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Phenotypic and genotypic characteristics of bacteria of genus *Arcanobacterium* with the emphasis on *Arcanobacterium phocae* isolated from epidemic necrotic pyoderma of fur animals

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eingereicht von

Mazen Alssahen
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"If you want to live a happy life, tie it to a goal, not to people or things."

Albert Einstein

This work is dedicated
to my
beloved parents and family

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Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

Mazen Alssahen

Abbreviations

A.	*Arcanobacterium*
A.	*Actinomyces*
Bp	Base pair
CAMP-Test	Christie-Atkins-Munch-Peterson-Test
CDC	Cholesterol-Dependent Cytolysin
CO2	Carbon dioxide
Da	Dalton, unit of molecular mass
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Microorganismen und Zellkulturen
et al.	et alii (and others)
fig.	Figure
FT-IR Spectroscopy	Fourier Transform Infrared Spectroscopy
g	Gram
gap	Glyceraldehyde 3-phosphate dehydrogenase encoding gene
h	Hour
ILY	Intermedilysin from *Streptococcus intermedius*
ily	*Streptococcus intermedius* intermedilysin encoding gene
ISR	16S-23S rDNA intergenic spacer region
kDa	Kilodalton
LAMP	Loop Mediated Isothermal Amplification Assay
LLO	Listeriolysin O from *Listeria monocytogenes*
llo	*Listeria monocytogenes* listeriolysin encoding gene
LPS	Lipopolysaccharides
mA	Milliampere
MALDI-TOF MS	Matrix-Assisted Laser Desorption Time of Flight Mass Spectrometry
Mg2+	Magnesium, ion
min	Minute
mm	Millimeter
MS	Mass Spectrometry
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
nm	Nanometer

PCR	Polymerase Chain Reaction
PHL	Phocaelysin from *Arcanobacterium phocae*
phl	*Arcanobacterium phocae* phocaelysin encoding gene
PLO	Pyolysin from *Trueperella pyogenes*
plo	*Trueperella pyogenes* pyolysin encoding gene
PLA	Pluranimaliumlysin from *Arcanobacterium pluranimalium*
pla	*Arcanobacterium pluranimalium* pluranimaliumlysin encoding gene
PLY	Pneumolysin from *Streptococcus pneumoniae*
ply	*Streptococcus pneumoniae pneumolysin encoding gene*
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpoB	Beta subunit of the RNA polymerase encoding gene
s	Second
SLO	Streptolysin O from *Streptococcus pyogenes*
slo	*Streptococcus pyogenes* streptolysin encoding gene
T.	*Trueperella*
Tab.	Table
tuf	Elongation factor tu encoding gene
U	Unit (international)
UV	Ultraviolet
Vol.	Volume
wgSNP	Whole genome single nucleotide polymorphism
°C	Degree Celsius
µl	Microliter
µm	Micrometer

Table of Content

Chapter 1- Introduction

Bacteria of genus *Arcanobacterium* are gram-positive, facultative anaerobic, asporogenous bacteria which can be found in a wide range of hosts and can be associated with diseases in animals and humans (Collins et al., 1982, Salam et al., 2020).

The genus is comprised of eleven classified species listed in the NCBI Taxonomy Browser and the German Collection of Microorganisms and Cell Culture.

(https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=28263),

(https://lpsn.dsmz.de/search?word=Arcanobacterium).

Arcanobacterium phocae (*A.*) was first recovered in 1997 from various tissues and fluids in common seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) of the coastal waters around Scotland, UK (Ramos et al., 1997). Later, Johnson et al. (2003) recovered *A. phocae* from sites of inflammation and from tissue samples taken during postmortem examination of various marine mammals. In further studies *A. phocae* has been suggested to be the aetiological agent of an emerging skin disease of fur animals named fur animal epidemic necrotic pyoderma (FENP) (Nordgren et al., 2014). These authors presumed a species shift of *A. phocae* from marine mammals to fur animals.

The aim of the present study was to identify and further characterize bacteria of genus *Arcanobacterium* which were isolated from necrotic pyoderma of fur animals using traditional and molecular methods. These bacteria were investigated phenotypically, by MALDI-TOF MS and FT-IR analyses and genotypically by sequencing various species-specific targets and potential virulence factor encoding genes and by whole genome sequencing.

Chapter 2- Review of literature

2.1 - Genus *Arcanobacterium*

2.1.1 - Taxonomic position

Bacteria of genus *Arcanobacterium* (*A.*) belong to order *Actinomycetales* and to the family *Arcanobacteriaceae* (Salam et al., 2020). The Latin name of genus *Arcanobacterium* is derived from the adjective "arcanus"- meaning secretive or mysterious and the word "bacterion" a small rod. The present classification of genus *Arcanobacterium* obtained from the taxonomy browser of National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=28263) and the prokaryotic nomenclature of the German Collection of Microorganisms and Cell Culture (https://lpsn.dsmz.de/search?word=Arcanobacterium) is shown in Tab. 1.

Genus *Arcanobacterium* was first described by Collins et al. in 1982. *Arcanobacterium haemolyticum,* previously known as *Corynebacterium haemolyticum,* was the first species classified to genus *Arcanobacterium*. This was based on the cell wall fatty acids and the peptidoglycan components of the bacteria which differ from species of genus *Corynebacterium.* This bacterium was originally cultivated from wound infections of American soldiers (MacLean et al., 1946).

In 1997, Ramos et al. classified the species *Arcanobacterium pyogenes*, *Arcanobacterium bernardiae* and *Arcanobacterium phocae,* which were isolated from various infections in animals and humans, blood and abscesses of human origin and from numerous tissues and body fluids of marine origin, respectively into this genus. Later, *Arcanobacterium pluranimalium* isolated from a porpoise and a deer (Lawson et al., 2001), *Arcanobacterium hippocoleae* isolated from vagina of a horse (Hoyles et al., 2002), *Arcanobacterium bialowiezense* and *Arcanobacterium bonasi,* which were recovered from prepuce of European bison bulls (Lehnen et al., 2006), and *Arcanobacterium abortisuis,* which was isolated from a placenta of a sow having miscarriage (Azuma et al., 2009), were assigned to this genus.

In further studies, according to a proposal of Yassin et al., 2011, genus *Arcanobacterium* was reclassified and included *Arcanobacterium haemolyticum*, *Arcanobacterium phocae*, *Arcanobacterium pluranimalium* and *Arcanobacterium hippocoleae. Arcanobacterium pyogenes*, *Arcanobacterium bialowiezense*, *Arcanobacterium bonasi* and *Arcanobacterium*

abortisuis were incorporated into the newly described genus *Trueperella* (*T*.) as *Trueperella pyogenes, Trueperella bialowiezensis, Trueperella bonasi and Trueperella abortisuis* (Yassin et al., 2011).

Furthermore, in 2012d, Hijazin et al. characterized *Arcanobacterium canis,* which was isolated from otitis externa of a dog, as a novel species of genus *Arcanobacterium*. Later, in 2013, Hijazin et al. described a new species of genus *Arcanobacterium,* which was recovered from specimen of a harbour seal and named *A. phocisimile*. In addition, *Arcanobacterium pinnipediorum,* which was also isolated from a harbour seal, has been characterized as a novel species of this genus (Sammra et al. 2015).

In 2017, Diop et al. described strain Marseille-P3428 belonging to a novel species of genus *Arcanobacterium*. This strain was isolated from a urine sample of a 54-day-old girl with rotavirus gastroenteritis and named *Arcanobacterium urinimassiliense*. However, in 2017 and 2020, Sammra et al. reported about two novel species of this genus which were named *Arcanobacterium wilhelmae* and *Arcanobacterium bovis*. These bacteria were isolated from vaginal discharge of a rhinoceros and from the milk of a cow with mastitis, respectively.

Furthermore, in 2019, Fall et al. identified *Arcanobacterium ihumii,* which was recovered from a vaginal swab of a woman living in Dielmo, a West African village, as a novel species of genus *Arcanobacterium*.

Table 1: Current classification of the family *Arcanobacteriaceae*, genus *Arcanobacterium* referring to the National Center for Biotechnology Information and the German Collection of Microorganisms and Cell Culture

Phylum	*Actinobacteria*
Class	*Actinobacteria*
Order	*Actinomycetales*
Family	*Arcanobacteriaceae*
Genus	*Arcanobacterium*
Species	*Arcanobacterium haemolyticum* (validly species)
Species	*Arcanobacterium hippocoleae* (validly species)
Species	*Arcanobacterium phocae* (validly species)
Species	*Arcanobacterium pluranimalium* (validly species)
Species	*Arcanobacterium canis* (validly species)
Species	*Arcanobacterium phocisimile* (validly species)
Species	*Arcanobacterium pinnipediorum* (validly species)
Species	*Arcanobacterium urinimassiliense* (not validly species)
Species	*Arcanobacterium wilhelmae* (validly species)
Species	*Arcanobacterium ihumii* (not validly species)
Species	*Arcanobacterium bovis* (validly species)

2.1.2 - Species of genus *Arcanobacterium*

2.1.2.1 - *A. phocae*

The first description of *A. phocae*, which was isolated from the peritoneal fluid of a common seal, was given by Ramos et al. in 1997. The species name is derived from ´*Phoca vitulina*´ the Latin word for ´common seal´. According to the studies of Ramos et al., 1997 six *A. phocae* strains were isolated from various tissues and fluids including lung, spleen, intestine, lymph nodes, mouth lesions, nasal swabs and peritoneal fluid of common seals and grey seals from coastal waters around Scotland. In a further study, 141 *A. phocae* strains were recovered from marine mammals that stranded along the central California coast (USA). This bacterium was cultured from tissue sites with abnormal discharge or evidence of inflammation in 66 California sea lions (*Zalophus californianus*), 50 Pacific harbour seals (*Phoca vitulina richardii*), 19 northern elephant seals (*Mirounga angustirostris*), 5 southern sea otters (*Enhydra lutris nereis*) and one common dolphin (*Delphinus delphis*) (Johnson et al., 2003).
In 2010, Giovannini described *A. phocae* strains, which were isolated from mixed infections of two California sea lions stranded along the southern cost of California. These strains were recovered from pleural fluid of one sea lion and from a wound from the second case.
A post mortem investigation on various soft tissue specimens of harbour seals from the North Sea of Schleswig-Holstein, Germany, collected between 1996 and 2014, reported about the isolation of *A. phocae* together with *Staphylococcus delphini*, *Bordetella bronchiseptica*, *Brucella* spp., *Clostridium perfringens*, *Escherichia coli*, *Erysipelothrix rhusiopathiae*, ß-hemolytic streptococci and *Staphylococcus aureus* (Siebert et al., 2017).
In 2007 multiple cases of pyoderma affecting fur animals spread across a wide region of Scandinavia, causing serious economic losses to fur industry. Nordgren et al. reported in 2014, during a retrospective case-control analysis, about the first isolation of *A. phocae* in fur animals. The primary fur animal species mentioned in this study were the captive mink (*Neovison vison*), blue foxes (*Vulpes lagopus*) and raccoon dogs (*Nyctereutes procyonoides*). *A. phocae* was isolated from the diseased animals together with *Staphylococcus* and *Streptococcus* species.
In addition, Chalmers et al. 2015 described that *A. phocae* has been involved, together with *Staphylococcus delphini* and *Streptococcus canis*, in the pathogenesis of cases of

pododermatitis of the Canadian mink (*Neovison vison*). Pododermatitis of farmed minks appeared as acute, severe ulceration of footpads with occasional further infection of nail beds. This could lead to a chronic hyperkeratotic purulent plantar dermatitis. A link between feeding of seal meat and the emergence of pododermatitis in minks was proposed (Chalmers et al., 2015).

In further studies, *A. phocae* was also characterized as main causative factor of a pyoderma of finnish fur animals named fur animal epidemic necrotic pyoderma (FENP) (Nordgren et al., 2016a). In a second study by experimental infection of mink, the typical signs and gross- and histopathological findings for FENP were detected when naive minks were infected with the tissue extract of minks with FENP using a subcutaneous or intradermal but not a peroral infection route (Nordgren et al., 2016b).

In 2017, Molenaar et al. described the isolation of *A. phocae* in minks in three cases of severe postvaccination wounds at the injection site. The minks were vaccinated with a killed vaccine Biocom P (United Vaccines Inc.; Madison, WI, USA). Furthermore, in 2017, Nonnemann et al. characterized *A. phocae* strains, which were isolated in Denmark from 15 adult and eight juvenile minks (*Neovison vison*), which suffered from dermatitis and from seven harbour seals (*Phoca vitulina*), two grey seals (*Halichoerus grypus*) and two otters (*Lutra lutra*), which were either found dead or had been euthanized due to animal welfare reasons. In addition, the results of a questionnaire suvery of detrimental effects on FENP in Finnland reported that FENP was introduced to Finland and spread further within the country via purchased fur animals and that risk factors of spread of FENP were type and size of the farm and contact with wildlife (Nordgren et al., 2017).

In 1997, Ramos et al. characterized *A. phocae* as facultative anaerobic, Gram-positive, non-motile, asporogenous, non-acid fast, short, rod-shaped bacterium which becomes fully mature after incubation on blood agar for 24 to 48 h at 37 °C. Furthermore, these authors and later in 2003, Johnson et al. described *A. phocae* to be ß-hemolytic on sheep blood agar. In addition, in 2009, Ülbegi-Mohyla et al. reported that *A. phocae* showed a relatively broad zone of hemolysis on sheep or rabbit blood agar. *A. phocae* additionally displayed a reverse CAMP-reaction within the zone of staphyloccal ß-hemolysin and a positive CAMP-like reaction with *Rhodococcus equi*, *Streptococcus agalactiae* and *Psychrobacter phenylpyruvicus* as indicator strains (Johnson et al., 2003; Ülbegi-Mohyla et al., 2009).

