-acetate, 1mM EDTA) and stained with GelRed Nucleic Acid Gel Stain (Biotium: Lot no. 17G0117-1052111), according to the producer's recommendations. A total volume of 12 µl (10 µl PCR product and 2 µl Gelpilot Loading dye 6x (PanReac AppliChem: Lot no. 8M011817) was added to each well. peqGOLD (6 µl) 100bp DNA Ladder Plus (VWR: Lot no. 00623455) was added in the first and last well. The electrophoresis proceeded for 1 hour at 100 volt, and the gel was visualized in the Syngene™ G: Box, with Genesys G: Box chemi-XRQ software (Syngene, UK).

4.3 Species identification

Purification of isolated DNA was performed by using the of ExoSAP-IT™ reagent (ThermoFisher: Lot.no 00710635), as described in the ExoSAP-IT™ PCR Product Cleanup Protocol in Thermo Fisher Scientific (2017). All isolates were amplified for the *gyrB* gene by using the gyrb13F/gyrB14R primers. Seventy isolates (appendix A), were verified for the gene and sequenced at Eurofins Genomics. The sequences were trimmed in SeqmanPro program, integrated in the DNASTAR software[2]. The results were compared to available sequences in the NCBI gene BLAST database, by using the BLAST program.[3] Multiple sequence alignment was performed using MegaAlign (DNASTAR), and a phylogenetic tree was constructed using the neighbor-joining method (Saitou & Nei, 1987). All sequences were included in the alignment.

[2] https://www.dnastar.com/software/
[3] https://blast.ncbi.nlm.nih.gov/Blast.cgi

4.4 In vitro lettuce juice experiment

4.4.1 Preparation of lettuce juice

RTE iceberg mixed salad and crispi lettuce from a national manufacturer were purchased from a local grocery store, stored at 4 °C, and analyzed within a few days. Both products shared the same expiration date (±2 days). Salad juice was prepared as described in Koukkidis, Haigh, Allcock, Jordan, and Freestone (2017), without modifications. The extracted juice was stored at -80 °C until subsequent analysis. One isolate from each producer (A, B, C, and D), a reference strain (*A. hydrophila* CCUG 14551) and a control sample (sterile water) were examined for growth characteristics of *Aeromonas* species isolated from lettuce products.

4.4.2 Culture media and growth analysis

Aeromonas cultures for inoculation were grown overnight in 5 ml of tryptone soy broth (TSB). The mixture was subjected to vortex mixing for 10s to release surface-attached bacteria, and thereafter incubated at 37 °C, under aerobic conditions. The following day, 100 µl of the bacterial solution and 1 ml of sterile water were pipetted into a separate, sterile 10 ml tube. The mixture was once again subjected to vortex mixing for 10s to release surface-attached bacteria. Absorbance was measured at 600nm in the UV-1800 Shimadzu UV Spectrophotometer (Cat. No. 206-25400-28).

4.4.3 Growth analysis

Different amounts of lettuce juice (%), sterile water (µl), and bacterial solution (µl) (total=1 ml) were pipetted in a 10 ml sterile tube, and subjected to vortex mixing for approximately 10s. The procedure was repeated for each isolate, and performed in both iceberg and crispi juice. All samples were incubated at 37 °C. Microbial growth was recorded by absorbance measurement at 600nm after 0 hour, 1 hour, 3 hours, 5 hours, 8 hours and 12 hours. Two hundred and sixty four tubes were divided between the four selected isolates, the reference strain, and the control sample.

The amounts of sterile water, bacterial solution, and lettuce juice that were added to each sample were as listed in table 5. The table lists the different concentrations that were used for one juice, and one isolate. The recipe was the same for each isolate, and was repeated for both juice samples. The control sample only contained 100 µl bacterial solution, and 900 µl sterile water. The concept was adapted and modified from Koukkidis et al. (2017).

Table 5. Volumes of juice, sterile water and bacterial solution added in one sample, per isolate.

		Time (hours)						
Juice (%)		0 h	1 h	3 h	5 h	8 h	12 h	Total
0,5	Water	895 µl	895 µl	895 µl	895 µl	895 µl	895 µl	5 370 µl
	Juice	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	30 µl
	Bacterial solution	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	600 µl
1	Water	890 µl	890 µl	890 µl	890 µl	890 µl	890 µl	5 340 µl
	Juice	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	60 µl
	Bacterial solution	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	600 µl
2	Water	880 µl	880 µl	880 µl	880 µl	880 µl	880 µl	5 280 µl
	Juice	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	300 µl
	Bacterial solution	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	600 µl
5	Water	850 µl	850 µl	850 µl	850 µl	850 µl	850 µl	5 100µl
	Juice	50 µl	50 µl	50 µl	50 µl	50 µl	50 µl	300 µl
	Bacterial solution	100 µl	100	100 µl	100 µl	100 µl	100 µl	600 µl

5 Results

5.1 Growth of *Aeromonas* isolates after storage at -80 °C.

Ninety percent of the analyzed isolates grew well under aerobic conditions. Further analysis of β-hemolytic activity showed presence of hemolysis in 21 % of the isolates. However there was a varying degree of recorded hemolysis among them (figure 7).

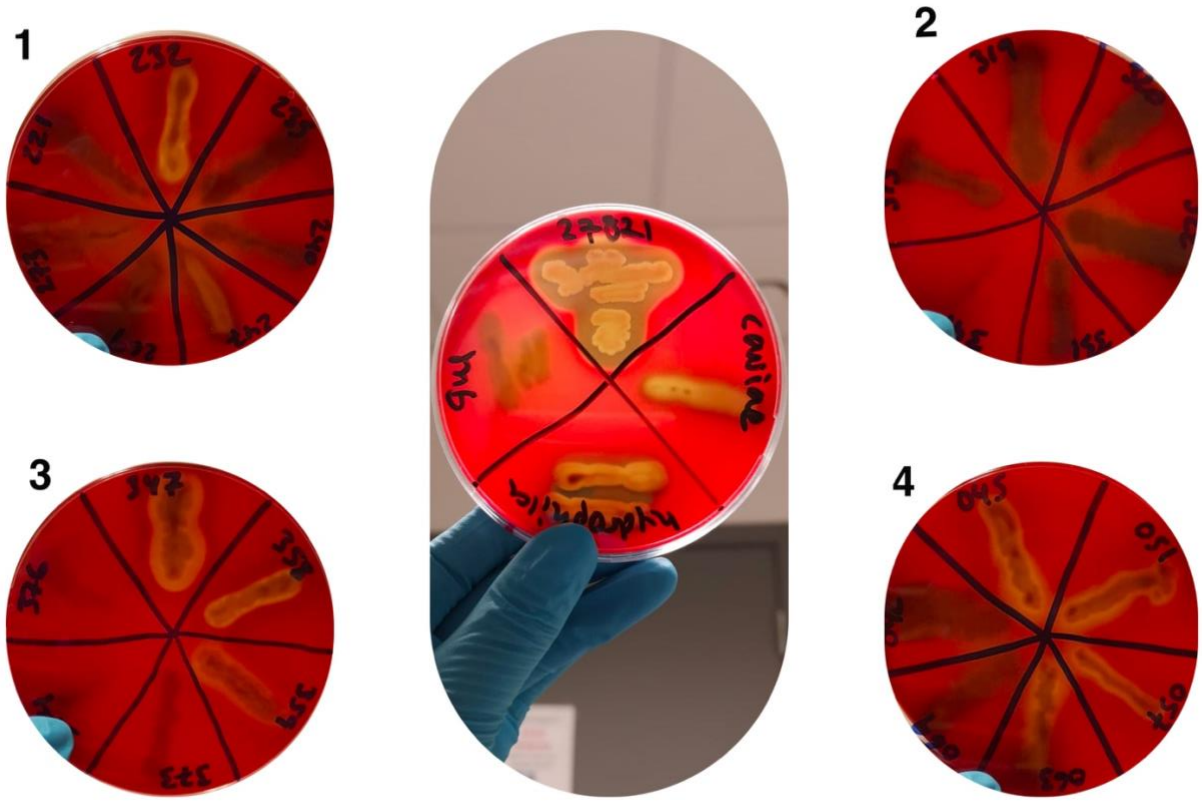

Figure 7. Examples of variety in the presence and degree of hemolysis among the isolates. In the middle are the four reference strains, *A. veronii* biovar *veronii* (CCUG 27821) showed extreme hemolysis. In the top left corner (1), there are signs of hemolysis in AER232. In the top left corner (2), there are no signs of hemolysis in the isolates. In the bottom left corner (4), AER347 showed extreme hemolysis. In the bottom right corner (4), there was notable hemolysis in some of the isolates. (Photo: NU)

5.2 Identification of *Aeromonas* isolates from four producers in Mid Norway.

5.2.1 Identification of isolates based on 16S rRNA and *gyrB* gene markers.

PCR analysis of the 16S rRNA gene confirmed bacterial growth for 97 % of the isolates that grew well under aerobic conditions. Likewise, 93 % of the isolates that successfully grew on TSA were verified for the *gyrB* gene. Positive bands were identified at approximately 1200 bp for 16S and 1000 bp for gyrB (figure 8). A complete overview of identification results based on sequencing of these two genes is presented in appendix A.