A. phocae has been investigated phenotypically (Ramos et al., 1997; Johnson et al., 2003; Ülbegi-Mohyla et al., 2009; Ülbegi, 2010), by proteomic mass spectrometry using MALDI-TOF MS analysis (Hijazin et al., 2012b; Wickhorst et al., 2016) and by Fourier transform infrared spectrometry (FT-IR) (Nagib et al., 2014), and genotypically by sequencing the 16S rRNA gene (Ramos et al., 1997; Johnson et al., 2003), the 16S-23S rDNA intergenic spacer region (ISR) (Hassan et al., 2008; Chalmers et al., 2015), the ß subunit of bacterial RNA polymerase encoding gene *rpoB* (Ülbegi-Mohyla et al., 2010), heat shock protein or chaperonin CPN60 encoding gene *cpn60* (Hijazin, 2012c), elongation factor tu encoding gene *tuf* and glyceraldehyde 3-phosphate dehydrogenase encoding gene *gap,* respectively (Wickhorst et al., 2016).

Gene *phl* encoding phocaelysin, a haemolysin of *A. phocae,* had been originally described by Ülbegi (2010). Gene sequences of *phl* of *A. phocae* DSM 10002^T and *A. phocae* DSM 10003 were submitted to NCBI GenBank under the accession numbers FN999907 and FN999908, respectively.

The complete genome sequence of *A. phocae* DSM 10002^T has been submitted to NCBI GenBank by Varghese and Submissions (2016) under accession number LT629804.

2.1.2.2 - *A. phocisimile*

The first description of the species *A. phocisimile*, which was isolated from vaginal swabs from two common seals (*Phoca vitulina*) in the German North Sea, was based on physiological and biochemical studies, chemotaxonomic analyses and by sequencing the 16S rRNA gene (Hijazin et al., 2013). Later, Sammra et al. (2014a) identified three additional *A. phocisimile* strains which were isolated from common seals and further characterized these strains and previously described type strain, using MALDI-TOF MS and genotypically by sequencing the 16S rRNA gene, ISR and the genes *rpoB* and *gap*. These bacteria were isolated together with other bacterial species. However, the pathogenic significance of the *A. phocisimile* strains remained unclear.

2.1.2.3 - *A. pinnipediorum*

In 2015, Sammra et al. described *A. pinnipediorum* as a novel species of genus *Arcanobacterium*. The type strain of this species was originally isolated in 2004 from an anal swab of a living male harbour seal of the German North Sea. The description was based on physiological and biochemical properties as well as on chemotaxonomic analyses and by sequencing the 16S rRNA gene. However, the pathogenic significance of *A. pinnipediorum* remained unclear. Later, the previously described type strain *A. pinnipediorum* was further characterized by MALDI-TOF MS and FT-IR analyses and by sequencing the genomic target ISR and the genes *rpoB*, *gap* and *tuf* (Sammra et al., 2018).

2.1.2.4 - *A. haemolyticum*

A. haemolyticum was first described in 1946 by MacLean et al. These authors recovered *A. haemolyticum* from wound infections of American soldiers. However, this bacterium was initially associated to genus *Corynebacterium* as *Corynebacterium haemolyticum*, together with *Corynebacterium pyogenes* and *Corynebacterium ovis* (MacLean et al., 1946). In further studies, Collins et al. (1982) classified *C. haemolyticum* to genus *Arcanobacterium* as *A. haemolyticum*, the first member of this novel genus.

A. haemolyticum has been described as a facultative anaerobic, Gram-positive, asporogenous, non-motile and non-acid fast, catalase-negative bacterium. The young colonies measured around 0,75 mm in diameter after 24 h of cultivation on blood agar under an optimal temperature of 37 °C, and developed to approximately 1.5-2.5 mm after 48 h, forming a complete zone of hemolysis on sheep blood agar (Collins et al., 1982; Lämmler and Hartwigk, 1995).

A. haemolyticum is worldwide known as a human pathogen, which is associated with pharyngitis and soft tissue infections (Banck and Nyman, 1986; Esteban et al. 1994; Alos et al. 1995; Goyal et al. 2005).

The infections caused by *A. haemolyticum* were similar to those caused by *Streptococcus pyogenes* and mainly affected adolescents and young adults (Banck and Nyman 1986; Miller et al. 1986; Balikci et al. 2011). *A. haemolyticum* was less likely to be responsible for invasive diseases, which mostly affected older patients with impaired immune systems (Brown et al.

2013). Systemic and chronic infections with *A. haemolyticum* had been described by Skov et al, (1998), Tan et al. (2006) and Therriault et al. (2008). In 2012. Garcia-de-la-Fuente et al. described 56 *A. haemolyticum* isolates from 51 northern Spanish patients with infections of the respiratory system. Furthermore, Sammra et al. (2014c) characterized six isolates from patients of two Danish hospitals phenotypically and genotypically.

The presence of *A. haemolyticum* in animal infections appears to be rare. *A. haemolyticum* had been isolated and described from bovine sperm (Richardson and Smith, 1968) and from pneumonia of a sheep (Roberts, 1969). In 2002, Tyrrell et al. found a strain of *A. haemolyticum* in a rabbit with a tooth root infection. In the same year 2002, Suvajdžić et al. characterized *A. haemolyticum* isolates from the lung of piglets. Seven *A. haemolyticum* isolates which were recovered from six horses with various infections, were described by Hassan et al. (2009). Bancroft-Hunt et al. characterized in 2010 an *A. haemolyticum* strain isolated as a pure culture from a necrotic fasciitis of a bull; in this study, the causal relationships between infection and illness could be established. The characterization of an *A. haemolyticum* isolated from a wound after castration of a horse was published in 2010 by Hijazin et al. In a further study Hijazin et al. (2012b) were able to identify 10 *A. haemolyticum* strains, including *A. haemolyticum* isolated from horses, using MALDI-TOF MS analysis. However, the type strain *A. haemolyticum* DSM 20595^{T} could be successfully identified by FT-IR analysis (Nagib et al., 2014). The first detection of *A. haemolyticum* in a wildlife host was in 2011 by Wragg et al. These authors investigated an *A. haemolyticum* strain, which was isolated from postmortem findings of a female European badger (*Meles meles*). In a further study, Sammra et al. (2014b) described an *A. haemolyticum* strain isolated from a purulent nasal discharge of a donkey.

The complete genome sequence of *A. haemolyticum* DSM 20595^{T} has been submitted to NCBI GenBank by Yasawong et al. (2010) under accession number CP002045.

2.1.2.5 - *A. hippocoleae*

The name of *A. hippocoleae* was derived from the Latin word ´´hippos´´ - horse and the word ´´colea´´- vagina, indicating the isolation of this species from the vagina of a horse. The first characterization of *A. hippocoleae* was in 2002 by Hoyles et al., who isolated *A. hippocoleae* together with *Corynebacterium* spp. and coagulase-negative staphylococci from the vaginal

discharge of a mare suffering from vaginitis. These authors described *A. hippocoleae* as facultatively anaerobic, non-acid fast, non-motile, non-sporulating, rod-shaped, Gram-positive bacterium. The sequence of the 16S rRNA gene of *A. hippocoleae* shared 94.8 % to 95.7 % sequence similarity to formerly described species of genus *Arcanobacterium* (Hoyles et al., 2002).

In 2003, Cai et al. described *A. hippocoleae,* which was isolated in pure culture from the urine of a horse. Furthermore, in 2008, Bemis et al. reported about the isolation of *A. hippocoleae* from the abdominal and pulmonary content of an American Quarterhorse foal after a still birth and from the mare´s placenta with signs of placentitis. In 2017, *A. hippocoleae* was isolated from an uterus swab of an apparently healthy mare. This strain was characterized phenotypically by biochemical properties, MALDI-TOF MS analysis and genotypically by sequencing various molecular target genes (Wickhorst et al., 2017b). The latest report on *A. hippocoleae* was in 2019 by Pègnè et al. These authors isolated *A. hippocoleae* from the genetical tract of 15 apparently healthy mares. The mares came from 11 breeding farms located in the French region of Brittany. *A. hippocoleae* was characterized phenotypically by biochemical properties, by MALDI-TOF MS and genotypically by sequencing the 16S rRNA gene, which confirmed that all 15 strains belong to the species *A. hippocoleae*, but, because of differences of 16S rRNA gene sequences and some other properties, could be separated in two groups within one species.

According to a phenotypic study of genus *Arcanobacterium* by Ülbegi-Mohyla et al. (2009), *A. hippocoleae,* cultivated on rabbit blood agar in a candle jar under microaerobic conditions, displayed a slightly enhanced zone of hemolysis in comparison to cultivation on sheep blood agar and showed positive CAMP-like hemolytic reactions with ß-hemolytic *S. aureus* and *A. haemolyticum* as indicator strains.

The first characterization of *A. hippocoleae* using MALDI-TOF MS was performed by Hijazin et al. (2012b). In 2014, Nagib et al. identified the type strain *A. hippocoleae* DSM 15539^T by FT-IR analysis.

The sequence of 16S rRNA gene of *A. hippocoleae* DSM 15539^T was submitted to NCBI GenBank by Hoyles et al. (2002) under accession number AJ300767.

2.1.2.6 - *A. pluranimalium*

A. pluranimalium was first described in 2001 by Lawson et al. The name of this bacterium was derived from the word ´´pluris´´- many and the word ´´animalium´´ - animals that refers to the strain isolation from various animals. *A. pluranimalium* was recovered from the spleen of a dead harbour porpoise and lung of a dead fallow deer. These isolates showed a sequence similarity of 100 % of 16S rRNA gene among each other and shared a sequence similarity between 93.9 % and 96.5 % to four other species of genus *Arcanobacterium* with the highest similarity to *A. phocae* (96.5 %). These authors described *A. pluranimalium* as facultatively anaerobic, Gram-positive, asporogenous, non-motile, non-acid fast bacterium.

A. pluranimalium type strain DSM 13483^T, cultivated on rabbit blood agar in a candle jar under microaerobic conditions, showed an enhanced zone of hemolysis compared to that after cultivation on sheep blood agar. Furthermore, *A. pluranimalium* displayed positive CAMP-like reactions with ß-hemolytic *S. aureus*, *R. equi* and *A. haemolyticum* as indicator strains (Ülbegi-Mohyla et al., 2009). Later, in 2010, Ülbegi-Mohyla characterized an *A. pluranimalium* strain isolated in a mixed culture from a dog with pyoderma.

In 2011, Foster and Hunt described *A. pluranimalium* strains, which were isolated from abortion material, semen, abscesses and viscera of sheep and from a milk sample of a cow suffering from mastitis. The identification of *A. pluranimalium* using MALDI-TOF MS analysis was first described in 2012 (Hijazin et al., 2012b). Furthermore, the type strain *A. pluranimalium* DSM 13483^T could be successfully identified by FT-IR analysis (Nagib et al., 2014). In 2013, *A. pluranimalium* was recovered from mastitis of a Holstein-Friesian cow in Switzerland (Moser et al., 2013). Later, in 2014, Risse et al. characterized an *A. pluranimalium* strain, which was isolated from a juvenile Giraffe following necropsy. Furthermore, in the same year 2014, Balbutskaya et al. characterized *A. pluranimalium* strains, which were isolated from various animal origin, phenotypically by biochemical properties, by MALDI-TOF MS analysis and genotypically by sequencing, as novel target, pluranimaliumlysin encoding gene *pla*.

In 2015, Abdulmawjood et al. developed a loop-mediated isothermal amplification (LAMP) assay based on pluranimaliumlysin encoding gene *pla* to identify *A. pluranimalium* strains, which were isolated from various animal origin. Later, in 2016, Wickhorst et al. identified three *A. pluranimalium* strains, which were isolated from bovine milk samples, by phenotypic

properties, MALDI-TOF MS analysis and genotypically by sequencing various molecular targets and by the newly developed LAMP assay based on gene *pla*. Pluranimaliumlysin appeared to be a constantly expressed putative virulence factor of *A. pluranimalium*, which could also be used for identification of this bacterial species. In addition, Ningrum et al., (2017) characterized an *A. pluranimalium* strain, which was isolated from a muskox (*Ovibos moschatus*), phenotypically and genotypically.

The sequence of gene *pla* was submitted to NCBI GenBank under accession number HE653976. The sequence of 16S rRNA gene was submitted to NCBI GenBank under accession number AJ250959.

2.1.2.7 - *A. canis*

A. canis was described in 2012d by Hijazin et al. as a novel species of genus *Arcanobacterium.*

This bacterium was isolated from an otitis externa of a seven year old, female English Bulldog. The name of this species was derived from the word (can´is. L. gen. n. *canis* of a dog) (Hijazin et al., 2012d). *A. canis* was Gram-positive, oxidase negative, non-motile, and appeared as non-spore-forming rod; CAMP-like reactions occurred with *R. equi* as an indicator strain (Hijazin et al., 2012d). The type strain *A. canis* P6775^{T} was identified by sequencing the 16S rRNA gene which was most closely related to the type strain *A. haemolyticum* (97.2 % sequence similarity) and to *A. hippocoleae* (96.5 % sequence similarity) and *A. phocae* (96.4% sequence similarity) (Hijazin et al., 2012d). One year later, four *A. canis* strains, which were isolated from infection of three dogs and one cat were further characterized by phenotypical properties, by MALDI-TOF MS and genotypical by sequencing the genomic targets 16S rRNA gene, ISR, 23S rDNA, *rpoB*, *gap* and *tuf* (Sammra et al., 2013). The sequence of the 16S rRNA gene of type strain *A. canis* DSM 25104^{T} was submitted to NCBI GenBank under accession number FR846134.

2.1.2.8 - *A. wilhelmae*

A. wilhelmae was described in 2017 by Sammra et al. as novel species of genus *Arcanobacterium*. The type strain was isolated during routine microbiological diagnostic from

the vaginal discharge of a rhinoceros (*Rhinoceros unicornis*) from Stuttgart Zoo Wilhelma, Germany. The name of this species derived from where this strain was isolated (wil.hel´mae. N.L. gen. n. *wilhelmae*. Wilhelma zoo in Stuttgart, Germany), (Sammra et al., 2017). This bacterium was Gram-positive, oxidase negative, non-motile, a non-spore-forming rod and showed no CAMP-like reactions with any indicator strain (Sammra et al., 2017). The type strain *A. wilhelmae* 647^T (*A. wilhelmae* DSM 102162^T) was identified by sequencing the 16S rRNA gene and shared sequence similarities between 95.4 % and 98.8 % with other type strains of genus *Arcanobacterium* and between 94.2% and 95.0 % with type strains of genus *Trueperella* (Sammra et al., 2017).

Later, in 2018, Sammra et al. further characterized, the previously described type strain *A. wilhelmae* DSM 102162^T by MALDI-TOF MS and FT-IR analyses and genotypically by sequencing the genomic targets ISR and the genes *gap, tuf* and *rpoB*.

The sequence of the 16S rRNA gene of type strain *A. wilhelmae* DSM 102162^T was submitted to NCBI GenBank under accession number NR_157610.

2.1.2.9 - *A. bovis*

A. bovis was described in 2020 by Sammra et al. as novel species of genus *Arcanobacterium*. The type strain was isolated during routine microbiological diagnostic from a milk sample collected from the left forequarter of a 7 year old, pure Simmental beef cow at post-mortem in the north of Scotland (Sammra et al., 2020). These authors described *A. bovis* as Gram-positive, oxidase negative, catalase positive, non-motile and as a non-spore-forming rod; CAMP-like reactions were observed with the indicator strains *S. aureus* and *Rhodococcus hoagii* (Sammra et al., 2020).

The type strain *A. bovis* C605018/01/1^T (DSM 107286^T) shared a 16S rRNA gene sequence similarity of 99.76 % to type strain *A. pluranimalium* DSM 13483^T and sequence similarities between 94.3% and 96.7 % to other type strains of genus *Arcanobacterium* (Sammra et al., 2020). The genome sequence of type strain *A. bovis* DSM 107286^T was submitted to GenBank as a whole genome shotgun sequencing project under accession number NZ_SJDT00000000.

2.1.2.10 - *A. urinimassiliense* and *A. ihumii*

A. urinimassiliense was described in 2017 by Diop et al. as novel species of genus *Arcanobacterium*. The type strain was recovered from the urogenital tract of a nearly two-month old female infant suffering from rotavirus gastroenteritis. This bacterium was irregular, non-motile, rod shaped, Gram-positive, catalase and oxidase negative. It appeared on blood agar in small beige colonies with a mean diameter of 200 µm. Bacterial cells were around 400-600 nm long and 300-600 nm wide (Diop et al., 2017). This novel species derived its name from the Latin words ´´*urina*´´for urine and ´´*Massilia*´´for Marseille in France, referring to the source origin and place from which the strain had been isolated. The type strain Marsielle-P3248^T was identified by sequencing the 16S rRNA gene, which shared 94.7 % sequence similarity to the type strain *A. phocae* DSM 10002^T as closest relative (Diop et al., 2017). The sequence of the 16S rRNA gene of type strain Marsellie-P3248^T was submitted to GenBank under accession number LT598574. The genome sequence of type strain Marsellie-P3248^T was submitted to GenBank under accession number NZ_LT632435. At present no further data are available about additional properties of this novel species. The German Collection of Microorganisms and Cell Culture (DSMZ) classified *A. urinimassiliense* as not validly published species (https://lpsn.dsmz.de/genus/arcanobacterium).

A. ihumii was described in 2019 by Fall et al. as novel species of genus *Arcanobacterium*. The type strain was originally isolated from a vaginal swab of a woman living in Dielmo, Afrika (Fall et al., 2019). The colonies of the type strain Marsielle-P5647^T were 1 mm in diameter. Growth was observed under anaerobic conditions at 37 °C but not under aerobic and microaerophilic conditions. This bacterium was Gram-negative **[sic]**, non-motile, oxidase-negative and catalase negative (Fall et al., 2019). This novel species was named *A. ihumii* according to where it had been isolated (i.hum.i'i, N.L. gen. n. ihumii, based on the acronym IHUMI, the Institut Hospitalo-Universitaire Mèditerranèe infection) (Fall et al., 2019). The sequence of 16S rRNA gene and the genome sequence of this species were submitted to GenBank under the accession numbers LT993248 and NZ_UWOS01000001, respectively. At present no further data are available about properties of this novel species. The German Collection of Microorganisms and Cell Culture (DSMZ) classified *A. ihumii* as not validly published species (https://lpsn.dsmz.de/genus/arcanobacterium).

2.2 - Identification by phenotypic and genotypic properties

2.2.1 – Growth properties, morphology, hemolysis, CAMP-like reactions and biochemical properties

Bacteria of genus *Arcanobacterium* are Gram-positive, irregular rod-shaped bacteria. In 1994, Holt et al. described bacteria of genus *Arcanobacterium* as non-motile, non-acid fast, non-spore forming and with a size of 0.3-0.8 x 1.0-5.0 µm. Furthermore, the cell shape of these bacteria was not uniform. They were pleomorphic and showed rod- or coccoid-like shape, usually arranged in V- or T-shape (Hirsh and Biberstein, 2004; Moore et al., 2010; Yassin et al., 2011). Bacteria of this genus were described as facultatively anaerobic and their growth is enhanced in CO_2-enriched atmosphere after 24 to 48 h incubation on 5% sheep blood agar plates at 37 °C under microaerobic conditions in a candle jar. Growth of the bacteria of genus *Arcanobacterium* occurred on ordinary media but enhanced on blood or serum containing media. At the temperature of 60 °C these bacteria become inactive in 15 min (Collins et al., 1982).

Bacteria were phenotypically assessed based on colony morphology and post-incubation hemolysis on 5 % sheep or rabbit blood agar plates (Ülbegi-Mohyla et al., 2009). Synergistic and antagonistic forms of hemolysis, the CAMP-like tests, were originally described by Skalka et al. in 1979 and was used for differentiation of bacteria of genus *Arcanobacterium* and *Trueperella* by Ülbegi-Mohyla et al. (2009). According to Christie, Atkins and Munch-Petersen (1944), the CAMP test was originally based on a thermolabile substance that is formed by CAMP-positive group B streptococci in the simultaneous presence of staphylococcal ß-hemolysin. Various tests are available for biochemical characterization of bacteria of genus *Arcanobacterium*, also including the catalase reaction. Catalase reduces hydrogen peroxide, releases oxygen, which leads to the formation of bubbles; in the negative case there was a clouding of the solution without bubble formation (Brückler et al., 1994). The detection of serolysis was carried out using Löffler medium. Due to the proteolytic activity, *T. pyogenes,* as positive control, showed a ditch formation in the positive case in the area of bacterial growth (Hartwigk and Marcus, 1962). The “API Coryne test system” from bioMérieux allows the simultaneous detection of nitrate reduction, the enzymes

pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, β-glucuronidase, β-galactosidase, α-glucosidase, N-acetyl-β-glucosaminidase, detection of esculin cleavage (β-glucosidase) and detection of the enzymes urease and gelatinase as well as the carbohydrate fermentation reactions of D-glucose, D-ribose, D-xylose, D-mannitol, D-maltose, D-lactose, D-sucrose and glycogen. Results can be evaluated directly with special software.

2.2.2 - Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS)

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a simple, rapid, cost-effective and high-throughput tool for the identification of microorganisms. The first description of mass spectroscopy (MS) was in the late 19th Century by J.J Thomson (1897). In addition, Griffiths in 1997 calculated the ratio of mass/charge of electrons. The MS technique has been used for the ionization and analysis of large molecular masses ranging between 100 Da and 100 KDa (Karas and Hillenkamp, 1988). The first report of successful bacterial chemotaxonomy using MALDI-TOF MS analysis for rapid identification of intact whole bacteria was in 1996 by Holland et al. According to Keys et al. in 2004 MALDI-TOF MS analysis has become a regular criterion in routine microbiology with composition to entire database for the identification of bacteria.

MALDI-TOF MS analyses were used in classification of various microorganisms such as bacteria and archaea (Seng et al., 2009; La Scola and Raoult, 2009) and for identification of several Gram-negative and Gram-positive bacteria, such as *Salmonella* spp. (Leuschner et al., 2004), *Campylobacter* spp. (Mandrell et al., 2005), *Listeria* spp. (Barbuddhe et al., 2008), *Clostridium* spp. (Grosse-Herrenthey et al., 2008), *Corynebacterium* spp. (Konrad et al., 2010), the *Staphylococcus intermedius* group (Decristophoris et al., 2011), *Streptococcus* spp. (Hinse et al, 2011), *Trueperella* spp. and *Arcanobacterium* spp. (Hijazin et al., 2012 a,b; Sammra et al., 2013, 2014 a, b, c; Wickhorst et al., 2016, 2017, 2019), viruses (Downard et al., 2009; Schwahn et al., 2009), yeasts, fungi (Ferroni et al., 2010; Cassagne et al., 2016) and molds (Alanio et al., 2011) and more recently also to analyze the viral compounds of corona virus disease COVID-19 (Mahmud and Garrett, 2020).

However, several studies have shown the possibility to obtain peptide spectra from protein extracts (Cain et al., 1994; Krishnamurthy et al., 1996). The main protein mass spectra

resulting from the analysis of the entire bacterial cells were from ribosomal proteins (Suh et al., 2005; Murray, 2010). The other identifiable proteins besides the ribosomal protein in a typical whole-cell MS were carbon storage regulators, ribosome modulation factors, cold-shock proteins, DNA-binding proteins and RNA chaperones (Dieckmann et al., 2008, 2010).
MALDI-TOF MS analysis was described as a process with three phases. First, the microbial sample is mixed and embedded into a suitable matrix material and applied to a metal plate, However, the matrix consist of α-cyano-4-hydroxycinnamic acid with 50% acetonitrile and 2,5 % trifluoroacetic acid (Dridi et al., 2011). Second, the matrix is let to dry until forming crystals, then transferred into a mass spectrometer and a pulsed laser irradiates the sample causing desorption and ionization of the biomolecules. Finally, the ions are sped up by an electrostatic field and forced out through a vacuum flight-tube reaching a detector at the tube terminal. The ions are separated based on their mass to charge ratio (*m/z*) where lighter ions reach faster to the detector (Fig. 1) forming a mass spectrum (Croxatto et al., 2012; Cobo, 2013).

$$tof = \alpha \sqrt{\frac{m}{z}}$$

Fig. 1: Time of flight (*tof*) formula where; (α) stands for a constant factor, (*m*) for the mass and (*z*) for charge of molecules (reformed in consonance with http://de.wikipedia.org/wiki/MALDI-TOF).

2.2.3 - Fourier Transform-Infrared Spectrometry (FT-IR)

Fourier Transform-Infrared Spectrometry (FT-IR) is a simple, quick, cost-efficient, highly reproducible fingerprint-like pattern and phenotypic cellular-based taxonomical method for identification and subtyping of microorganisms at species and different subspecies level (Kuhm et al., 2009; Johler et al., 2016; Dinkelacker et al., 2018; Martak et al., 2019). FT-IR technique was developed for microbial analysis in 1991 by Helm et al. and Naumann et al., respectively. FT-IR is a technique for identification of various bacterial species, such as *Listeria* spp. (Holt et al., 1995), *Streptococcus* spp. (Goodacre et al., 1996), *Bacillus* spp. (Beattie et al., 1998; Lin et al., 1998) and for identification of bacteria causing urinary tract infections (Goodacre et al., 1998). Furthermore, in 2014, Nagib et al. identified *T. pyogenes*

strains isolated from bovine clinical mastitis using FT-IR analysis. In 2018, Sammra et al. used FT-IR analysis to identify and further characterize two type strains of genus *Arcanobacterium*, namely *A. pinnipediorum* and *A. wilhelmae*.

2.2.4 - 16S rRNA gene

The first identification and analyzation of ribosomes as actively pervasive units was performed in 1987 by Woese et al. The bacterial ribosome 70S, due to its sedimentation coefficient, can be divided into the two subunits 50S and 30S. The genes of ribonucleic acid in prokaryotes are systematically arranged together in the consequent order 16S-23S-5S to form an rRNA operon which acts as basic transcription and functional unit (Gürtler and Stanisich, 1996; Pisabarro et al., 1998; Roth et al., 1998; Klappenbach et al., 2000). In 1980, Ellwood and Nomura described *E. coli* as a standard model for 16S rRNA studies in prokaryotes constituting seven operons.

The 16S rRNA gene contains species specific regions and is large enough (about 1500 bp), for molecular identification to study relationships between different bacteria (Weisburg et al., 1991; Patel, 2001; Cai et al., 2003). The cut-off value of 16S rRNA gene at the species level was a sequence similarity equal or more than 98.7 % (Stackebrandt and Ebers, 2006), and for genera level a sequence similarity equal or more than 95% (Stackebrandt and Goebel, 1994). A comparison of 16S rRNA gene sequence to global molecular targets such as RNA polymerase subunits and heat shock protein revealed that the phylogenetic trees drawn from the sequences of these target genes demonstrate a remarkable similarity to that derived from 16S rDNA sequencing results (Goh et al., 1996; Ghebremedhin et al., 2008).

The nucleotide sequences could be submitted to the European Molecular Biology Laboratory (EMBL) (Cochrane et al., 2008; http://www.ebi.ac.uk), to DNA Data Bank of Japan (DDBJ) (Kaminuma et al., 2011; http://www.ddbj.nig.ac.jp) and to GenBank (Benson et al., 2011; http://www.ncbi.nml.nih.gov/genbank).

2.2.5 - 16S-23S rDNA intergenic spacer region (ISR)

The 16S-23S rDNA intergenic spacer region (ISR) is an extent of DNA which is located between the 16S and the 23S rRNA genes. The ISR has various different transfer RNA (tRNA) genes as functional units. Furthermore, ISR of some Gram-negative bacteria like *E. coli* contain $tRNA^{Ala}$ (alanine), $tRNA^{Ile}$ (Isoleucine) and $tRNA^{Glu}$ (Glutamine) (Condon et al., 1995). Sequencing of ISR is an useful tool for taxonomic studies of prokaryotes (Amann et al., 1995) and for characterization and differentiation of bacteria to the species level (Jensen et al., 1993; Wang et al., 1997; Zhang et al., 1997; García-Martínez et al., 1999). An universal ISR primer pair has been developed from conserved regions of the 16S and 23S ribosomal RNA genes, amplifying the ISR of different bacterial pathogens including *S. aureus*, *Enterococcus faecium*, *E. coli* and *Enterobacter* spp. (Kostman et al., 1995). This primer pair was also used to amplify and analyze the ISR of various bacterial species, such as species of genus *Streptococcus*, genus *Arcanobacterium* (Chanter et al., 1997; Hassan et al., 2008; Ülbegi, 2010, Sammra et al., 2018; Wickhorst et al., 2016) and genus *Trueperella* (Eisenberg et al., 2012; Hijazin et al., 2012a, e; Wickhorst et al., 2018; Ahmed et al., 2020).