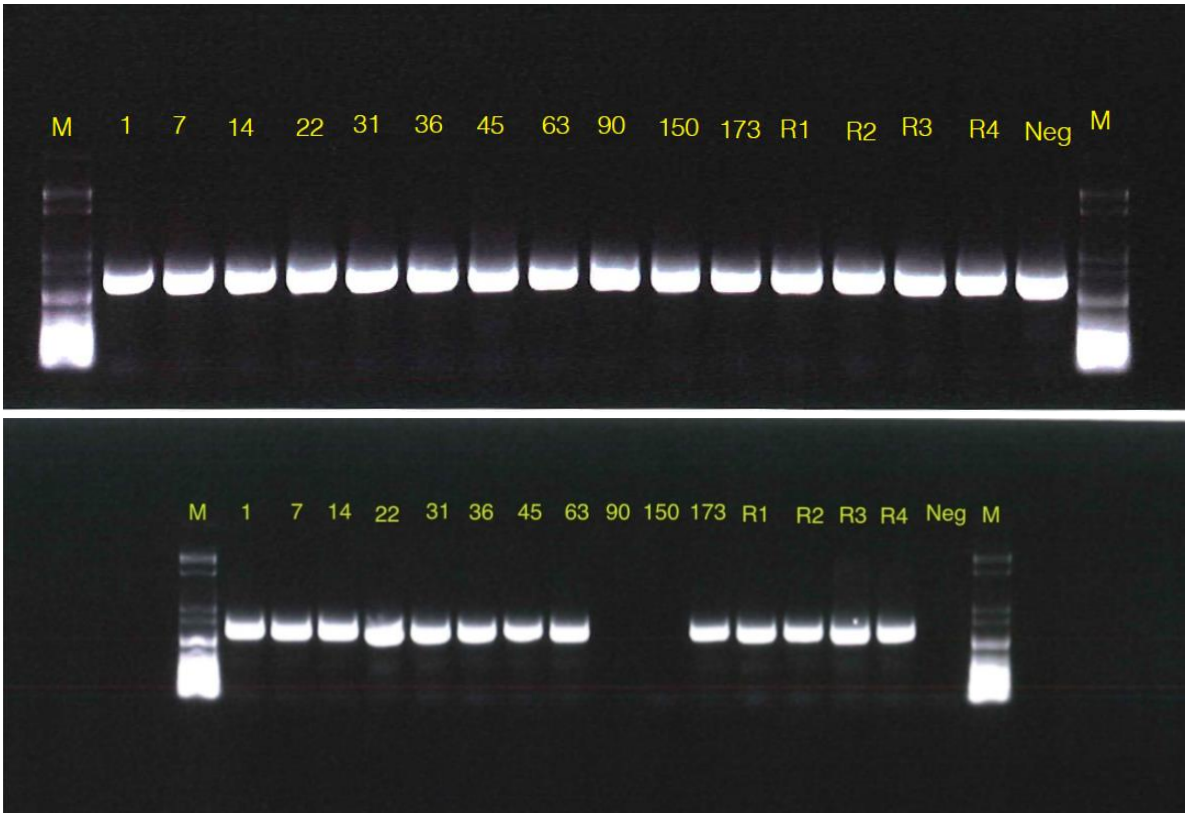

Figure 8. A sample of isolates, and reference strains, that show positive and negative verification of the isolates on an intraspecies level, for the 16S rRNA and gyrB gene analysis. The numbers on top of each well represent the *Aeromonas* isolate analyzed. The top row represents results for the 338F/1492R 16S rRNA primers, and the bottom row represents the gyrB3F/14R primers. The wells marked R1, R2, R3, and R4, represent the reference strains *A. caviae* CCUG 28939, *A. hydrophila* CCUG 14551, *A. veronii* sobria *veronii* CCUG 30360, and *A. veronii* biovar *veronii* CCUG 27821, respectively. The "Neg" well represents the negative control *S. aureus*. (Photo: NU)

5.2.2 Sequencing results.

Ninety one percent of all presumptive *Aeromonas* isolates were positively verified as *Aeromonas* species (figure 9). The sequencing results showed a 60 % prevalence of *A. media* within the isolates. *A. salmonicida*, including its subspecies *pectinolytica* was verified in 18 % of the sequenced strains. The gram-negative bacteria *Enterobacter cloacae*, *Rahnella aquatilis*, and *Stenotrophomonas maltophilia* were each identified with 1 % prevalence, respectively.

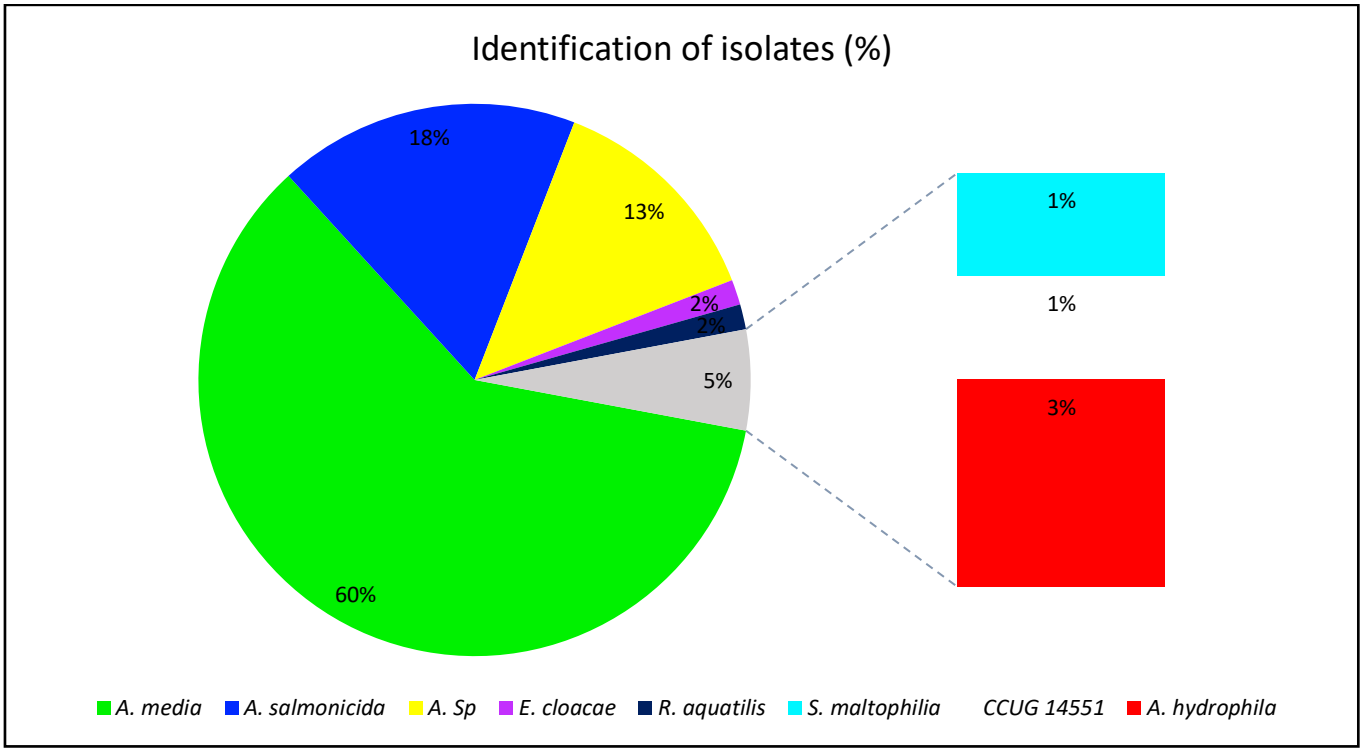

Figure 9. Identification results of *Aeromonas* strains isolated from lettuce, shown by percentage.

A complete matrix of the sequencing results, including the strains' query score and identification accuracy (%) is presented in appendix B.

5.3 Distribution of *Aeromonas* species between the four producers.

All verified *Aeromonas* species were widely distributed between the four producers. (figure 10). The predominant *A. media* was mostly present in producer B. *A. salmonicida* was observed in all producers, except producer C. Producer A was the only one that had all three verified species distributed in their products, the remaining three (producer B, C and D) had prevalence of *S. maltophilia*, *E. cloacae*, and *R. aquatilis*, respectively.

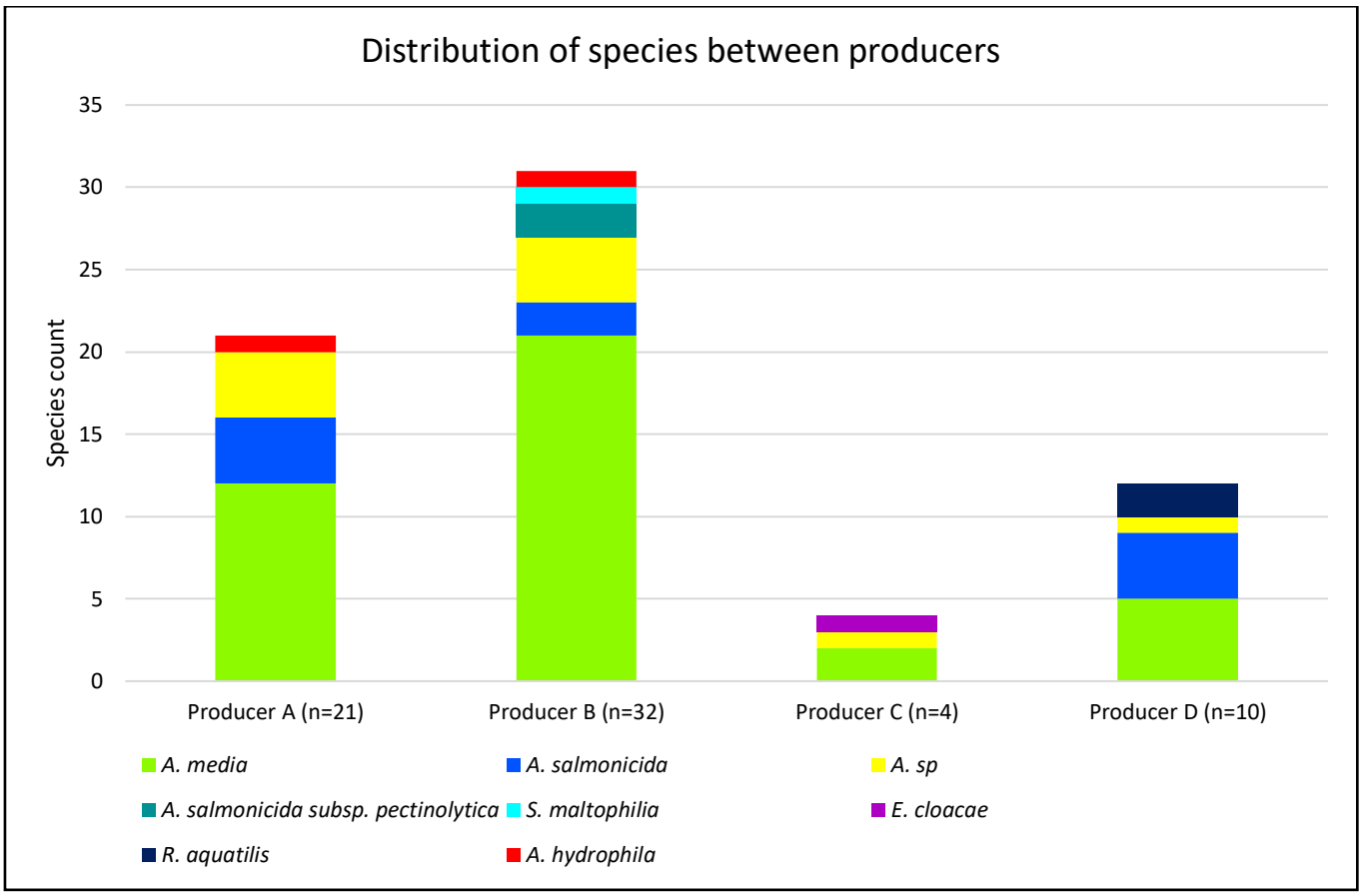

Figure 10. The prevalence and distribution of *Aeromonas* species between the four producers.