2.2.6 - 23S rRNA gene

The 23S rRNA gene contains a larger number of sequence variations in comparison to the 16S rRNA gene and it measures in *E. coli* 2,904 bp (Lewin, 1998). The variable regions of 23S rRNA gene could be used for the design of species-specific primer pairs which allowed an identification of *Campylobacter* spp. (Eyers et al., 1993) and *Pasteurella multocida* (Miflin and Blackall, 2001). Highly specific universal primers, which could amplify 23S rRNA gene sequence from different bacterial species, was developed by Hunt et al., (2006). However, in 2009, Pei et al. studied the diversity of 23S rRNA genes within individual prokaryote genomes. Sequencing of 23S rRNA gene was also used to identify *Actinomyces weissii* (Hijazin et al., 2012c) and bacteria of genus *Arcanobacterium*, such as *A. haemolyticum* (Sammra et al., 2014b).

2.2.7 - RNA polymerase encoding gene *rpoB*

The RNA polymerase (RNAP) is an important enzyme in all living microorganisms and it is responsible for RNA transcription. Furthermore, it plays a key role in the gene expression process (Borukhov et al., 2003; Adèkambi et al., 2009). Also antimicrobial resistances appeared to be associated with the *rpoB* gene, such as the resistance against the antibiotic rifampicin, which is used in the treatment of *Mycobacterium tuberculosis* (Telenti et al., 1997; Fluit et al., 2001). The RNA polymerase beta-subunit encoding gene *rpoB* is a competent phylogenetic marker to rectify the limitations in the 16S rRNA gene arising from the existence of multiple operons within a single genome (Drancourt and Raoult, 2002; Da Mota et al., 2004; Walsh et al., 2004). Gene *rpoB* was used as a discriminative tool (Dahllöf et al., 2000; Case et al., 2007). The sequencing of *rpoB* gene enabled a robust and accurate molecular identification of bacteria (Rowland et al., 1993). Furthermore, these authors used *rpoB* gene to study the taxonomic relationship of *S. aureus*. However, gene *rpoB* was also used in molecular identification of aquatic and soil bacteria (Peixoto et al., 2002; Taylor et al., 2004). In addition, gene *rpoB* could be used in phylogenetic studies of Gram-positive and Gram-negative bacteria (Morse et al., 2002). In 2004, Khamis et al. described an universal oligonucletide primer pair for amplification of *rpoB*. However, in 2010, Ülbegi and Ülbegi-Mohyla et al. used gene *rpoB* to identify various species of genera *Arcanobacterium* and *Trueperella* and for identification of two *T. pyogenes* strains isolated from a bearded dragon and a gecko. In addition, in 2012, Deperrois-Lafarge and Meheut used *rpoB* gene to study bacterial pathogens in dairy products. Furthermore, due to hyper variable regions of gene *rpoB*, it was used in restriction fragment length polymorphism (PCR-RFLP) technique for the discrimination between *C. pseudotuberculosis* and *T. pyogenes* (Pavan et al., 2012). In addition, gene *rpoB* was used for further characterization of *A. canis*, which was isolated from otitis externa of a dog (Sammra et al., 2013), for *A. pluranimalium* isolated from milk samples and for *A. hippocoleae* isolated from an uterus of a healthy mare, respectively (Wickhorst et al., 2016; 2017b). In addition, in 2014, Sammra et al. used gene *rpoB* together with some other genomic targets for characterization of *A. phocisimile* strains, which were isolated from harbour seals. Furthermore, gene *rpoB* was used for genotypic analysis of *A. pluranimalium* isolated from a muskox (*Ovibos moschatus*) (Ningrum et al., 2017) and for genotypic

characterization of *A. pinnipediorum* and *A. wilhelmae*, which were isolated from a harbour seal and from the genital tract of a rhinoceros, respectively (Sammra et al., 2018).

2.2.8 - Glyceraldehyde 3-phosphate dehydrogenase encoding gene *gap*

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has a vital glycolytic role inside the cellular cytoplasm of eukaryotes, prokaryotes and archaea. This gene is a highly conservative 37 kDa tetrametric housekeeping protein, it acts as the sixth step catalyzer breaking down glucose for energy and carbon supply (Sirover, 1997).

GAPDH has an extensive moonlighting effect, relying on its structural heterogeneity and location within the living cell (Fillinger et al., 2000; Sirover, 2011). The glyceraldehyd 3-phosphate dehydrogenase encoding gene was used in several studies as useful tool for identification and genetic classification of *Staphylococcus* spp. (Yugeuros et al., 2000, 2001; Ghebremedhin et al. 2008) and for genotypic identification of *Lactobacillus* species, such as *Lactobacillus* (*L.*) *plantarum* and *Lactobacillus* (*L.*) *sakei* (Naterstad et al., 2007). More recently, sequencing of gene *gap* could successfully be used to identify and characterize bacteria of genera *Trueperella* and *Arcanobacterium*, such as *T. pyogenes*, which was isolated from a brain abscess of an adult roebuck (Wickhorst et al., 2018) and *A. hippocoleae*, which was recovered from an uterine swab of an apparently healthy mare (Wickhorst et al., 2017b). The sequence of gene *gap* could also be used for identification and characterization of *Actinomyces hyovaginalis* from a giraffe with suppurative arthritis (Wickhorst et al., 2017a). In 2014a, Sammra et al. used the target gene *gap* together with other target genes to confirm the species identity of an *A. haemolyticum* strain, which was isolated from a donkey. In the same year, sequencing gene *gap* was used to characterize *A. haemolyticum* strains, which were isolated from six patients of two hospitals in Denmark (Sammra et al. 2014c) and an *A. pluranimalium* strain, which was recovered from a juvenile Giraffe following necropsy (Risse et al., 2014). In addition, an *A. pluranimalium* strain, which was isolated from an euthanized muskox suffering from severe weakness, was characterized by sequencing gene *gap* and some other target genes (Ningrum et al. 2017). However, gene *gap* was also used to identify and further characterize of *A. canis*, *A. phocisimile*, *A. wilhelmae* and *A. pinnipediorum*, respectively (Sammra et al., 2013, 2014a, 2018).

2.2.9 - Elongation factor tu encoding gene *tuf*

Elongation factor tu, consisting of 393 amino acids, is one of the most highly conservative and abundant polypeptide in prokaryotes (Wittinghofer et al., 1983; Weijland et al., 1993).
Gene *tuf* has already been included in taxonomic studies for identification of *Lactobacillus* and *Bidifobacterium* species (Ventura et al., 2003) and species of genus *Yersinia* (Isabel et al., 2008). The design of *tuf* specific oligonucleotide primer by Wickhorst et al in 2016 allowed the partial amplification and sequencing of gene *tuf* for various bacteria of genus *Arcanobacterium* and *Trueperella*. This primer pair was also used to identify and characterize a *T. pyogenes* strain isolated from a brain abscess of an adult roebuck (Wickhorst et al., 2018), for identification of an *A. hippocoleae* strain isolated from a mare and for identification of an *A. hyovaginalis* strain recovered from an adult giraffe, respectively (Wickhorst et al., 2017 b, a).
In 2014b, Sammra et al. used the target gene *tuf* together with other target genes to confirm the species identity of an *A. haemolyticum* strain, which was isolated from a donkey and to characterize *A. haemolyticum* strains, which were isolated from six patients of two hospitals in Denmark (Sammra et al. 2014c).
In addition, sequencing of target gene *tuf* had been used together with other genomic targets to identify an *A. pluranimalium* strain, which was isolated from a juvenile Giraffe following necropsy (Risse et al., 2014) and an *A. pluranimalium* strain, which was isolated from a euthanized muskox (Ningrum et al. 2017). However, gene *tuf* was also used to further characterize *A. wilhelmae* and *A. pinnipediorum,* respectively (Sammra et al., 2018).

2.2.10 – Heat shock protein or chaperonin CPN60 encoding gene *cpn60*

Chaperones CPN60, GroEL or HSP60 are monomeric components of the bacterial chaperone system. They have a supporting function in the formation of the spatial structures of the proteins and protect the subunits from overheating during the assembly of complexes (Saibil, 2013).
Sequencing of gene *cpn60* from various bacterial species resulted in the design of degenerate primers, which could amplify a PCR product of a 540-567 bp region of this gene from various bacteria (Goh et al., 1996). A huge amount of data of *cpn60* sequences of prokaryotes and

eukaryotes and archaea could be found in the chaperonin database cpnDB (http://www.cpndb.ca/) (Hill et al., 2004). Goh et al. suggested in 1996 that the gene *cpn60* could be used as an alternative molecular target for species identification of *Staphylococcus* spp. (Goh et al., 1996). Sequencing of gene *cpn60* was also used as tool for phylogenetic studies of various bacteria, such as *Streptococcus* spp. (Alber et al., 2004), *Lactobacilus* spp. (Baliotta et al., 2008) and *Aeromonas* spp. (Miñana-Galbis et al., 2009). In 2012, Hijazin et al. designed a set of oligonucleotide primer using gene *cpn60* of *T. pyogenes* obtained from GenBank to amplify and sequence gene *cpn60* of various species of genera *Arcanobacterium* and *Trueperella*. Furthermore, in 2016, Abdulmawjood et al. used the sequence of gene *cpn60* of *T. pyogenes* for development of a loop-mediated isothermal amplification assay (LAMP) for molecular identification of *T. pyogenes* strains from various origins. Furthermore, the gene *cpn60* LAMP assay was used for identification of *T. pyogenes* strains isolated together with *Brucella abortus* from a cat and a dog with recurrent cases of mastitis and abortion on a dairy farm in Egypt (Wareth et al., 2018).

2.2.11 - Pore forming toxin encoding genes

Pore forming toxins are bacterial cytotoxic proteins, which perforate the plasma membrane of the host cell and the intracellular organelle membrane (Iacovache et al., 2010).
These toxins play an important role as putative virulence factors of various pathogens, such as *Streptococcus pneumoniae*, *S. aureus* and *E. coli* (Los et al., 2013).

2.2.11.1 - Arcanolysin encoding gene *aln*

In 2011, Jost et al. described arcanolysin (ALN) as a cholesterol-dependent cytolysin (CDC), which is a hemolysis-causing factor of *A. haemolyticum*. These authors successfully cloned the arcanolysin-encoding gene *aln*. The weight of ALN appeared to be 64 kDa and contains a signaling sequence, a putative PEST sequence and a variant undecapeptide at the domain 4 location, a considerable segment for the toxin functioning (Jost et al., 2011). Furthermore, ALN consist of 587 amino acids with 44 amino acids as protein-secretion signaling amino acids. However, the arcanolysin encoding gene *aln* measures 1,764 bp (Lucas 2009; Lucas et al., 2010).

2.2.11.2 - Pluranimaliumlysin encoding gene *pla*

The first description of pluranimaliumlysin encoding gene *pla* was in 2013 by Moser et al. These authors designed and developed PCR primers to identify and characterize an *A. pluranimalium* strain, which was isolated from bovine mastitis. Later, in 2014, Balbutskaya et al. used the sequence of gene *pla* for identification and characterization of *A. pluranimalium* strains, which were isolated from various animal origins. In the same year, an *A. pluranimalium* strain, which was isolated from a juvenile giraffe following necropsy, had been genotypically characterized by sequencing gene *pla* and other target genes (Risse et al., 2014). However, in 2015, a loop mediated isothermal (LAMP) assay based on gene *pla* was successfully used to identify 28 *A. pluranimalium* strains, which were isolated from various animal origins (Abdulmawjood et al., 2015). Furthermore, three *A. pluranimalium* strains, which were isolated from bovine milk samples, were genotypically identified and characterized by sequencing gene *pla* together with other target genes and by using a LAMP assay based on gene *pla* (Wickhorst et al., 2016).

2.2.11.3- Phocaelysin encoding gene *phl*

Phocaelysin encoding gene *phl* was first described by Ülbegi 2010. This author used the sequence analysis based on gene *phl* for identification and characterization of the two *A. phocae* reference strains, *A. phocae* DSM 10002^{T} and *A. phocae* DSM 10003 (accession number FN999907 and FN999908, respectively). In addition, this author designed specific oligonucleotide primer with the primer sequences *phl*-F 5`- CTT AGT TGA GGG AAA GAA CCG AAA GAC-3` and *phl*-R 5`- TCG AGT TCA CCA TGG ATT CGT CGT AAG-3` and *phl*2- F 5`- TAA TCT TCG TCG TGG CGA CTT GTC CGT CA-3` (Ülbegi 2010).

2.2.11.4 - Pyolysin encoding gene *plo*

Pyolysin is a primary virulence factor of *T. pyogenes* and a heat-labile and oxygen-stable protein which displayed cytotoxic effects on polymorphonuclear leucocytes and on pTK2 kidney cells (Ding and Lämmler, 1996; Funk et al., 1996). In 1997, Billington et al. described

pyolysin (PLO) as a novel member of the thiol-activated cytolysin family. These authors cloned the gene encoding PLO and presented data that PLO had 1.605 bp and encoded a protein of 57.9 kDa.

The importance of PLO in the pathogenesis process was confirmed by using specific antibodies, which neutralized the hemolysin activity of *T. pyogenes* after infection of mice, which survived from death (Billington et al., 1997).

However, PLO appeared to be produced by all *T. pyogenes* isolates investigated to date and its production seems to be limited with stationary phase cultures (Ding and Lämmler, 1996; Billington et al., 1997; Jost et al., 1999; Silva et al., 2008).

Sequence analysis of gene *plo* was used to identify and characterize *T. pyogenes* strains, which were isolated from various animals, such as livestock and companion animals (Risseti et al., 2017), an Eurasian lynx (Alssahen et al., 2020) and from an okapi and a royal python (Ahmed et al., 2020).