5.4 Distribution of *Aeromonas* species between lettuce products.

The distribution of verified *Aeromonas* species also varied within the different types of lettuce, as shown in figure 11. The product with the highest count of bacterial count was ruccula mix, which was supplied by both producer A and B. *A. media* was prevalent in 100 % of the product "babyleaf spinach". In contrast, the bacterium was not detected in two of the products (everyday mix and romano mix). *A. salmonicida* was detected in 7 of the lettuce products, and absent in four of the products supplied by producers B, C, and D.

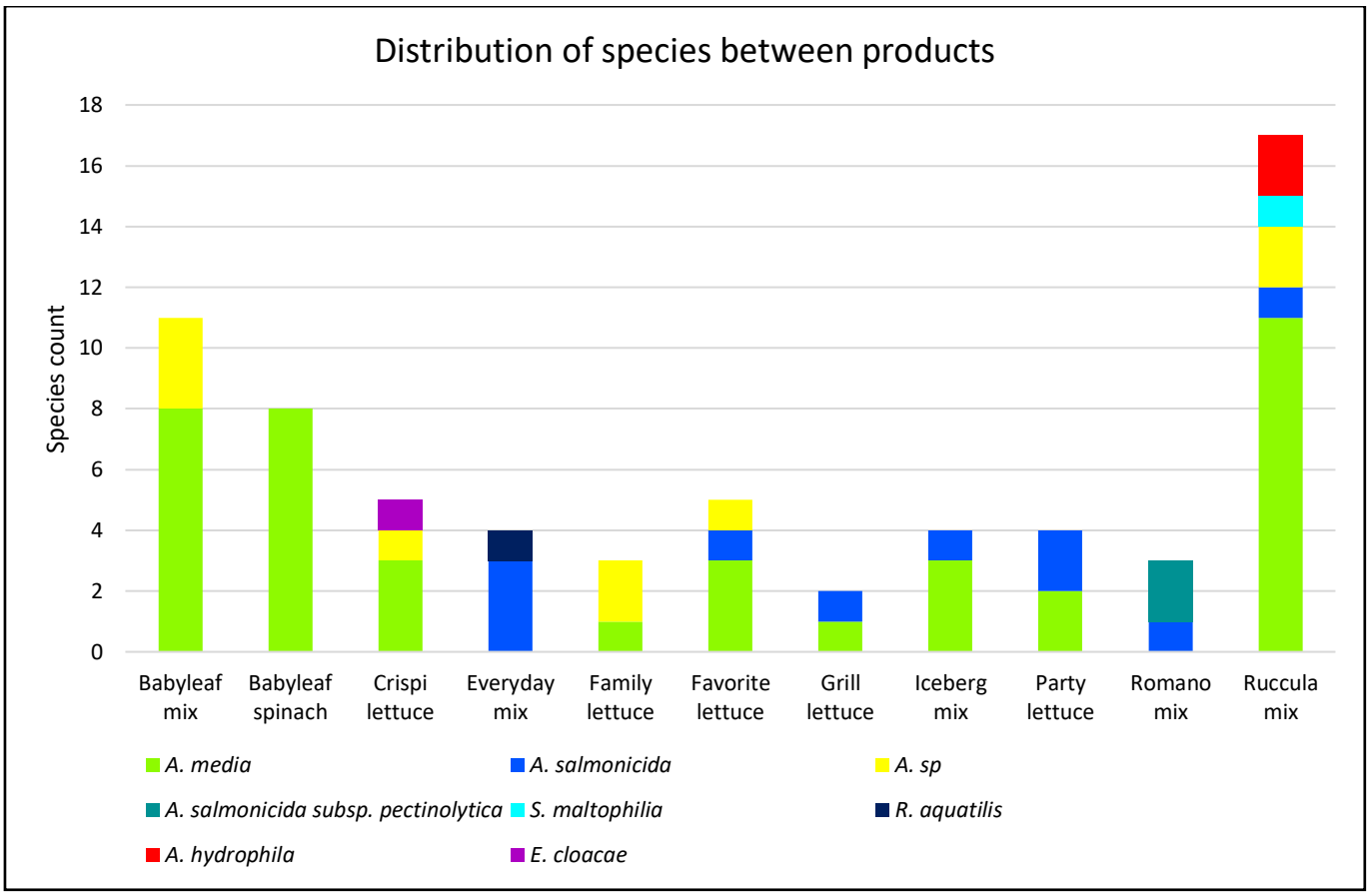

Figure 11. The prevalence and distribution of *Aeromonas* species isolated from various lettuce products.

5.5 The prevalence of virulence-associated genes.

The virulence genes associated with *Aeromonas* were present in nearly all species. None of the shiga-like toxin genes were present in the verified species, but one reference strain (*A. veronii* biovar *veronii* CCUG 27821) was positive for the stx-2 gene. *A. hydrophila* (CCUG 14551) was the only reference strain that harbored all virulence genes (*act*, *ast*, *alt*). The remaining references only harbored the *ast* and *alt* genes (table 6).

Seventy nine percent of the verified *A. media* species harbored the *act* gene, in contrast to 40 % prevalence in the *A. salmonicida* species and 71 % prevalence in *A. sp*. The *ast* gene was verified in 100 % of the *A. salmonicida subsp. pectinolytica* species, *A. sp*. and *A. hydophila*. The latter species and *A. salmonicida subsp. pectinolytica* were the only species where the *ast* gene was 100 % verified. All *A. salmonicida* species were hemolytic, including its subspecies *pectinolytica*. Although a majority of the *A. media* species were non-hemolytic, 4 % showed signs of hemolysis. None of the *A. sp*. were hemolytic (table 6).

Table 6. Virulence associated genes and β-hemolysis visualized on blood agar for *Aeromonas* species isolated from lettuce.

	Number (percentage) of isolates and reference strains harboring virulence associated genes.						
Species	n	act	ast	alt	stx-1	stx-2	β-hemolysis
A. media	39	34 (79)	36 (92)	38 (97)	0	0	2 (4)
A. salmonicida	10	4 (40)	8 (80)	8 (80)	0	0	10 (100)
A. salmonicida subsp. pectinolytica	2	0	2 (100)	2 (100)	0	0	2 (100)
A. sp	7	5 (71)	7 (100)	6 (86)	0	0	0
A. hydrophila	2	1 (50)	2 (100)	2 (100)	0	0	0
Rahnella aquatilis	1	NA	NA	NA	NA	NA	0
Enterobacter cloacae	1	NA	NA	NA	NA	NA	1 (100)
Stenotrophomonas maltophilia	1	NA	NA	NA	NA	NA	0
*A. caviae**	1	0	1 (100)	1 (100)	0	0	1 (100)
*A. hydrophila**	1	1 (100)	1 (100)	1 (100)	0	0	1 (100)
*A. veronii biovar sobria**	1	0	1 (100)	1 (100)	0	0	0
*A. veronii biovar veronii**	1	0	1 (100)	1 (100)	0	1 (100)	1 (100)

*reference strains.

NA,not analyzed.

5.6 Phylogenetic analysis.

A phylogenetic tree (figure 12) was constructed from the nucleotide alignment. The tree was divided in two main subsections, one subsection contained 51 leaves representing *A. media* and the other 12 leaves representing *A. salmonicida*. There was a distinct clustering of species with their representative type of strain. The alignment of *A. sp.* was unevenly distributed between the two clusters, where the majority were aligned with *A. media*. Moreover, the *A. hydrophila* strains were also clustered with the *A. media* subsection. The phylogenetic analysis involved 68 nucleotide sequences which ranged from 503 to 1147 nt.

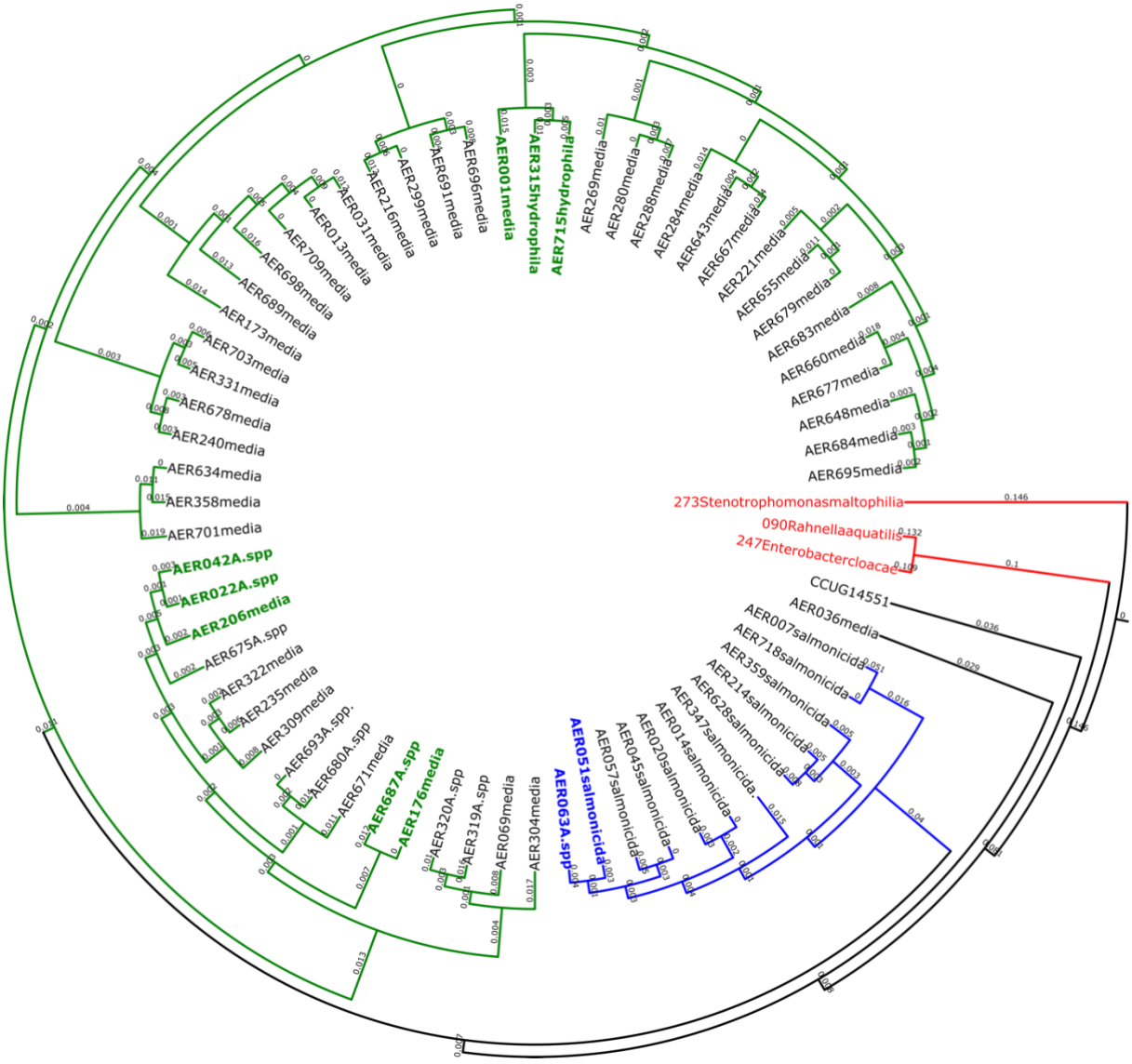

Figure 12. Circular neighbor-joining tree based on *gyrB* sequences showing the relationship among 63 *Aeromonas* isolates inter-and-intra-species evolutionary relationships of *Aeromonas* species isolates isolated from lettuce. The numbers near the nodes indicate the bootstrap values (percentage of 1000 replicates).