2.2.12 - Loop Mediated Isothermal Amplification (LAMP) Assay

The loop mediated isothermal amplification (LAMP) assay was developed in 2000 by Notomi et al. These authors described the LAMP assay as a novel method which amplifies the DNA under isothermal conditions using four primers with a high specificity, efficiency and rapidity. A LAMP assay requires a strand-displacing polymerase, usually Bst-polymerase and four or six primers that recognize a total of six or eight target sequences. The reaction is initialized by two internal primers that bind to the target sequence. Finally, a loop-shaped single strand is released by means of two further primers, which is the template for the actual DNA synthesis. In the subsequent LAMP cycle, the two inner primers hybridize to the product loops and initialize the actual cycle. Different size of polymers of the original DNA segments were formed. The reaction can be accelerated by two additional loop primers and continues with accumulation of 10^9 copies of the original target DNA in less than one hour (Nagamine et al., 2002). The end products can be presented in different ways, such as gel-electrophoresis with coloring by an intercalation agent such as ethidium bromide or gel red. The result can then be made visible under UV light (Notomi et al., 2000). Another detection option of the LAMP amplicons is the turbidity. During the LAMP amplification reaction the magnesium pyrophosphate is formed. This product has white turbidity, which can be detected with a real-

time turbidity meter or even by the naked eye after adding Mg2+ (Mori et al., 2001). For the detection of double-stranded DNA the dye SYBR Green I could be also used. After the amplification process is completed 1 µl SYBR Green I is added to the LAMP products (Parida et al., 2008). In case of a positive reaction the reaction mixture turns green, whereas in case of a negative result there is no color change and the reaction mixture remains orange (Njiru et al., 2008). Alternatively, similar to qPCR, the reactions could be followed directly by using real time fluorometer (Genie® II device) (Optigene, United Kingdom). This is a compact portable battery-operated device with two independent heating blocks. The detection of the target DNA takes place at an isothermal temperature by means of fluorescence measurement and the entire amplification process, which is usually completed within 30 minutes can be observed in real time on a screen.

LAMP assays were used for detection and identification of various microorganisms and parasites, such as *Mycobacterium tuberculosis* (George et al., 2011), *Trypanosoma rhodesiense* and *Trypanosoma gambiense* (Njiru et al., 2008), *A. pluranimalium* (Abdulmawjood et al., 2015; Wickhorst et al., 2016), *T. pyogenes* (Abdulmawjood et al., 2016; Wickhorst et al., 2018; Alssahen et al., 2020; Ahmed et al., 2020) and more recently to identify the novel corona virus SARS-CoV-2 (Park et al., 2020).

2.2.13 - Whole genome sequencing

In 1975, Sanger and Coulson described a rapid method for determining nucleotide sequences in single-stranded DNA by bacteriophage. However, determination of the complete DNA sequence of an organism`s genome at a single time was named a whole genome sequencing. The conventional genomic sequencing of *Haemophilus influenza* in 1995 by Sanger was the starting point of the genomic era (Fleischmann et al., 1995). However, during the Human Genome Project (HGP) the shotgun sequencing approach was developed and here the large sizes of DNA were successfully sequenced based on the sequence overlapping concept (Zhang et al., 2011). Whole genome sequencing is a very useful research tool, but it had been also introduced to the clinics (Gilissen et al., 2014). Genome analyses of bacteria and archaea were also performed by shotgun sequencing (Fleischmann et al., 1995).

The methods and high-throughput sequencing technologies of whole genome had been described in 2009 by Mukhopadhyay, using technologies such as illumine dye sequencing,

pyrosequencing and SMRT sequencing. Furthermore, the properties of nanopore technology and its ability for generating long reads could be applied in whole genome sequencing (Kwong et al., 2015). A newly developed technology of genome sequencing, which is named third-generation sequencing is to produce substantially longer reads (Bleidorn, 2016). The process of whole genome analysis could be divided into three main phases: (1) amplification and sequencing (2) alignment or assembling (3) annotation (Duan et al., 2010; Edwards and Holt, 2013; Ekblom and Wolf, 2014).

The next generation sequencing (NGS) was widely used in taxonomic studies of various bacteria such as *Asinibacterium* species (Brzoska et al., 2019) and for novel species of genus *Arcanobacterium* (Sammra et al., 2018, 2020). Because of the importance whole genome sequencing had also been used to diagnose and to investigate epidemiologically the novel corona virus SARS-CoV-2 (Stefanelli et al., 2020).

2.2.13.1 - Whole-genome single nucleotide polymorphism (wgSNP)

The most common type of genetic variation is the whole genome single nucleotide polymorphism (wgSNP), which is the substitution of a single nucleotide at a specific position in the genome (Gupta et al., 2001). The linkage disequilibrium (LD) of the SNPs in the region of genome had been explained as non-random association of alleles at different loci in the population (Lewontin, 1964). However, the distribution of SNPs in the genome occurs more frequently in the non-coding region than in the coding regions (Barreiro et al., 2008). Furthermore, the mutation or the exchange of genetic material of different organisms could play an important role to determine the SNP density in the genome (Nachman, 2001). The application of wgSNP could be widely used in human medicine to determine the genetic variation associated with the diseases or feature (Zahng et al., 2004). In addition, wgSNP analysis was used to study the methicillin-resistant *S. aureus* hospital clone EMRSA-15 (Holmes et al., 2014). In the same year, wgSNP analysis had been successfully used to follow the evolution of *Mycobacterium tuberculosis* (Cabal et al., 2014). Furthermore, 55 isolates of foodborne outbreaks of *Salmonella enterica* could be successfully sequenced and through wgSNP analyzed. The results revealed well supported clades, with less than four-SNP pairwise diversity, that were conformable with epidemiologically determined outbreaks (Taylor et al., 2015). In the same year, the phylogenetic analysis based on SNP analysis was

used to resolve the interspecies relationships between *Brucella melitensis* and to differ between the vaccine and endemic strains (Tan et al., 2015). In 2016, Dal Pozzo et al. investigated *Coxiella burnetii* in caprine and bovine Belgian farms using SNP analysis.

Chapter 3 - Publications

Appendix 1

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Identification of *Arcanobacterium phocae* isolated from fur animals by phenotypic properties, by MALDI-TOF MS analysis and by detection of phocaelysin encoding gene *phl* as probable novel target

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ABSTRACT

In the present study 12 *Arcanobacterium phocae* strains isolated from fur animals in Finland, including foxes, minks and Finnraccoons, could successfully be identified phenotypically, by matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) and genotypically by sequencing 16S rDNA and phocaelysin (PHL) encoding gene *phl*. The PHL of all 12 *A. phocae* strains in the present study and reference strains *A. phocae* DSM 10002^T and *A. phocae* DSM 10003 displayed, as typical members of the cholesterol dependent cytolysin-group of toxins, the variant undecapeptide sequence EATGLAWDPWW which appeared to be most closely related to arcanolysin of *Arcanobacterium haemolyticum* and pyolysin of *Trueperella pyogenes*. In addition, gene *phl* could be determined with a newly designed loop-mediated isothermal amplification (LAMP) assay. The detection of mass spectra by MALDI-TOF MS and the LAMP assay based on gene *phl* might help to reliably identify *A. phocae* in future and also elucidate the role this species plays in infections of fur animals.

1. Introduction

According to Yassin et al. (2011) genus *Arcanobacterium* (*A.*) consists of four species, namely *A. haemolyticum*, *A. phocae*, *A. pluranimalium* and *A. hippocoleae*. More recently *A. canis* (Hijazin et al., 2012d), *A. phocisimile* (Hijazin et al., 2013), *A. pinnipediorum* (Sammra et al., 2015), *A. wilhelmae* (Sammra et al., 2017) and *A. urinimassiliense* (Diop et al., 2017) were described as novel species of this genus.

A. phocae was first isolated and characterised in 1997 in mixed culture from various tissues and fluids in common seals (*Phocae vitulina*) and grey seals (*Halichoerus grypus*) of the coastal waters around Scotland, UK (Ramos et al., 1997). However, this bacterium was recovered from pneumonic and septicemic seals but its pathological significance was unclear. Later, Johnson et al. (2003) recovered *A. phocae* from sites of inflammation from live stranded California sea lions (*Zalophus californianus*), Pacific harbour seals (*Phoca vitulina richardii*) and northern elephant seals (*Mirounga angustirostris*) and from sites of inflammation and tissue samples taken during postmortem examinations of the above mentioned marine mammals and from stranded southern sea otters (*Enhydra lutris nereis*) and a common dolphin (*Delphinus delphis*) of the central California coast (USA). In 2010, Giovannini (2010) isolated *A. phocae* from mixed infections of two California sea lions stranded along the southern California coast. *A. phocae* was isolated from pleural fluid of one sea lion and from a wound from the second case.

In addition, *A. phocae* has been involved, together with *Staphylococcus delphini* and *Streptococcus canis,* in the pathogenesis of cases of pododermatitis of the Canadian mink *(Neovison vison)* (Chalmers et al., 2015). Pododermatitis of farmed minks appeared as acute, severe ulceration of footpads with occasional further infection of

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nail beds. This could lead to a chronic hyperkeratotic purulent plantar dermatitis. A link between feeding of seal meat and the emergence of pododermatitis in minks was proposed (Chalmers et al., 2015). Furthermore, *A. phocae* has been suggested to be the aetiological agent of an emerging skin disease of fur animals called fur animal epidemic necrotic pyoderma (FENP). FENP, as multifactorial disease, is an emerging disease among Finnish fur animals in which *A. phocae* as well as other infectious factors, such as a novel *Streptococcus* sp., are involved (Nordgren et al., 2014). This previous study suggested a species shift of *A. phocae* from marine mammals to fur animals. In Finland, Nordgren et al., 2016 also studied experimentally possible routes of transmission by an infection of minks with *A. phocae,* either alone or concurrently with a novel *Streptococcus* sp. Typical signs and gross- and histopathological findings for FENP were detected when naïve minks were infected with the tissue extract of minks with FENP using a subcutaneous or intradermal but not a peroral infection route. However, predisposing factors such as the environment, the general condition of the animals, temperature and skin trauma might also contribute to the development of FENP (Nordgren et al., 2016). Molenaar et al., 2017 described the isolation of *A. phocae* in minks in three cases of severe postvaccination wounds at the injection site.

The identification of hitherto described *A. phocae* was performed with phenotypic methods (Ramos et al., 1997; Johnson et al., 2003; Ülbegi-Mohyla et al., 2009; Ülbegi, 2010; Hijazin et al., 2013), MALDI-TOF MS analysis (Hijazin et al., 2012b, 2012c, 2012d), Fourier transform infrared (FT-IR) spectroscopy (Nagib et al., 2014) and genotypically by sequencing 16S rDNA (Ramos et al., 1997; Johnson et al., 2003), 16S-23S rDNA intergenic spacer region (Hassan et al., 2008; Chalmers et al., 2015) and the genes *rpo*B (Ülbegi-Mohyla et al., 2010), *cpn*60 (Hijazin et al., 2012a), *tuf* and *gap* (Wickhorst et al., 2016). Also, a real-time PCR was applied for detecting *A. phocae* directly from DNA extraction from mink footpad tissue without the interference of other bacterial species (Chalmers et al., 2015). More recently a complete genome sequence of *A. phocae* DSM 10002^T was described by Varghese and Submissions (2016) (accession number: LT629804).

The present study was designed to investigate the usefulness of phenotypic properties, MALDI-TOF MS analysis and phocaelysin encoding gene *phl* as a novel target gene for identifing of *A. phocae* strains isolated from fur animals.

2. Materials and methods

2.1. Bacterial strains

The strains investigated in the present study included 12 *A. phocae* isolated from fur animals in Finland (Nordgren et al., 2014), type strain *A. phocae* DSM 10002^T, *A. phocae* DSM 10003 and other reference strains of genus *Arcanobacterium* and *Trueperella* (Hijazin et al., 2012d, 2013; Sammra et al., 2015). The origin and date of isolation of the *12 A. phocae* strains isolated from fur animals are summarised in Supplementary material (Table S1 and Table 1). Two of the *A. phocae* strains (strain 66 and 83) came from the same farm, all the other strains came from different farms.

2.2. Phenotypical characterisation of the bacteria

The bacterial culturing of the *A. phocae* strains was carried out on 5% sheep blood agar for 48 h at 37° C in a candle jar. The strains were investigated phenotypically, by determining haemolysis, synergistic or reverse CAMP-like reactions, with a commercial identification system (API-Coryne test system, bioMérieux, Nürtingen, Germany), tablets containing substrates (Medco Diagnostika GmbH, München, Germany), with 4-methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany) and some other biochemical tests described previously (Ülbegi-Mohyla et al., 2009; Hijazin et al., 2013).

Table 1
Phenotypical properties of the 12 *A. phocae* strains in the present study, type strain *A. phocae* DSM 10002^T and *A. phocae* DSM 10003.

Biochemical properties	*A. phocae* (n = 12)	*A. phocae* DSM 10002^{T*}	*A. phocae* DSM 10003*
Haemolysis on SBA	+(+) (10); + (2)	+(+)	+(+)
CAMP-like haemolytic reactions**			
Staphylococcus aureus β-haemolysin	− (12)	−	−
Streptococcus agalactiae	+ (12)	+	+
Rhodococcus equi	+ (12)	+	+
Reverse CAMP reaction	+ (12)	+	+
Nitrate reduction	$-^1$ (12)	$-^1$	$-^1$
Pyrazinamidase	$-^1$ (8); $+^1$ (4)	$-^1$	$-^1$
Pyrrolidonyl arylamidase	$+^1$ (10); $-^1$ (2)	$-^1$	$-^1$
Alkaline phosphatase	$+^1$ (12)	$+^1$	$+^1$
β-Glucuronidase	$-^{1,3}$ (12)	$-^{1,3}$	$-^{1,3}$
β-Galactosidase	$+^{1,3}$ (12)	$+^{1,3}$	$+^{1,3}$
α-Glucosidase	$+^{1,2,3}$ (12)	$+^{1,2,3}$	$+^{1,2,3}$
N-Acetyl-β-Glucosaminidase	$+^{1,3}$ (12)	$+^{1,3}$	$+^{1,3}$
Esculin	$-^1$ (12)	$-^1$	$-^1$
Urease	$-^1$ (12)	$-^1$	$-^1$
Gelatine	$-^1$ (12)	$-^1$	$-^1$
Fermentation of:			
Glucose	$+^1$ (12)	$+^1$	$+^1$
Ribose	$+^1$ (9); $-^1$ (3)	$+^1$	$+^1$
Xylose	$-^1$ (11); $(+)^1$(1)	$-^1$	$-^1$
Mannitol	$-^1$ (12)	$-^1$	$-^1$
Maltose	$+^1$ (12)	$+^1$	$+^1$
Lactose	$+^1$ (12)	$+^1$	$+^1$
Saccharose	$+^1$ (7); $-^1$ (5)	$+^1$	$+^1$
Glycogen	$+^1$ (9); $-^1$ (3)	$+^1$	$+^1$
Catalase	+ (10); − (2)	+	+
Serolysis on Loeffler agar	− (12)	−	−
Starch hydrolysis	+ (12)	+	+

* Results according to Ülbegi-Mohyla et al. (2009); Ülbegi (2010) and Hijazin et al. (2013); () = number of strains showning positive or negative reactions; SBA = sheep blood agar.