5.7 Growth characteristics of selected isolates from the four producers.

Growth characteristics of four selected isolates, one from each producer, were examined in an in vitro salad juice experiment. Iceberg lettuce juice, and crispi lettuce juice were used in this storage experiment. The results are presented below. A complete matrix of the results is presented in appendix C.

5.7.1 Growth characteristics of the selected isolates.

5.7.1.1 *AER679 -Producer A*

Bacterial growth that was registered for this isolate varied for each sample. The initial point for several of the samples was relatively high in both juices. The growth of the isolate was higher in samples with juice (figure 13). There were only small differences between the different concentrations. At the end of the storage period, samples with 5 % juice grew well. The isolate performed in a similar way in the two different juices.

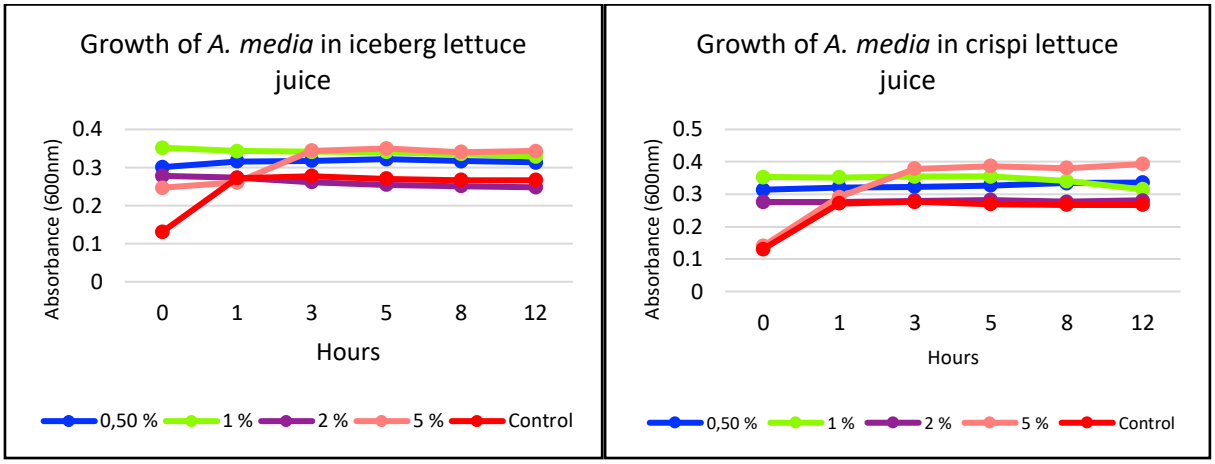

Figure 13. The growth of *A. media* in iceberg and crispi lettuce juice, measured by absorbance (600nm). Bacterial growth was recorded at different hourly intervals.

5.7.1.2 *AER628 -Producer B*

Bacterial growth that was registered for this isolate also varied for each sample. There were only small differences between the different concentrations. At the end of the storage period, samples with 2 % juice grew well. The isolate performed in a similar way in both juices. The growth of the isolate was higher in samples with juice, compared to the control sample (figure 13).

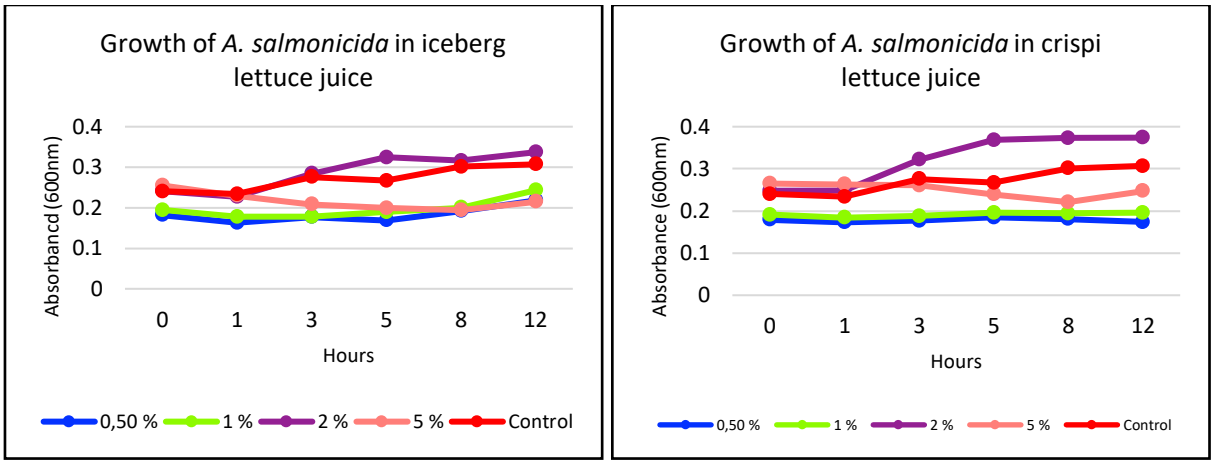

Figure 14. The growth of *A. salmonicida* in iceberg and crispi lettuce juice, measured by absorbance (600nm). Bacterial growth was recorded at different hourly intervals.

5.7.1.3 *AER042 -Producer C*

Bacterial growth that was registered for this isolate showed stagnant or declining growth for each sample. One of the samples had an relatively high initial point compared to the others (1 %, iceberg juice). At the end of the storage period, samples with 5 % juice grew well. The isolate performed in a similar way in both juices. The growth of the isolate was higher in samples with juice, compared to the control sample (figure 13).

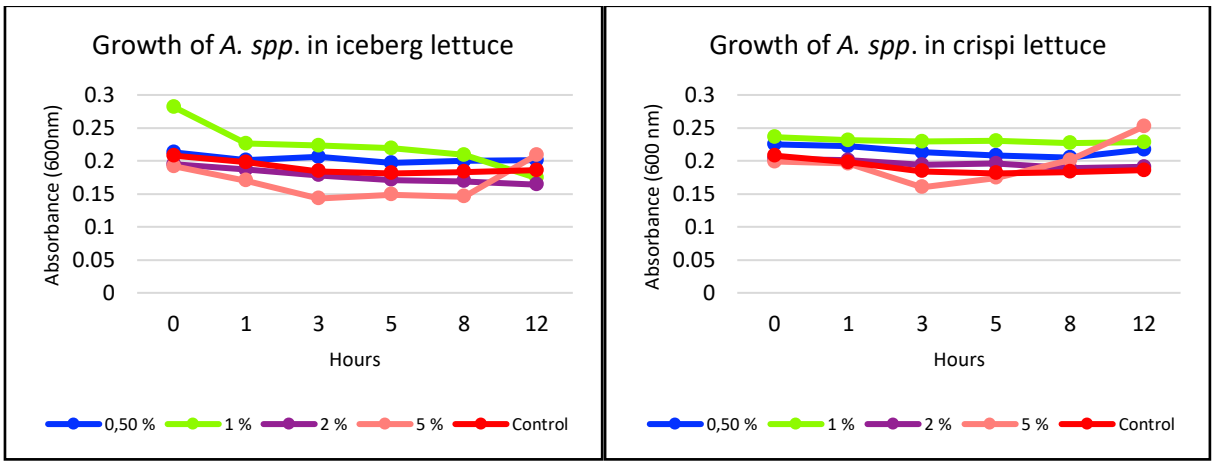

Figure 15. The growth of *A. spp*. in iceberg and crispi lettuce juice, measured by absorbance (600nm). Bacterial growth was recorded at different hourly intervals.

5.7.1.4 *AER703 -Producer D*

Bacterial growth that was registered for this isolate varied for each sample. Samples with 2 % juice grew in tandem with the control sample. The initial point for a few of the samples was relatively high in both juices. There were only small differences between the different concentrations. At the end of the storage period, samples with 0,5 % and 5 % juice grew well. The isolate performed in a similar way in both juices. The growth of the isolate was slightly higher in samples with juice, compared to the control sample (figure 16).

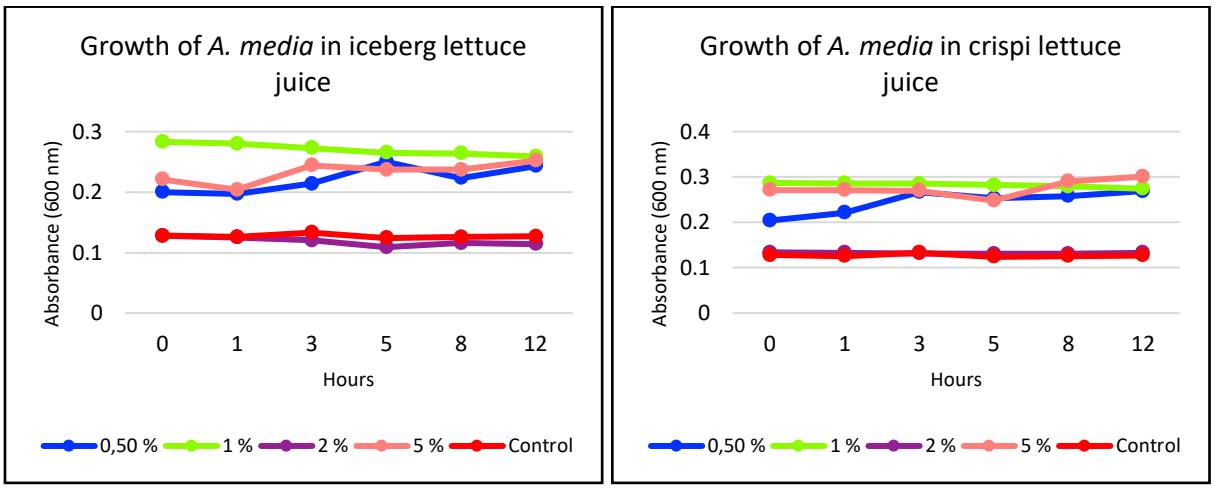

Figure 16. The growth of *A. media* in iceberg and crispi lettuce juice, measured by absorbance (600nm). Bacterial growth was recorded at different hourly intervals.

6 Discussion

The *Aeromonas* genus is an aquatic bacteria that has been isolated from diverse environments, including rivers, lakes, ponds, drinking water, and ground water. Some Aeromonas species are virulent in humans, fish, worms and other living organisms. The ecology of enteric human pathogens associated with fresh produce has received special attention, as customer demands for fresh salads are continuously increasing and the presence of potentially pathogenic bacteria within plant-associated microbiota can affect produce safety. (Brandl, Cox, & Teplitski, 2013; Critzer & Doyle, 2010; Teplitski, Warriner, Bartz, & Schneider, 2011)

In this study, *Aeromonas* strains isolated from different lettuce products supplied by four local producers were characterized and identified by biochemical and molecular methods. Two major *Aeromonas* species were identified; *A. media* and *A. salmonicida*, and were found to be unevenly distributed between the four producers. Furthermore, *A. sp*. was verified but could not be placed in a define taxon. *A. hydrophila* was also identified, but there were indications that these strains might in fact belong to the *A. media* complex.

6.1 Growth and identification of Aeromonas species

Seventy five (90 %) of the bacterial samples that had been stored at −80 °C for several years grew well under aerobic conditions, which supports the theory that DNA is the most stable component in biological samples. (Holland, Smith, Eskenazi, & Bastaki, 2003; Paoli, 2005)

PCR analysis of the 16S rRNA gene sequencing of Aeromonas isolates in this work was successful to determine the isolates on species level, as 97 % of the isolates were verified for this gene. This is in accordance to previous studies, where 16s rRNA gene sequencing has, for a number of reasons, been the most common housekeeping genetic marker used to identify strains at the genus level. (J. Michael Janda & Abbott, 2007; Patel, 2001) However, the use of this marker in phylogenetic analysis of *Aeromonas* spp. has revealed that the genus composes a very tight group of species, where some of them differ by a few nucleotides. (Küpfer et al., 2006; Martinez-Murcia et al., 1992)

It was therefore necessary to include housekeeping genes (*rpoB* and *gyrB*) that provide a more accurate detection of the *Aeromonas* spp. on genospecies level. Sequencing by the *gyrB* gene was successful in 93 % of the strains in this study. In addition, seventeen selected isolates were analyzed for the *rpoB* gene by PCR analysis, and 16 (94 %) were verified for the gene by approximately 550 bp. (data shown in appendix A). Furthermore, *Aeromonas* isolates were correctly identified by phenotypic method, thus indicating that the biochemical and molecular methods used in this study are acceptable tools for

differentiating *Aeromonas* genospecies from other present non-aeromonas microorganisms, confirming previous work on *Aeromonas* and similar findings in other bacteria genera. (Fukushima, Kakinuma, & Kawaguchi, 2002; Soler et al., 2004; Yáñez et al., 2003)

The predominant verified species *A. media*, has previously been isolated from water samples and described as a non-motile rod-shaped bacteria with a temperature range for growth between 4 and 37 °C. (Allen et al., 1983; Wan et al., 2007) However, previous biochemical analysis of *A. media* has shown phenotypic resemblance between *A. media* species and *A. caviae* species, thereby placing *A. media* in the *A. caviae* complex. (Abbott, Cheung, & Janda, 2003; Ørmen et al., 2005) The literature contains numerous studies where the most frequent mesophilic *Aeromonas* spp. isolated from RTE vegetables are *A. hydrophila*, *A. schubertii*, *A. trota* and *A. caviae.* (Kaur et al., 2017; McMahon & Wilson, 2001; Nishikawa & Kishi, 1988)

Therefore, one can speculate whether the verified *A. media* strains in this study, in fact belong to the *A. media* species. To verify these findings, it is necessary to include further analysis that distinguish *A. media* from other present *Aeromonas* spp. For instance, the use of selective primer pairs and multiplex PCR (mPCR) methods that are designed to target specific *Aeromonas spp.* has been described as a successful tool for precise identification of *Aeromonas* species at genospecies level (Persson et al., 2015). Selective primers were designed to target the *rpoB* and *gyrB* gene in *A. hydrophila*, *A. media*, *A. caviae*, and *A. veronii*. Each primer pair was specifically designed to target each species accordingly. The results showed low intraspecies divergence for both genes in all four examined species. (Persson et al., 2015) One of the many difficulties in the identification of *Aeromonas* strains to species level, has been the continuous use of traditional biochemical methods. However, because some *Aeromonas* species display heterogeneous biochemical properties, the correct identification rate for biochemical tests has been shown to be very low.

Furthermore, the number of *Aeromonas* species is continuously expanding, therefore the use of molecular methods becomes more necessary in order to obtain correct species identification. Multiplex PCR is an approach that would be able to give precise identification of *Aeromonas* species, as it allows amplification of target genes that select for a specific species. In addition, this method allows for amplification of several target DNA sequences from several foodborne pathogens, which can be visualized by agarose gel electrophoresis. (Villamizar-Rodriguez et al., 2015) This can be further useful in improvement of routine diagnostics and shed new light on the controversial clinical and epidemiological aspects of the *Aeromonas* genus.

Another approach that would provide precise identification of *A. media* is multilocus sequence typing (MLST). MLST allows comparison of several housekeeping genes (typically seven or more) which are present in all isolates of a particular species. (Reyes & Zervos, 2010) *A. media* differs from other *Aeromonas* spp. by a few distinctive genetic features (Talagrand-Reboul et al., 2017), and sequencing with MLST can help highlight these features, thereby distinguishing *A. media* from other present *Aeromonas* species. It is a method that originally was designed to assess genetic interrelationships in evolutionary studies, and has exceled in identifying broad population-based interrelationships. (Reyes & Zervos, 2010)

Phylogenetic analysis based on *gyrB* sequences resulted in a neighbor-joining tree, that showed robust and well-separated clusters. The largest subsection consisted of *A. media*, *A. sp.* and *A. hydrophila*. One cluster in particular aligned two *A. sp*. (AER022 and AER206) with one *A. media* (AER042), with a range of nucleotide substitution of 0.5 % between the three isolates. This suggests that these isolates either belong to the *A. media* species or *A. sp*. because of close phenotypic resemblance between them. Moreover, two of the isolates, *A. sp*. (AER042) and *A. media* (AER206) were both supplied by producer B and were isolated from the same product, but analyzed at different times. It is therefore remarkable that only one of them was identified as *A. media*. *A. hydrophila* has been categorized as a major human pathogen of the *Aeromonas* genus, and has frequently been linked to the contamination of RTE vegetables. Despite its pathogenic importance, only two isolates of this species were identified in this work, and both of them were clustered with *A. media*. This further supports the theory that a small margin of error must be considered when the results demonstrate a very large proportion of *A. media* in the samples.

The other distinct subsection consisted of several *A. salmonicida* species and one *A.* sp. *A. salmonicida* has previously been described as non-motile psychrophilic fish pathogen, and has been isolated from a variety of fish products. (Bartkova, Kokotovic, Skall, Lorenzen, & Dalsgaard, 2017; Hoel et al., 2017; Sorum, Holstad, Lunder, & Håstein, 2000) This species contains five recognized subspecies: *salmonicida, achromogenes*, *masoucida*, *smithia*, and *pectinolytica*. (B. Austin & Austin, 2007) The latter is described as mesophilic, with the ability to thrive at a wide range of temperatures, including 37 °C. The first four are psychrophilic subspecies which are restrained to low temperatures. (Vincent, Rouleau, Moineau, & Charette, 2017) As no previous cases of *A. salmonicida* isolated from vegetables has been reported, and because of the incubation temperature used in this study (37 °C), there is a possibility that the isolates identifies as *A. salmonicida* in fact were of the mesophilic nature and thus part of the *pectinolytica* species. However, further analysis are necessary to verity such associations.

The prevalence of the *R. aquatilis*, *S. maltophilia*, and *E. cloacae* in this study was an interesting finding that must be examined further. The common denominator for all three bacteria genera is that they are emerging pathogens that can cause human disease. *R. aquatilis* has been associated with products grown in or on the soil, and has previously been isolated from fresh water. (Harrell, Cameron, apos, & Hara, 1989) *S. maltophilia* and *E. cloacae* are environmental emerging multi-drug resistant organisms. *S. maltophilia* has been described in clinical cases associated with respiratory infections in humans, and *E. cloacae* has been associated with a wide variety of infections such as pneumonia, urinary tract infections and septicemia. (Annavajhala, Gomez-Simmonds, & Uhlemann, 2019; Brooke, 2012; Sanders & Sanders, 1997; Wisplinghoff et al., 2004) Therefore, because of they can be ubiquitous in the environment, and have proven to be multi-drug resistant, it is necessary to further examine their role in RTE vegetables as an increasing production and consumption of such products is expected in the future.

6.2 Virulence characteristics of the identified isolates.

The *act* gene, which encodes a cytotoxic enterotoxin was observed in 79 %, 40 %, 71 % and 50 % of *A. media*, *A. salmonicida*, *A. sp*., and *A. hydrophila*, respectively. Sen and Rodgers (2004) and Yano et al. (2015) have previously shown high prevalence of this gene in strains from drinking water (70 %) and seafood (75 %). (Sen & Rodgers, 2004; Yano et al., 2015) In contrast to previous findings in Hoel et al. (2017), where the *act* gene was not detected in their *A. media* strains, a majority of the *A. media* strain in this work were verified for this gene.

The proportion of isolates harboring the *ast* and *alt* genes, was higher in all species. However, the impact these two genes have on pathogenesis has not been completely recognized. Puthucheary and Puah (2012) have hypothesized that the *A. caviae* and *A. veronii* species lack the *ast* gene. However, the *A. caviae* and *A. veronii* reference strains amplified for this work were verified for both genes, in both species. Moreover, in regards to previously mentioned similarities between the *A. caviae* and *A. media* species, it is worth mentioning that 92 % and 97 % of the *A. media* species were verified for the *ast* and *alt* gene, respectively. Furthermore, PCR amplification of these genes showed multiple bands for some of the isolates when visualized in an agarose gel (data not shown). Collectively, these findings suggest intraspecies heterogenicity for said samples in regards to the *ast* and *alt* genes. Thus indicating that the hypothesis on the lack of specific genes cannot be attributed to certain species.