** Synergistic or reverse CAMP-like reaction with indicator strains; the reactions are shown as follows: +(+) = enhanced positive reaction; + = positive reaction; (+) = weak reaction; − = negative reaction; ^{1} = Api-Coryne test system (bioMérieux, Nürtingen, Germany); ^{2} = tablets containing substrates (Medco Diagnostika GmbH, München, Germany); ^{3} = 4-methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany).

2.3. Proteomic analysis by MALDI–TOF MS

The 12 *A. phocae* strains were analysed by MALDI-TOF MS as described previously (Hijazin et al., 2012b, 2012c, 2012e) using the Bruker Daltonik MALDI Biotyper software package version 4.0 (Bruker Daltonik, Bremen, Germany) as follows: A few colonies of freshly cultured bacteria were suspended in 75% ethanol. After centrifugation, the pellet was resuspended in 30 µl 70% formic acid and with the same volume of pure acetonitrile. The suspension was centrifuged and 1 µl of the supernatant was transferred to a polished steel MALDI target plate (Bruker Daltonik) and allowed to dry at room temperature. This was done for 8 spots per sample. The sample was overlaid with 1 µl matrix (10 mg α -cyano-4-hydroxy-cinnamic acid ml^{-1} in 50% acetonitrile/2.5% trifluoroacetic acid). Mass spectra were acquired using a microflex mass spectrometer (Bruker Daltonik) in the linear mode and a mass range of 2–20 kDa using the automated functionality of flexControl 3.4 software (Bruker Daltonik). At least 20 of the 24 acquired raw spectra were used to generate a main spectrum.The software calculates a similarity score [log (score)] by considering the proportion of matching

peaks to a main spectrum of the database and also the proportion of the matching peaks to the unknown mass spectrum. A third factor reflects the intensity symmetry of the matching peaks. The logarithmized score values range from 0 (no similarity) to 3 (absolute identity). Log (score) values ≥ 2 are rated as identification of bacteria at the species level. Log (score) values ≥ 1.7 and < 2.0 are considered as identification of microorganisms at least on the genus level. Log score values < 1.7 indicate that a spectrum is not suitable for identification by the MALDI Biotyper.

2.4. Genotypical identification of the bacteria

For phylogenetic analysis the 16S rDNA of the 12 *A. phocae* strains was determined by amplification and sequencing as described previously (Sammra et al., 2014) as follows: The DNA template was extracted from freshly cultivated bacterial colonies using the DNeasy Blood and Tissue Kit in accordance with the manufacturer's instructions (Qiagen, Hilden, Germany). A 1403 nucleotides sequence of bacterial 16S rDNA was amplified with forward primer 16S rDNA UNI-L (5′-AGA GTT TGA TCA TGG CTC AG-′3) and reverse primer 16S rDNA UNI-R (5′-GTG TGA CGG GCG GTG TGT AC-′3). The PCR amplifications were performed in 30 µl reaction mixtures with 1 µl for each primer (10 pmol/µl), 15 µl ready to use HotGoldStar Mix (Eurogenetec, Seraing, Belgium) and 10 µl Aqua dest. Finally, 3 µl DNA template was added to each reaction tube. For DNA amplification the following PCR program was used: One step of 10 min at 95 °C; 30 cycles, with one cycle consisting of 30 s at 95 °C, 60 s at 58 °C, and 60 s at 72 °C; and one step of 7 min at 72 °C. The PCRs were performed with the GeneAmp PCR System 2400 thermocycler, Perkin-Elmer (Norwalk, USA). For detecting PCR products, 5 µl of the amplified DNA was run on a 1.5% agarose gel (Biozym, Hess-Oldendorf, Germany) and visualised under UV light ImageMaster VDS (Amersham Biosciences, New Jersey, USA). The remaining amplicon (ca. 15 µl) was used for sequencing with primer 16S rDNA-533F (5′-GTG CCA GCM GCC GCG GTA A-′3) and 16S rDNA-907-R (5′-CCG TCA ATT CMT TTG AGT TT-′3) from Seqlab-Sequence Laboratories (Göttingen, Germany). The sequences were analysed using FinchTV (version, 1.4.0), alignment and dendrogram analysis by the Clustal W method using DNASTAR Lasergene version 8.0.2 (DNASTAR Inc., Madison, USA).

In addition, the previously sequenced phocaelysin encoding gene *phl* of *A. phocae* DSM 10002^T (accession number: LT629804 region: 1304165 up to 1305874) was used for the design of *phl* specific oligonucleotide primer. The oligonucleotide primers were selected using Primer-BLAST-program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) obtained from National the Center for Biotechnology Information (Bethesda, Maryland, USA). The forward primer phl3-F with the sequence 5′-AGC AGC TAT CGC TTC GTT CA-′3 and the reverse primer phl3-R with the sequence 5′-CAT GGG TTC AAT GTC GTG CC-′3 were used with the following PCR program: One step of 3 min at 94° C; 30 cycles, with one cycle consisting of 30 s at 94 °C, 40 s at 37 °C, and 60 s at 72 °C; and one step of 5 min at 72 °C. The PCRs were performed and the amplicons sequenced as described above.

Finally, a loop-mediated isothermal amplification (LAMP) assay coupled with a real-time fluorometer based on gene *phl* was developed and used as follows: For template preparation freshly cultivated bacterial colonies were suspended in a tube containing 500 ml HYPLEX® LPTV buffer tube (AmplexDiagnostics, Gießen, Germany) and boiled for 10 min using a heatblock. Subsequently, 2 µl of this suspension was used as template. For LAMP assay a set of six oligonucleotide primers were designed using LAMP Designer software, ver. 1.10 (PREMIER Biosoft, Palo Alto, CA, USA) including two outer primers (forward primer pho-F3 and backward primer pho-B3), two inner primers (forward inner primer pho-LoopF and backward inner primer pho-LoopB) and two loop primers (pho-FIP and pho-BIP) (Table S2 and Table 2). The design of the oligonucleotide primer was based on the sequence of gene *phl* of *A. phocae* type strain DSM 10002^T (accession no. FN999907). The oligonucleotide primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany). For investigating the specificity the oligonucleotide sequences were submitted to the NCBI GenBank using LAMP Designer software. The LAMP reaction was carried out with a total volume of 20 µl of the reaction mixture containing 0.4 µl each of pho-F3 and pho-B3 primer (25 pmol/µl) equivalent to 0.5 µM final concentration, 0.8 µl each of pho-LoopF and pho-LoopB primer (25 pmol/µl) equivalent to 1.0 µM final concentration, 1.6 µl each of pho-FIP and pho-BIP primer (25 pmol/µl) equivalent to 2.0 µM final concentration, 2.4 µl sterile Aqua dest. and 10 µl 2 x Isothermal Master Mix Iso-001 (Optigene, Horsham, UK). Subsequently, 2 µl DNA preparation was added as a template. The LAMP assay was run at 65° C for 30 min with a melting curve analysis step (annealing curve 98° C to 80° C ramping at 0.05° C per s) in a portable real-time fluorometer (Genie II®, Optigene, West Sussex, UK) in accordance with the manufacturers instructions.

Table 2
Inclusivity and exclusivity test of the *A. phocae* LAMP assay based on phocaelysin encoding gene *phl* for 12 *A. phocae* strains isolated from fur animals, for type strain *A. phocae* DSM 10002^T, *A. phocae* DSM 10003 and for various other species of genus *Arcanobacterium* and *Trueperella*.

Bacterial species and code no.	Detection time mm:ss	Melting temperature °C
A. phocae 41	6:45	84.4
A. phocae 91	8:10	84.3
A. phocae 128	8:00	84.3
A. phocae 132	6:15	84.3
A. phocae 66	7:30	84.4
A. phocae 83	7:45	84.3
A. phocae 108	7:45	84.5
A. phocae 122	7:00	84.3
A. phocae 127	6:30	84.3
A. phocae 870/4	6:30	84.5
A. phocae 89	7:15	84.4
A. phocae 96	7:45	84.3
A. phocae DSM10002^T	5:45	84.1
A. phocae DSM 10003	6:00	84.7
A. phocisimile DSM 26142^T	–	–
A. phocisimile 4112	–	–
A. pinnipediorum DSM 28752^T	–	–
A. pluranimalium DSM 13483^T	–	–
A. canis DSM 25104^T	–	–
A. haemolyticum DSM 20595^T	–	–
A. hippocoleae DSM 15539^T	–	–
A. wilhelmae DSM 102162^T	–	–
T. abortisuis DSM 19515^T	–	–
T. bernardiae DSM 9152^T	–	–
T. bialowiezensis DSM 17162^T	–	–
T. bonasi DSM 17163^T	–	–
T. pyogenes DSM 20630^T	–	–
T. pyogenes DSM 20594	–	–

3. Results and discussion

Among the 12 *A. phocae* strains investigated in the present study 10 strains displayed a relatively wide zone of complete haemolysis and two strains a slightly reduced hemolysis on 5% sheep blood agar plates. In addition, all 12 *A phocae* strains showed a synergistic CAMP-like haemolysis with *Streptococcus agalactiae* and *Rhodococcus equi* as indicator strains and a reverse CAMP reaction in the area of incomplete staphylococcal β-haemolysis. The biochemical properties mainly corresponded to properties of type strain *A. phocae* DSM 10002^T and *A. phocae* DSM 10003 (Table 1).

Comparable to previously conducted MALDI-TOF MS analysis of various species of genus *Arcanobacterium* and *Trueperella* (Hijazin et al., 2012c), a single *T. bernardiae* strain of animal origin (Hijazin et al., 2012e), *A. pluranimalium* from a juvenile giraffe and from bovine milk samples (Risse et al., 2014; Wickhorst et al., 2016), *A. haemolyticum* from a donkey (Sammra et al., 2014), *T. pyogenes* from a roebuck

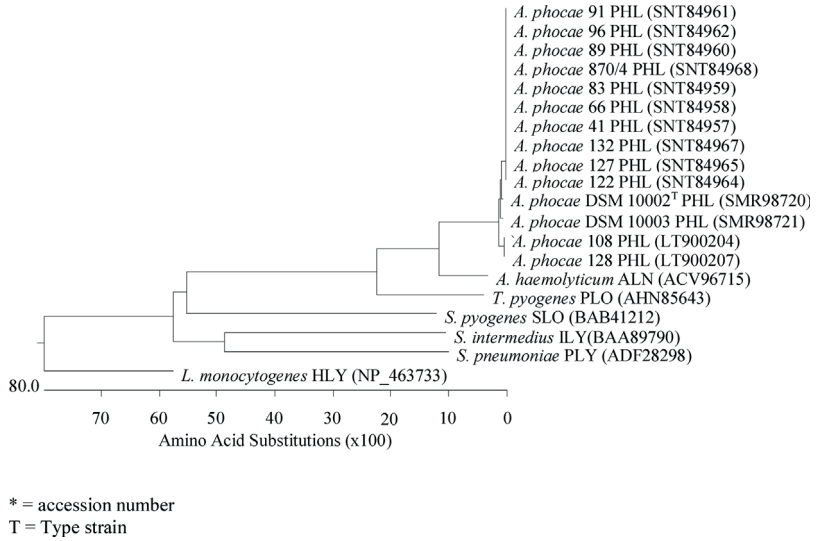

Fig. 1. Phylogenetic relationship among amino acid sequences of PHL of the 12 *A. phocae* strains in the present study, PHL of reference strains *A. phocae* 10002^T and *A. phocae* 10003, ALN of *A. haemolyticum*, PLO of *T. pyogenes*, SLO of *S. pyogenes*, ILY of *S. intermedius*, PLY of *S. pneumoniae* and HLY of *L. monocytogenes* obtained from NCBI GenBank.

(Wickhorst et al., 2017a) and an *A. hippocoleae* strain isolated from a healthy mare (Wickhorst et al., 2017b), the MALDI-TOF MS analysis of the present study allowed the identification of all 12 *A. phocae* strains to the species level with log [score] values between 2.2 to 2.4 for the 12 *A. phocae* strains indicating that MALDI-TOF MS could also be used to reliably identify *A. phocae* strains isolated from fur animals (Fig. S3 and Fig. 1). As shown by numerous authors, MALDI-TOF MS appears to be a powerful tool for species classification of a broad spectrum of Gram-positive and Gram-negative bacteria (Seng et al., 2009; Murray, 2010; Bizzini et al., 2011).

The 12 *A. phocae* strains were additionally identified by sequencing the 16S rRNA gene with approximate sizes of 1350 bp (strain 41: acc. no. LT854821; strain 91: LT854825; strain 128: LT854830; strain 132: LT854831; strain 66: LT854822; strain 83: LT854823; strain 108: LT854827; strain 122: LT854828; strain 127: LT854829; strain 870/4: LT854820; strain 89: LT854824; strain 96: LT854826) with a sequence similarity between 99.6% and 100% among them and a sequence similarity to 16S rDNA of type strain *A. phocae* DSM 10002^T (FN562994) and *A. phocae* DSM 10003 (FN562995) between 99.0% and 99.5%, respectively. The dendrogram analysis of the 16S rRNA gene is presented in Fig. S4 and Fig. 2. It was of interest that the 16S rDNA sequences of *A. phocae* 128 and *A. phocae* 108 isolated from a fox and a mink, respectively differed slightly in their 16S rDNA sequences compared to the sequences of type strain *A. phocae* DSM 10002^T, *A. phocae* DSM 10003 and the remaining *A. phocae* investigated in the present study. However, *A. phocae* 128 and *A. phocae* 108 were also negative in pyrrolidonyl arylamidase and catalase reaction and showed a reduced hemolysis on sheep blood agar. A positive catalase reaction is generally known as a typical property of *A. phocae* (Ramos et al., 1997).