Act has been linked to *aerA* and the production of hemolysis, especially in *A. hydrophila*. (Tomàs, 2012) The findings of this work were not consistent with this claim, as the *A. hydrophila* strains showed no signs of hemolysis, although the *act* gene was present in 50 % of the strains. β-hemolysis was displayed in all the *A. salmonicida* strains, including its subspecies *pectinolytica*. However, none of the *pectinolytica* strains were verified for the *act* gene. Furthermore, only four (n=10) of the *A. salmonicida* strains harbored the *act* gene. This suggests that β-hemolysis cannot be attributed to a single factor. On the other hand, the prevalence of *aerA* was not incorporated in this work. There are thus not sufficient results to claim that the absence or presence of hemolysis in the strains was solely due to the prevalence or lack of the *act* gene. However, previous findings in Hoel et al. (2017) displayed hemolysis in all *A. dhakensis* strains, even though the *act* gene was absent in this species. In light of such contradicting findings, the lack of observed hemolysis in this work might be a result of altered gene expressions and/or posttranslational processes not analyzed here.

The *Aeromonas* isolates in this work are, based on the present results, considered to be potential pathogens. However, the pathogenicity of *Aeromonas* species is associated with several virulence factors, and as noted above, some analysis that determine the genus' pathogenicity were not incorporated in this work. Shiga-like toxins (stx-1 and stx-2) have been associated with hemolytic-uremic syndrome, and detected in clinical and environmental isolates of *A. hydrophila*, *A. caviae*, *and A. veronii* biovar *sobria*. (M. J. Figueras et al., 2011; Palma-Martinez et al., 2016; Snowden et al., 2006) None of the isolates in this work harbored these genes. Moreover, no cases have been reported where stx-1 and/or stx-2 have detected in foodborne isolates.

6.3 Growth characteristics of selected isolates from the four producers.

The findings in the in vitro salad juice experiment showed high discrepancies among the isolates, and their growth kinetics in the two different juices. The literature on which the experiment was inspired by, was targeted towards growth of *Salmonella* in salad juices, under alternate conditions. (Koukkidis et al., 2017)

Previous studies have investigated the growth of *A. hydrophila* and *L. monocytogenes* on fresh vegetables stored under a controlled atmosphere. (Berrang, Brackett, & Beuchat, 1989, 1989b) Moreover, the survival and growth of *E. coli* 0157:H7 on salad vegetables has been investigated by Abdul-Raouf, Beuchat, and Ammar (1993). However, there is little information available about the growth of *Aeromonas* spp. in salad juice. The selected isolates involved in this study showed stagnant or declining growth in iceberg lettuce juice, and slightly better growth kinetics when inoculated in crispi lettuce juice.

Interestingly, a combination of low concentrations of water and high concentrations of juice (850 µl and 5 %, respectively) enhanced the growth of *A. media* (AER679 and AER703) in iceberg lettuce juice. This was also the case for isolate AER679 in crispi lettuce juice. However, the oppsosite was observed for *A. salmonicida* (AER628). The samples with less amounts of juice (0,5 % and 2 %, respectively) showed increasing growth for this species in iceberg lettuce juice. In regards to crispi lettuce juice, said isolate showed promising growth kinetics in the samples with 0,5 %, 1 %, and 2 % lettuce juice concentrations. Growth of *A. sp*. in both lettuce juices varied at every other reading.

Various factors may have contributed to the variating growth results found in this study. For instance, the storage conditions, the type of lettuce product, the selected *Aeromonas* strain and its characteristics, and the concentration amounts applied, may have been contributing factors. As noted above, no cases have been reported regardig storage expriments targeting the growth of *Aeromonas* spp. in salad juices. Therefore, the conditions used in this storage experiment were solely based on previous knowledge about *Aeromonas* spp. in general.

One can speculate that a storage experiment stimulating more ideal storage conditions might have provided more accurate results. For instance, the samples were incubated at 37 °C over a 12 hour period. Although this temperature is optimal for growth of *Aeromonas* spp., it neither reflects realistic storage temperatures for vegetables at the grocery store, nor in the consumer's home. Ideally, a storage experiment where samples are stored at 4 °C over the product's designated shelf life might have provided more accurate results. Furthermore, several lettuce products could have been included, for broader observations on the isolates behaviours when exposed to different environments.

One can also speculate on whether the products' natural components were contributing factors to the lack of- or minimal growth observed in this study. Previous studies have detected significant levels of antioxidant activities and phenolic components in lettuce (Caldwell, 2003; Cao, Sofic, & Prior, 1996; Chu, Sun, Wu, & Liu, 2002; Vinson, Hao, Su, & Zubik, 1998) When plants are exposed to ecological pressures such as pathogen attacks, cutting and/or shredding, post-harvest processing, and storage, their cell walls break down and release phenolic compounds that can inhibit bacterial growth. Parente et al. (2013) showed that lettuce simultaniosly contains a large amount of total polyphenols as well as high antioxidant activity. Likewise, Liu et al. (2007) found that, among 25 types of lettuce products, leaf lettuce contained the highest total phenotic count. The concentration of phenolic acid in lettuce has proved to be sensitive to environmental conditions. (Liu et al., 2007; Parente et al., 2013)

The total phenolic content in the lettuce products involved in this work was not measured in advance. It is therefore possible that the noted stagnant growth, or declining growth, observed in some of the isolates may be attributed to significant levels of phenolic compounds that were initially present, and thus inhibited the growth of the *Aeromonas* isolates. However, further analysis are necessary to verify such associations.

7 Conclusion

This work assessed the growth and identification of *Aeromonas* strains isolated from lettuce, after several years of storage at below 80 degrees. Biochemical and molecular methods that were performed were successful in identifying the isolates at genus and genospecies level. A majority of the isolates harbored several of the virulence-associated genes, thus showing virulence properties compared to those in clinical studies.

The characterization and identification of *Aeromonas* isolates showed high prevalence of *A. media*. These findings must be verified by subsequent analysis which specifically target this species to support this study's work. The prevalence and identification of *A. salmonicida* suggests that mesophilic strains of this genus can be present in minimally processed vegetables, but further analysis are necessary to support these findings.

The growth of *Aeromonas* spp. seems to be highly affected by several factors, including natural microbiota, plant composition content, storage conditions, and pre- and post-harvesting processes. However, further studies that assess the growth of *Aeromonas* species under stimulated storage experiments are necessary to support the findings in this work.

8 Future perspectives

To obtain a more definite identification of the isolates at hand, specific primers targeting each species and mPCR methods can be included in further studies. Furthermore, several virulence-associated factors can be included to reaffirm the findings in this work, and the overall virulence properties associated with *Aeromonas* spp. in foodborne isolates. MLST can also be included, as it is a method that originally was designed to assess genetic interrelationships in evolutionary studies, and has exceled in identifying broad population-based interrelationships.

For better knowledge of growth of *Aeromonas* spp. in salad juice, a follow up study under similar conditions, or more realistic conditions must be included. The product's naturally occuring phenols can be measured in advance to examine it's original condition before the experiment is repeated. Furthermore, a sensory study can be performed in conjuction with said experiment, where properties such as general appearance, color, texture and aroma can been assesssed during storage, thereby stregnthening the results.

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Appendix A. *Aeromonas* strains isolated from different lettuce products. A summary of all isolates analyzed in this work.

Sample ID	Isolate nr. AER	Selected isolate	Producer	Lettuce type	# isolate	Aerob growth #1	Aerob growth #2	Hemolysis	Cell pellet	260/280 ratio	DNA (ng/µl)	338F/1492R	gyrB3F/14R	*rpoB*	*act*	*ast*	*alt*	*stx-1*	*stx-2*
Aeromonas caviae (CCUG 25939)					1	+		+	+	2,185/2,017	23,02	+	+		-	-	+	-	-
Aeromonas hydrophila (CCUG 14551)					1	+		+	+	2,106/2,043	28,01	+	+		+	+	+	-	-
Aeromonas veronii biovar sobria (CCUG 30360)					1	+		-	+	2,209/1,949	27,63	+	+		-	+	+	-	-
Aeromonas veronii biovar veronii (CCUG 27821)					1	+		+++++	+	2,153/2,867	22,32	+	+		-	+	+	-	+
Sal 16	1-3	AER 001	A	Favorite lettuce	1	+	+	-	+	2/1,758	13,99	+	+		-	+	+	-	-
Sal 28	7	AER007	B	Iceberg mix	1	+	+	+	+	1,391/1,419	9,94	+	+		+	+	+	-	-
Sal 29	8-10	AER 008	B	Iceberg mix	1	-	-	-	-	-	-	-	-		-	-	-	-	-
Sal 30	13	AER 013	B	Iceberg mix	1	+	-	-	+	1,803/1,805	24,06	+	+		+	+	+	-	-
Sal 32	14-19 (17&18)	AER 014	B	Romano mix	1	+	+	+	+	1,778/1,371	12,63	+	+	+	-	+	+	-	-
Sal 33	20/21	AER 020	B	Romano mix	1	-	-	+	+	2,195/1,862	18,97	+	+		-	+	+	-	-
Sal 39	22	AER 022	A	Favorite lettuce	1	+	+	-	+	2,087/1,714	14,17	+	+		+	+	+	-	-
Sal 52	26-30/29-30	AER 026	B	Iceberg mix	1	-	-	-	-	-	-	-	-		-	-	-	-	-
Sal 53	31-33	AER 031	B	Iceberg mix	1	+	+	-	+	1,565/1,677	11,48	+	+		+	-	+	-	-
Sal 54	36-40	AER 036	B	Iceberg mix	1	+	+	-	+	1,63/1,6	12,98	+	+		+	+	+	-	-
Sal 57	42 (41 or 43)	AER 042	C	Crispi	1	+	-	-	+	1,911/1,822	22,05	+	+		+	+	+	-	-
Sal 58	45/46	AER 045	D	Everyday mix	1	+	+	+	+	1,778/1,548	12,44	-	+		-	+	+	-	-
Sal 59	51 (or 52-54)	AER 051	D	Everyday mix	1	+	-	+	+	2/1,911	20,87	+	+	+	+	+	+	-	-
Sal 60	57 (or 58-60)	AER 057	D	Everyday mix	1	+	-	+	+	1,774/1,902	22,34	+	+		+	+	+	-	-
Sal 67	63	AER 063	A	Party lettuce	1	+	+	+	+	1,481/1,391	9,42	+	+	+	-	+	+	-	-
Sal 71	69	AER 069	A	Party lettuce	1	+	-	-	+	2/1,586	14,52	+	+		+	+	+	-	-
Sal 81	90 (91 or 92)	AER 090	D	Everyday mix	1	+	+	-	-	2,526/1,725	17,88	+	+		NA	NA	NA	NA	NA
Sal 100	149 (150)	AER 150	B	Classic lettuce	1	+	+	-	-	2,105/1,333	9,97	+	-	-	NA	NA	NA	-	-
Sal 112	172-174	AER 173	D	Babyleaf spinach	1	+	+	-	+	1,091/1,455	3,65	+	+		+	+	+	-	-
Sal 113	175 (176 or 177)	AER 176	D	Babyleaf spinach	1	+	-	-	+	1,902/1,93	24,6	+	+		+	+	+	-	-
Sal 124	188/189	AER 188	D	Babyleaf spinach	1	-	-	-	-	-	-	-	-		-	-	-	-	-
Sal 125	190/191	AER 190	D	Babyleaf spinach	1	-	-	-	-	-	-	-	-		-	-	-	-	-
Sal 141	206	AER 206	C	Crispi	1	+	+	-	+	53,657/59278	56,47	+	+		+	+	+	-	-
Sal 147	214	AER 214	A	Favorite lettuce	1	+	+	+	+	79,937/147,244	113,59	+	+	+	-	+	+	-	-
Sample ID	**Isolate**	**Selected isolate**	**Producer**	**Lettuce type**	**# isolate**	**Aerob growth #1**	**Aerob growth #2**	**Hemolysis**	**Cell pellet**	**260/280 ratio**	**DNA (ng/µl)**	**338F/1492R**	**gyrB3F/14R**	**rpoB**	**act**	**ast**	**alt**	**stx-1**	**stx-2**

Appendix A. *Aeromonas* strains isolated from different lettuce products. A summary of all isolates analyzed in this work.

	nr. AER																		
Sal 149	216/218	AER 216	C	Crispi	1	+	+	+	+	42,794/105,434	74,12	+	+		+	+	+	-	-
Sal 150	221/225	AER 221	C	Crispi	1	+	+	-	+	16,285/55,101	35,69	+	+		+	+	+	-	-
Sal 152	232	AER 232	B	Ruccula mix	1	+	+	++	+	9,937/82,813	46,38	+	+	+	NA	NA	NA	-	-
Sal 153	235/237	AER 235	B	Ruccula mix	1	+	+	-	+	7,791/6,755	7,27	+	+		+	+	+	-	-
Sal 154	240/241	AER 240	B	Ruccula mix	1	+	+	-	+	52,615/47,938	50,28	+	+		+	+	+	-	-
Sal 159	247	AER 247	C	Crispi	1	+	+	+	+	110,24/117,9	114,07	+	+		NA	NA	NA	-	-
Sal 171	268/269	AER 269	B	Ruccula mix	1	+	+	-	+	44,801/56,701	50,75	+	+		-	+	+	-	-
Sal 172	273	AER 273	B	Ruccula mix	1	+	+	+	+	120,167/99,56	109,86	+	+		NA	NA	NA	-	-
Sal 174	280	AER 280	B	Babyleaf spinach	1	+	+	-	+	105,664/157,365	131,51	+	+		+	+	+	-	-
Sal 175	283/284	AER 284	B	Babyleaf spinach	1	+	+	-	+	66,946/37,5	52,22	+	+	+	-	+	+	-	-
Sal 179	288/289	AER 288	A	Favorite lettuce	1	+	+	-	+	2,032/1,946	44,51	+	+	+	-	+	+	-	-
Sal 181	299	AER 299	A	Favorite lettuce	1	+	+	-	+	93,497/19,746	56,2	+	+		-	+	+	-	-
Sal 188	304/306	AER 304	B	Ruccula mix	1	+	+	-	+	43,759/40,206	41,98	+	+		+	+	+	-	-
Sal 189	309/310	AER 309	B	Ruccula mix	1	+	+	-	+	39,707/45,064	42,38	+	+		+	+	+	-	-
Sal 190	315	AER 315	B	Ruccula mix	1	+	+	-	+	104,607/68,191	86,4	+	+		+	+	+	-	-
Sal 218	319/320	AER 319	A	Family lettuce	1	+	+	-	+	2,239/2,039	60,12	+	+		+	+	+	-	-
Sal 218	319/320	AER 320	A	Family lettuce	1	+	+	-	+	2,032/1,966	31,1	+	+	+	+	+	+	-	-
Sal 219	322	AER 322	A	Family lettuce	1	+	+	-	+	2,37/1,532	17,9	+	+		+	+	+	-	-
Sal 216	331	AER 331	A	Grill lettuce	1	+	+	-	+	1,559/1,672	26,78	+	+		+	+	+	-	-
Sal 234	341/342	AER 341	A	Grill lettuce	1	-	+	-	+	1,977/2,159	40,2	+	-	+	NA	NA	NA	-	-
Sal 235	347/348	AER 347	A	Grill lettuce	1	+	+	+++++	+	1,6/1,565	7,79	+	+		-	-	+	-	-
Sal 237	359/358	AER 358	A	Party lettuce	1	+	+	+	+	2,217/1,88	44,07	+	+		-	-	-	-	-
Sal 237	359/358	AER 359	A	Party lettuce	1	+	+	+	+	1,6/1898	17,07	+	+		-	+	-	-	-
Sal 227	373/374	AER 373	B	Grill mix	1	+	+	-	+	2,175/2,07	83,61	+	-	+	NA	NA	NA	-	-
Sal 227	373/374	AER 374	B	Grill mix	1	+	+	-	+	1,771/1,831	44,64	+	-		NA	NA	NA	-	-
Sal 228	376/377	AER 376	B	Grill mix	1	+	+	-	+	1,617/1,934	32,85	+	-		NA	NA	NA	-	-
Sal 226	601/602	AER 601	B	Iceberg mix	1	-	-	-	-	-	-	-	-		-	-	-	-	-
Sal 204	628/629	AER 628	B	Ruccula mix	1	+	+	+	+	1,222/1,72	14,14	+	+	+	-	-	-	-	-
Sal 205	632/633/634	AER 634	B	Ruccula mix	1	+	-	+	+	2,053/1,897	24,72	+	+		-	+	+	-	-
Sample ID	Isolate nr. AER	Selected isolate	Producer	Lettuce type	# isolate	Aerob growth #1	Aerob growth #2	Hemolysis	Cell pellet	260/280 ratio	DNA (ng/µl)	338F/1492R	gyrB3F/14R	*rpoB*	*act*	*ast*	*alt*	*stx-1*	*stx-2*

Appendix A. *Aeromonas* strains isolated from different lettuce products. A summary of all isolates analyzed in this work.

Sal 223	640	AER 640	C	Salanova	1	+	+	-	+	1,6/1,684	7,27	+	-		NA	NA	NA	-	-
Sal 251	641/642/664/665	AER642	B	Babyleaf mix	1	-	-	-	-	-	-	-	-		-	-	-	-	-
Sal 252	643/644/645/646/647	AER643	B	Babyleaf mix	1	+	+	+	+	2,072/2,02	43,17	+	+	+	+	+	+	-	-
Sal 253	648/649/650/651	AER648	B	Babyleaf mix	1	+	+	-	+	1,987/2,4	43,9	+	+		+	+	+	-	-
Sal 254	652/653/654/666	AER652	B	Babyleaf mix	1	-	-	-	-	-	-	-	-		-	-	-	-	-
Sal 255	660/661/662/663	AER 660	B	Babyleaf mix	1	+	+	-	+	2,027/2,154	45,68	+	+		+	+	+	-	-
Sal 256	655/656/657/658/659	AER 655	B	Babyleaf mix	1	+	+	-	+	1,97/2,084	83,35	+	+		+	+	+	-	-
Sal 257	667/668/669/670	AER 667	B	Ruccula mix	1	+	+	-	+	2,036/1,905	30,27	+	+		+	+	+	-	-
Sal 258	671/672/673/674	AER 671	B	Ruccula mix	1	+	+	-	+	1,758/2,076	42,08	+	+		-	+	+	-	-
Sal 259	675/676	AER 675	B	Ruccula mix	1	+	+	-	+	1,79/1,859	50,35	+	+		-	+	+	-	-
Sal 264	677	AER 677	B	Babyleaf spinach	1	+	+	-	+	2,175/2,423	84,8	+	+		+	+	+	-	-
Sal 265	678	AER 678	B	Babyleaf spinach	1	+	+	-	+	1,967/2,141	99,71	+	+	+	+	+	+	-	-
Sal 266	679	AER 679	A	Rucula mix	1	+	+	-	+	2,185/2,047	45,69	+	+		+	+	+	-	-
Sal 266	683	AER 683	A	Rucula mix	1	+	+	-	+	2,149/2,141	68,57	+	+		+	+	+	-	-
Sal 267	696/681	AER 696	A	Ruccula mix	1	+	+	-	+	2,244/2,017	66,3	+	+		+	+	+	-	-
Sal 268	694/695	AER 695	A	Ruccula mix	1	+	+	-	+	2,037/2,269	63,54	+	+		+	-	+	-	-
Sal 272	687	AER 687	B	Babyleaf mix	1	+	+	-	+	1,804/1,829	19,19	+	+		-	+	+	-	-
Sal 273	689/690	AER 689	B	Babyleaf mix	1	+	+	-	+	1,986/1,867	55,03	+	+		+	+	+	-	-
Sal 274	682/684	AER 684	B	Babyleaf mix	1	+	+	-	+	1,968/2,237	32,32	+	+		+	+	+	-	-
Sal 278	692a/692b/693	AER 693	B	Babyleaf mix	1	+	+	-	+	2,032/1,72	41,04	+	+		+	+	+	-	-
Sal 279	680/685/686	AER 680	B	Babyleaf mix	1	+	+	-	+	2,103/1,98	75,64	+	+	+	+	+	-	-	-
Sal 280	691	AER 691	B	Babyleaf mix	1	+	+	-	+	1,873/2,105	24,57	+	+	+	+	+	+	-	-
Sal 287	708/709/710/711/712	AER 709	D	Babyleaf spinach	1	+	+	-	+	2,069/1,818	81,11	+	+		+	+	+	-	-
Sal 288	698/699/700	AER 698	D	Babyleaf mix	1	+	+	-	+	2,128/2,047	76,36	+	+		+	+	+	-	-
Sal 289	703/704/705	AER 703	D	Babyleaf spinach	1	+	+	-	+	1,973/1,928	39,27	+	+		+	+	+	-	-
Sal 296	713/714/715/716/717	AER 715	A	Ruccula mix	1	+	+	-	+	1,905/1,731	31,06	+	+		-	+	-	-	-
Sal 297	706/707	AER 706	A	Ruccula mix	1	+	+	-	+	1,86/2,184	56,3	+	+		ND	ND	ND	-	-
Sal 298	701/702	AER 701	A	Ruccula mix	1	+	+	-	+	2,01/2,039	62,82	+	+	+	+	+	+	-	-
Sal 305	718	AER 718	D	Romano mix	1	+	+	+	+	2,08/2,118	34,76	+	+		-	+	+	-	-
Sal 307	719/720	AER 720	D	Romano mix	1	+	+	+	+	2,323/1,745	21,91	+	+		ND	ND	ND	-	-

Appendix B. Identification results of *Aeromonas* strains isolated from different lettuce products.