The 12 *A. phocae* strains in the present study could also be characterised using oligonucleotide primer with specificity to phocaelysin encoding gene *phl*. The selection of the oligonucleotide primer was based on the previously published gene sequence (Varghese and Submissions, 2016). Gene *phl* of the 12 *A. phocae* strains yielded between 96.0% and 100% sequence identity among them and between 95.5% and 97.2% sequence identity to the *phl* sequences of type strain *A. phocae* DSM 10002^T (LT854832) and *A. phocae* DSM 10003 (LT854833), respectively. The accession numbers of gene *phl* of the 12 *A. phocae* strains were for strain 41: LT900198; strain 91: LT900202; strain 128: LT900207; strain 132: LT900208; strain 66: LT900199; strain 83: LT900200; strain 108: LT900204; strain 122: LT900205; strain 127: LT900206; strain 870/4: LT900209; strain 89: LT900201and strain 96: LT900203.

Phylogenetic analysis of the amino acid sequences of PHL of the 12 *A. phocae* strains in the present study, PHL of the reference strains *A. phocae* 10002^T (acc. no. SMR98720) and *A. phocae* 10003 (SMR98721), arcanolysin (ALN) of *A. haemolyticum* (ACV96715), pyolysin (PLO) of *T. pyogenes* (AHN85643) and of other pore-forming toxins including streptolysin O (SLO) of *Streptococcus pyogenes* (BAB41212), intermedilysin (ILY) of *Streptococcus intermedius* (BAA89790), pneumolysin (PLY) of *Streptococcus pneumoniae* (ADF28298) and listeriolysin O (HLY) of *Listeria monocytogenes* (NP_463733) obtained from NCBI GenBank revealed a close relation of PHL to ALN (74.3%) and PLO (61.8%), respectively, less pronounced to the other pore-forming toxins (Fig. 1).

A detailed analysis of the primary structure of amino acids of PHL of *A. phocae* DSM 10002^T compared to ALN of *A. haemolyticum* DSM 205195^T, described by Jost et al. (2011), yielded a variant undecapeptide which both lacked the conserved cysteine residue and displayed a typical tryptophan spacing (WxxWW) which differed from the consensus sequence (WxWW). PHL and ALN also showed the conserved threonine leucine pair in domain 4, according to Jost et al. (2011), known to be responsible for the interaction with membrane cholesterol (Fig. 2A). It was of interest that despite some sequence variation of gene *phl*, all 12 *A. phocae* strains in the present study and the reference strains *A. phocae* DSM 10002^T and *A. phocae* DSM 10003 uniformly displayed the undecapeptide sequence EATGLAWDPWW which differs from the undecapeptide sequence of ALN of *A. haemolyticum*, PLO of T. *pyogenes*, undecapeptides of other CDCs and the consensus sequence (Fig. 2B).

Comparable to gene *plo* of *T. pyogenes*, which appeared to be a constant characteristic of all investigated *T. pyogenes* (Billington et al., 1997; Ülbegi-Mohyla et al., 2010; Hijazin et al., 2011) and gene *pla* as typical characteristic of all investigated *A.* pluranimalium (Moser et al., 2013; Balbutskaya et al., 2014, Risse et al., 2014; Wickhorst et al., 2016; Ningrum et al., 2017), *phl* of *A. phocae* seems to be also constantly present in all strains of this species and could be used for molecular identification of *A. phocae*. Further studies will focus on providing more information on the consistent presence and the pathogenic importance of this hitherto unknown virulence factor of *A. phocae*.

The application of LAMP assays for detecting food-borne bacterial pathogens and toxicants as well as mycotoxin-producing food-borne fungi was reviewed by Niessen et al. (2013). This technique was also used previously to identify *A. pluranimalium* and *T. pyogenes* (Abdulmawjood et al., 2015; Abdulmawjood et al., 2016). LAMP assays were described as an alternative to using PCR-based methods with a

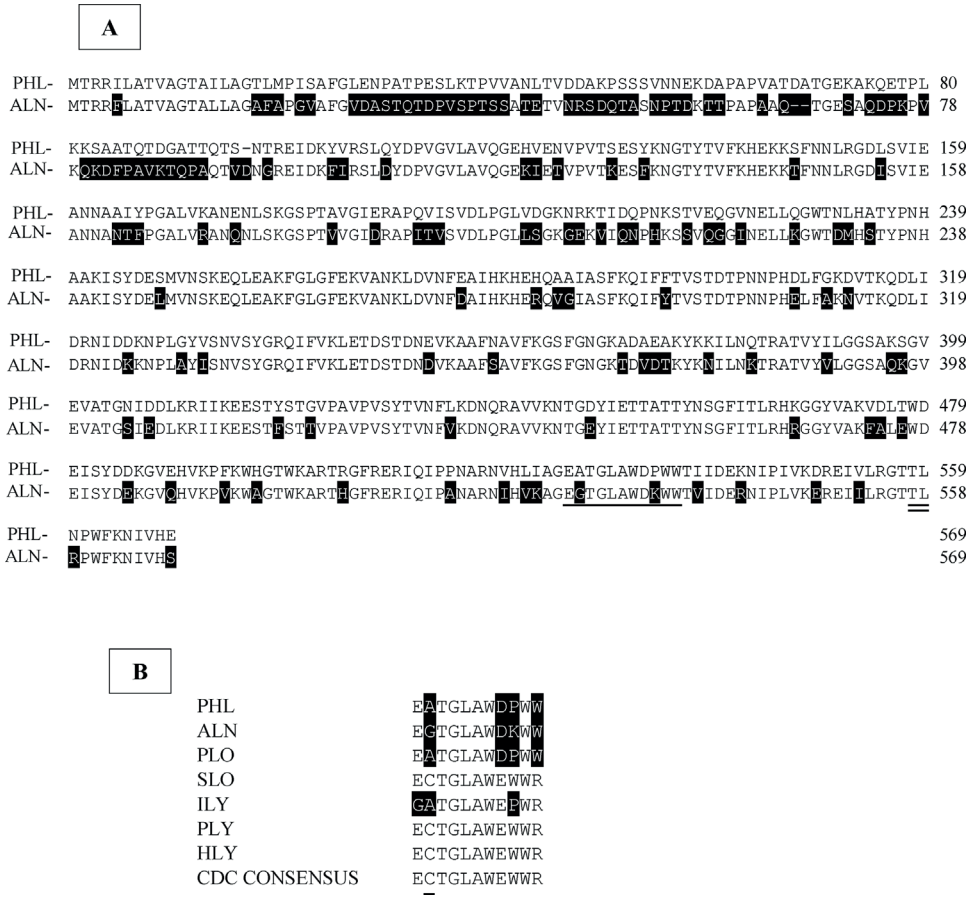

Fig. 2. (A) Alignment of amino acid sequences of PHL of *A. phocae* DSM 10002^T (acc. no. SDU80356) and ALN of *A. haemolyticum* DSM 20595^T (ACV96715) with undecapeptide (single underlined) and cholesterol interacting TL motif (double underlined). Differences in amino acid sequences are shown in marked letters. (B) Undecapeptide sequences of PHL, ALN, PLO, SLO, ILY, PLY, HLY and the consensus CDC. The cysteine conserved in thiol-activated CDCs but absent in PHL is underlined in the consensus sequence. Differences from consensus depicted in marked letters. Abbreviations as in Fig. 3.

high sensitivity and specificity, a shorter reaction time and a comparably low susceptibility for inhibitors.

The newly developed LAMP assay in the present study, targeting the phocaelysin encoding gene *phl*, provided a rapid and reliable identification of all 12 *A. phocae* isolated from various fur animals, type strain *A. phocae* DSM 10002^T and *A. phocae* DSM 10003. No cross reaction could be observed with various other species of genus *Arcanobacterium* and closely related genus *Trueperella* (Fig. 3, Table 2). This could be demonstrated using a real-time fluorometer. Melting curve analysis, which is termed by Genie II® as anneal curve analysis, revealed no significant differences among the various *A. phocae* strains. The melting temperature of the *A. phocae* specific amplicon was 84.4 °C (± 0.1 °C) (Table 2).

The phenotypic properties, the MALDI-TOF MS analysis and the

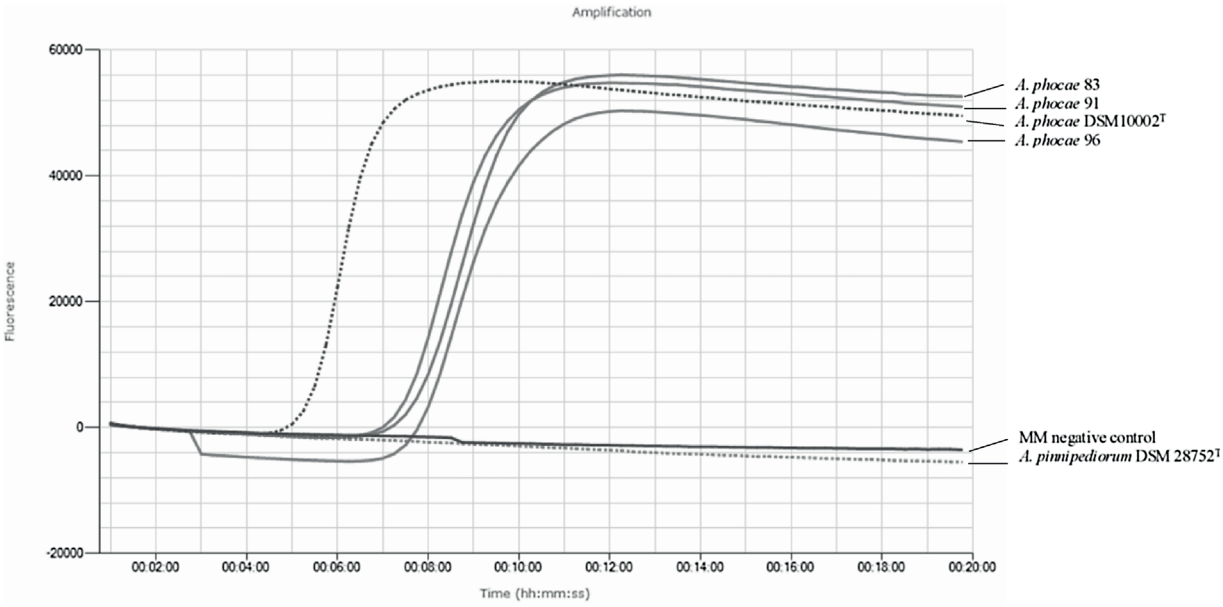

Fig. 3. Typical amplification signal of LAMP products of three *A. phocae* isolates and the type strains *A. phocae* DSM 10002^T and *A. pinnipediorum* DSM 28752^T. A master mix (MM) was used as negative control.

novel target gene *phl* might improve the accurate identification of *A. phocae* and help to elucidate the role this species plays in infections of seals, fur animals, such as minks, foxes and Finnraccoons, other animals and possibly humans.

Conflict of interest statement

Markus Timke has potential conflicts of interest as an employee of Bruker Daltonik GmbH, which produces the matrix-assisted laser desorption ionisation-time of flight mass spectrometry system used in this study, as well as the MALDI Biotyper and the software packages. The other authors declare that they have no competing interests. This research did not receive any specific funding.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetmic.2018.01.017.

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Appendix 1.1- Supplemental tables and figures of Appendix 1

Supplemental table 1

Origin of the 12 *A. phocae* strains investigated in the present study.

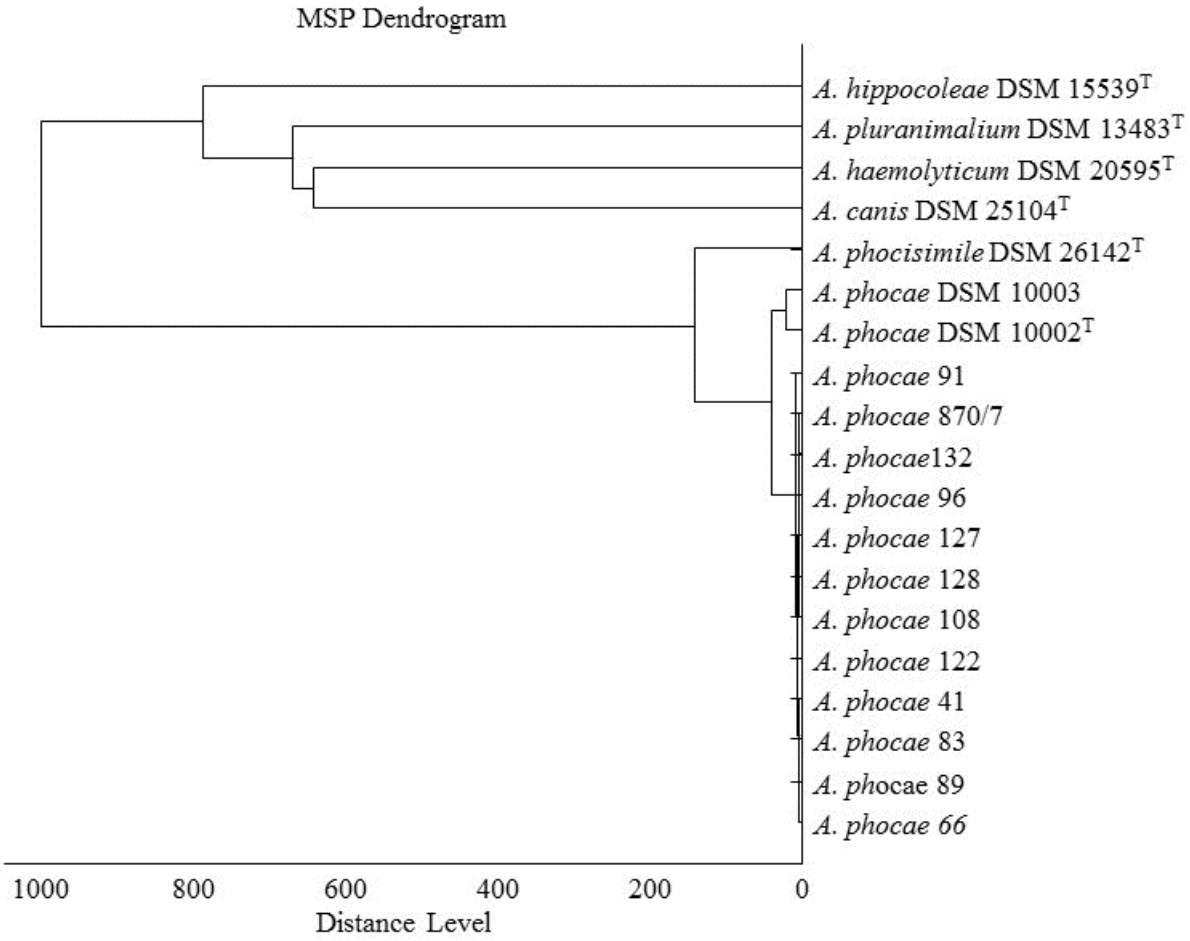
Animal species	*Strain code and year of isolation*	*Organ*
Fox (*Vulpes lagopus*)	41-2009	eye
	91-2010	skin
	128-2015	uterus
	132-2015	eye
Mink (*Neovison vison*)	66-2010	eye
	83-2010	paw
	108-2012	skin
	122-2013	lung
	127-2014	lung
	870/4-2016	skin
Finnraccoons (*Nyctereutes procyonoides*)	89-2010	paw
	96-2011	paw

Supplemental table 2

Phocaelysin encoding gene *phl* specific oligonucleotide primers used for the *A. phocae* LAMP assay in the present study.