Sample	Seq. ID	Seq. length (bp)	Description	Max score=total score	E-value	ID (%)
AER001	22FE01	1101	*A. media*	1792	0	96
AER007	22FE00	935	*A. salmonicida*	1371	0	96
AER013	22FD99	775	*A. media*	1273	0	100
AER014	22FE04	775	*A. salmonicida subsp. pectinolytica*	1415	0	100
AER020	22FE03	732	*A. salmonicida subsp. pectinolytica*	1336	0	100
AER022	22FE02	1090	*Aeromonas sp.*	1927	0	98
AER031	22FE07	1115	*A. media*	1683	0	95
AER036	22FE06	1114	*A. media*	1572	0	94
AER042	22FE05	1089	*Aeromonas sp.*	1923	0	99
AER045	22FE10	721	*A. salmonicida*	1327	0	100
AER051	22FE09	1101	*A. salmonicida*	1779	0	100
AER057	22FE08	1084	*A. salmonicida*	1956	0	99
AER063	22FE13	1128	*A. salmonicida*	1831	0	99
AER069	22FE12	729	*A. media*	1258	0	98
AER090	22FE11	770	*R. aquatilis*	1413	0	99
AER173	22FE16	1119	*A. media*	1810	0	97
AER176	22FE15	631	*A. media*	1116	0	99
AER206	22FE14	1101	*A. media*	1892	0	98
AER214	22FE19	1092	*A. salmonicida*	1916	0	99
AER216	22FE18	1115	*A. media*	1777	0	96
AER221	22FE17	1094	*A. media*	1786	0	97
AER235	22FE22	1100	*A. media*	1786	0	97
AER240	22FE21	1093	*A. media*	1892	0	98
AER247	22FE20	1147	*Enterobacter cloacae*	1679	0	94
AER269	22FE25	1088	*A. media*	1825	0	97
AER273	22FE24	775	*S. maltophilia*	1402	0	99
AER280	22FE23	715	*A. media*	1264	0	98
AER284	22FE28	1111	*A. media*	1759	0	96
AER288	22FE27	1119	*A. media*	1814	0	96
AER299	22FE26	773	*A. media*	1387	0	99
AER304	22FE31	1114	*A. media*	1700	0	97
AER309	22FE30	1079	*A. media*	1912	0	99
AER315	22FE29	1115	*A. hydrophila*	1792	0	96
AER319	22FE34	1135	*Aeromonas sp.*	1648	0	94
AER320	22FE33	1145	*Aeromonas sp.*	1677	0	96
AER322	22FE32	1082	*A. media*	1892	0	97
AER331	22FE37	1092	*A. media*	1864	0	98

Appendix B. Identification results of *Aeromonas* strains isolated from different lettuce products.

AER347	22FE36	1131	*A. salmonicida*	1796	0	96
AER358	22FE35	1132	*A. media*	1687	0	95
AER359	22FE40	1124	*A. salmonicida*	1810	0	97
AER628	22FE39	1095	*A. salmonicida*	1929	0	99
CCUG 14551	22FE38	1086	*A. hydrophila*	1969	0	99
AER634	22FE43	675	*A. media*	1177	0	98
AER643	22FE42	732	*A. media*	1291	0	98
AER648	22FE41	1089	*A. media*	1905	0	98
AER655	22FE46	1114	*A. media*	1788	0	98
AER660	22FE45	1074	*A. media*	1836	0	99
AER667	22FE44	1115	*A. media*	1845	0	97
AER671	22FE49	1076	*A. media*	1845	0	98
AER675	22FE48	1084	*Aeromonas sp.*	1921	0	100
AER677	22FE47	503	*A. media*	913	0	99
AER678	22FE52	1113	*A. media*	1855	0	97
AER679	22FE51	715	*A. media*	1308	0	100
AER680	22FE50	1100	*Aeromonas sp.*	1308	0	98
AER683	22FE55	1108	*A. media*	1812	0	98
AER684	22FE54	1100	*A. media*	1866	0	99
AER687	22FE53	1093	*Aeromonas sp.*	1901	0	99
AER689	22FE58	1096	*A. media*	1746	0	96
AER691	22FE57	1080	*A. media*	1820	0	98
AER693	22FE56	755	*Aeromonas sp.*	1362	0	99
AER695	22FE61	1100	*A. media*	1857	0	97
AER696	22FE60	1122	*A. media*	1805	0	97
AER698	22FE59	1139	*A. media*	1578	0	94
AER701	22FE64	1177	*Aeromonas sp.*	1478	0	98
AER703	22FE63	1099	*A. media*	1903	0	98
AER706	22FE62	0	No sequence		0	
AER709	22FE67	705	*A. media*	1236	0	98
AER715	22FE66	1104	*A. hydrophila*	1832	0	97
AER718	22FE65	764	*A. salmonicida*	1376	0	100
AER720	22FE70	0	No sequence		0	

Appendix C. Growth characteristics of selected isolates from the four producers in lettuce juice.

Observed growth for AER679 in lettuce and crispi juice, presented by absorbance values measured at 600nm. The lowercase superscript letters show which juice the value belongs to; iceberg (i) and crispi (c).

	Hours					
	0	1	3	5	8	12
0.5 %	0,30^{i}/0,31^{c}	0,32^{i}/0,32^{c}	0,32^{i}/0,32^{c}	0,32^{i}/0,33^{c}	0,32^{i}/0,33^{c}	0,31^{i}/0,34^{c}
1 %	0,35^{i}/0,35^{c}	0,34^{i}/0,35^{c}	0,34^{i}/0,36^{c}	0,34^{i}/0,36^{c}	0,34^{i}/0,34^{c}	0,33^{i}/0,31^{c}
2 %	0,28^{i}/0,28^{c}	0,27^{i}/0,28^{c}	0,26^{i} /0,28^{c}	0,26^{i}/0,28^{c}	0,25^{i}/0,28^{c}	0,25^{i}/0,28^{c}
5 %	0,25^{i} /0,14^{c}	0,22^{i}/0,29^{c}	0,34^{i}/0,38^{c}	0,35^{i} /0,39^{c}	0,34^{i} /0,38^{c}	0,34^{i}/0,39^{c}
Control	0,13	0,27	0,28	0,27	0,27	0,27

Observed growth for AER628 in lettuce and crispi juice, presented by absorbance values measured at 600nm. The lowercase superscript letters show which juice the value belongs to; iceberg (i) and crispi (c).

	Hours					
	0	1	3	5	8	12
0.5 %	0,18^{i} /0,17^{c}	0,16^{i} /0,17^{c}	0,18^{i} /0,18^{c}	0,17^{i} /0,19^{c}	0,19^{i} /0,18^{c}	0,22^{i} /0,17^{c}
1 %	0,19^{i} /0,19^{c}	0,18^{i} /0,18^{c}	0,18^{i} /0,19^{c}	0,19^{i} /0,20^{c}	0,20^{i} /0,19^{c}	0,24^{i} /0,20^{c}
2 %	0,24^{i} /0,25^{c}	0,23^{i} /0,25^{c}	0,28^{i} /0,32^{c}	0,32^{i} /0,37^{c}	0,32^{i} /0,37^{c}	0,34^{i} /0,37^{c}
5 %	0,26^{i} /0,26^{c}	0,23^{i} /0,26^{c}	0,21^{i} /0,26^{c}	0,20^{i} /0,24^{c}	0,19^{i} /0,22^{c}	0,22^{i} /0,25^{c}
Control	0,24	0,23	0,28	0,27	0,30	0,31

Appendix C. Growth characteristics of selected isolates from the four producers in lettuce juice.

Observed growth for AER042 in lettuce and crispi juice, presented by absorbance values measured at 600nm. The lowercase superscript letters show which juice the value belongs to; iceberg (i) and crispi (c).

	Hours					
	0	1	3	5	8	12
0.5 %	0,21^{i} /0,23^{c}	0,20^{i} /0,22^{c}	0,21^{i} /0,21^{c}	0,18^{i} /0,21^{c}	0,20^{i} /0,21^{c}	0,20^{i} /0,22^{c}
1 %	0,28^{i} /0,24^{c}	0,23^{i} /0,23^{c}	0,22^{i} /0,23^{c}	0,22^{i} /0,23^{c}	0,21^{i} /0,23^{c}	0,17^{i} /0,23^{c}
2 %	0,19^{i} /0,20^{c}	0,19^{i} /0,20^{c}	0,18^{i} /0,19^{c}	0,17^{i} /0,20^{c}	0,17^{i} /0,19^{c}	0,16^{i} /0,19^{c}
5 %	0,19^{i} /0,20^{c}	0,17^{i} /0,20^{c}	0,14^{i} /0,16^{c}	0,15^{i} /0,17^{c}	0,15^{i} /0,20^{c}	0,21^{i} /0,25^{c}
Control	0,21	0,20	0,18	0,18	0,18	0,18

Observed growth for AER703 in lettuce and crispi juice, presented by absorbance values measured at 600nm. The lowercase superscript letters show which juice the value belongs to; iceberg (i) and crispi (c).

	Hours					
	0	1	3	5	8	12
0.5 %	0,20^{i} /0,20^{c}	0,20^{i} /0,22^{c}	0,21^{i} /0,27^{c}	0,25^{i} /0,25^{c}	0,22^{i} /0,26^{c}	0,24^{i} /0,27^{c}
1 %	0,28^{i} /0,29^{c}	0,28^{i} /0,29^{c}	0,27^{i} /0,29^{c}	0,27^{i} /0,28^{c}	0,26^{i} /0,28^{c}	0,26^{i} /0,27^{c}
2 %	0,13^{i} /0,13^{c}	0,13^{i} /0,13^{c}	0,12^{i} /0,13^{c}	0,11^{i} /0,13^{c}	0,12^{i} /0,13^{c}	0,11^{i} /0,13^{c}
5 %	0,22^{i} /0,27^{c}	0,21^{i} /0,27^{c}	0,24^{i} /0,27^{c}	0,24^{i} /0,25^{c}	0,24^{i} /0,29^{c}	0,25^{i} /0,30^{c}
Control	0,13	0,13	0,13	0,12	0,12	0,13