Primer name	*Primer sequence (*5'-3'*)*	*Primer length*	*Melting temperature º C*
pho-F3	GCTAAGATCTCTTACGACGAAT	22 bp	56.5
pho-B3	CATTCGATACGTAGCCGAG	19 bp	56.7
pho-LoopF	GCTGCTTGATGTTCATGCTTAT	22 bp	56.5
pho-LoopB	CCGCACGATTTATTCGGTAAAG	22 bp	58.4
pho-FIP	AAGATCTGCTTGAACGAAGCGAGCGAATAAGCTCGATGTCA	41 bp	72.4
pho-BIP	GTTTCCACCGATACGCCCAATACCGATCAATAAGGTCCTGC	41 bp	74.4

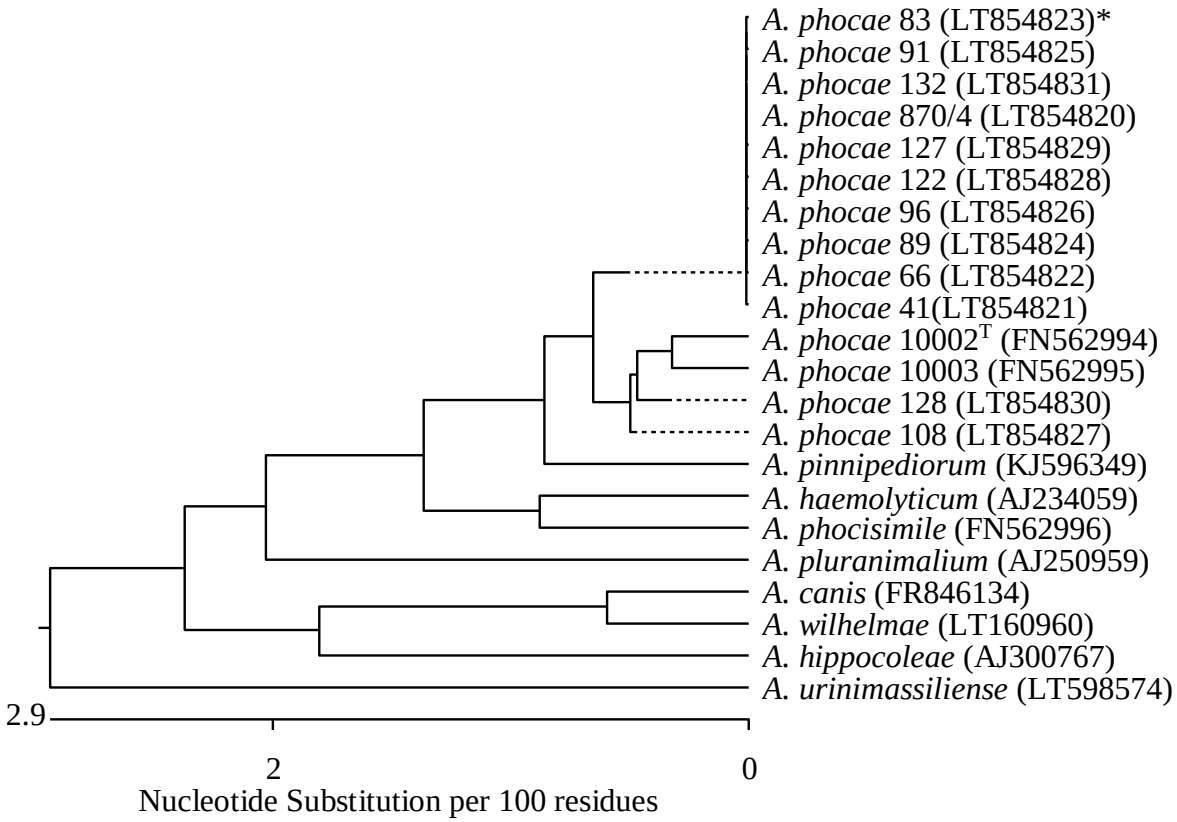
Supplemental figure 3



T = Type strain

Fig. 3. MALDI Biotyperdendrogram analysis of the 12 *A. phocae* strains of the present study, type strain *A. phocae* DSM 10002^T, *A. phocae* DSM 10003 and from various other species of genus *Arcanobacterium.*

Supplemental figure 4



* = Accession number

T = Type strain

Fig. 4. Dendrogram analysis of the 16S rRNA gene sequences of the 12 *A. phocae* strains of the present study, type strain *A. phocae* DSM 10002^{T}, *A. phocae* DSM 10003 and from various other species of genus *Arcanobacterium* obtained from NCBI GenBank.

Appendix 2

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Epidemiological analysis of *Arcanobacterium phocae* isolated from cases of mink dermatitis of a single farm

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wgSNP

ABSTRACT

The present study was designed to identify nine *Arcanobacterium phocae* strains isolated from cases of mink dermatitis of a single farm in Finland and characterize the strains for epidemiological relationships. All nine strains and previously described *A. phocae* used for comparative purposes were identified and further characterized phenotypically, by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), by Fourier Transform Infrared Spectroscopy (FT-IR) and genotypically by detection of phocaelysin encoding gene *phl* with a previously developed loop-mediated isothermal amplification (LAMP) assay and by sequencing 16S rRNA gene and gene *phl*, the elongation factor tu encoding gene *tuf* and the β subunit of bacterial RNA polymerase encoding gene *rpoB*. Genetic relatedness among isolates was determined using whole-genome single nucleotide polymorphism (wgSNP) analysis. The wgSNP results, partly the MALDI-TOF MS and FT-IR analyses and sequencing of the genes, revealed that the nine *A. phocae* strains recovered from a single farm showed close sequence similarities among each other and differed from previously investigated *A. phocae* strains isolated from other farms and animals in Finland and from the *A. phocae* type strain. This indicated a close epidemiological relationship of the *A. phocae* strains isolated from a single farm and that the nine *A. phocae* strains of the present study might have developed from a common ancestor.

1. Introduction

Arcanobacterium phocae was first isolated and characterized in 1997 in mixed culture from various tissues and fluids in common seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) of the coastal waters around Scotland, UK (Ramos et al., 1997). However, this bacterium was recovered from pneumonic and septicemic seals but its pathological significance was unclear. Later, Johnson et al. (2003) recovered *A. phocae* from sites of inflammation from live stranded California sea lions (*Zalophus californianus*), Pacific harbor seals (*Phoca vitulina richardii*) and northern elephant seals (*Mirounga angustirostris*) and from sites of inflammation and tissue samples taken during postmortem examination of the above mentioned marine mammals and from stranded southern sea otters (*Enhydra lutris nereis*) and a common dolphin (*Delphinus delphis*) of the central California coast (USA). In 2010, Giovannini (2010) isolated *A. phocae* from mixed infections of two California sea lions stranded along the southern California coast. *A. phocae* was isolated from pleural fluid of one sea lion and from a wound of the second case. In 2014, Nordgren et al. (2014) recovered *A. phocae* in tissues of minks, finnraccoons, foxes with fur animal epidemic necrotic pyoderma (FENP). In some cases it was found together with a *Streptococcus* species. In addition, *A. phocae* has been involved, together with *Staphylococcus delphini* and *Streptococcus canis,* in the pathogenesis of cases of pododermatitis of the Canadian mink (*Neovison vison*) (Chalmers et al., 2015). Pododermatitis of farmed mink appeared as acute, severe ulceration of footpads with occasional further infection of nail beds. This

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could lead to a chronic hyperkeratotic purulent plantar dermatitis. A link between feeding of seal meat and the emergence of pododermatitis in mink was proposed by Chalmers et al. (2015). Also Nonnemann et al., 2017 described that the association between *A. phocae* in mink and seals might be caused by the use of seal meat as a source of high protein feed for farmed mink.

In FENP immunological and environmental factors seem to contribute to the disease and the bacteria spread from farm to farm (Aaltonen et al., 2016; Nordgren et al., 2016b). In Denmark Hammer et al. (2016) studied experimentally the role of *A. phocae* and *Streptococcus halichoeri*, suspected to be the causal factors of FENP, in ten healthy male white color type minks, yielding a possible synergistic effect between both bacterial species. In Finland Nordgren et al. (2016a) also studied experimentally possible routes of transmission of infections of mink with *A. phocae*, either alone or concurrently with a novel *Streptococcus* species. Typical signs, and gross- and histopathological findings for FENP were detected when naïve mink were infected with the tissue extract of mink with FENP using a subcutaneous or intradermal but not a perorally infection route. The isolation of *A. phocae* in mink in three cases of severe postvaccination wounds at the injection site was described by Molenaar et al. (2017). More recently Alssahen et al. (2018) characterized *A. phocae* strains isolated from fur animals in Finland, including foxes, finnraccoons and minks.

The identification of hitherto described *A. phocae* was performed with phenotypic methods (Ramos et al., 1997; Johnson et al., 2003; Ülbegi-Mohyla et al., 2009; Ülbegi-Mohyla, 2010), MALDI-TOF MS analysis (Hijazin et al., 2012; Alssahen et al., 2018), Fourier transform infrared spectroscopy (FT-IR) (Nagib et al., 2014) and genotypically by determination of gene *phl* with a loop mediated isothermal amplification (LAMP) assay and by sequencing gene *phl* (Alssahen et al., 2018) and by sequencing 16S rRNA gene (Ramos et al., 1997; Lawson et al., 2001; Johnson et al., 2003; Alssahen et al., 2018), the elongation factor tu encoding gene *tuf* (Wickhorst et al., 2016) and the β subunit of bacterial RNA polymerase encoding gene *rpoB* (Ülbegi-Mohyla et al., 2010; Sammra et al., 2013). More recently a complete genome sequence of *A. phocae* DSM 10002^T was described by Varghese and Submissions (2016) (accession number: LT629804). The present study was designed to determine epidemiological relationships of nine *A. phocae strains* isolated from cases of mink dermatitis of a single farm in Finland.

2. Materials and methods

2.1. Bacterial strains

The nine *A. phocae* strains (*A.*48, *A.*49, *A.*50, *A.*51, *A.*67, *A.*68, *A.*69, *A.*70, *A.*107) investigated in the present study were isolated from cases of dermatitis from nine minks of a single farm in Finland, over a period of two years from May 2010 to August 2012. For comparative purpose, four previously characterized *A. phocae* strains isolated from foxes, finnraccoon and mink (*A.*41, *A.*96, *A.*108, *A.*128) of other farms in Finland (Alssahen et al., 2018), the type strain *A. phocae* DSM 10002^T and reference strains of other species of genus *Arcanobacterium* (Sammra et al., 2015) were included. The four control strains included the previously described catalase positive *A. phocae* strains *A. phocae A.*41 and *A. phocae A.*96 and the catalase negative *A. phocae* strains *A. phocae A.*108 and *A. phocae A.*128 (Alssahen et al., 2018). The origin and date of isolation of the nine *A. phocae* strains isolated from minks of a single farm and the four strains isolated from foxes, finnraccoon and mink from three additional farms were summarized in Table 1.

2.2. Phenotypical characterization of the bacteria

All *A. phocae* strains were identified by cultural properties, phenotypically by determination of haemolysis and synergistic or reverse CAMP-like reactions, by a commercial identification system (API-Coryne, bioMérieux, Nürtingen, Germany) (Ülbegi-Mohyla et al., 2009; Alssahen et al., 2018), by Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI TOF MS, Bruker Biotyper database 7.854, Bruker Daltonik, Bremen, Germany) analysis (Wickhorst et al., 2019) and by Fourier transform infrared spectroscopy (FT-IR, Bruker Tensor with HTS-XT, Bruker Optik, Ettlingen, Germany), (Nagib et al., 2014).

Table 1
Origin of the nine *A. phocae* strains investigated in the present study and the four previously characterized *A. phocae* strains isolated from foxes (*Vulpes lagopus*), finnraccoon (*Nyctereutes procyonoides*) and mink (*Neovison vison*).

Animal species	Strain code	Farm	Sample drawing	Origin
Mink	*A. phocae A.*48	A	10.05.2010	Paw
Mink	*A. phocae A.*49	A	10.05.2010	Skin
Mink	*A. phocae A.*50	A	10.05.2010	Skin
Mink	*A. phocae A.*51	A	10.05.2010	Tail
Mink	*A. phocae A.*67	A	18.11.2010	Eyelid
Mink	*A. phocae A.*68	A	18.11.2010	Skin
Mink	*A. phocae A.*69	A	22.11.2010	Eye
Mink	*A. phocae A.*70	A	22.11.2010	Skin
Mink	*A. phocae A.*107	A	30.08.2012	Paw
Fox	*A. phocae A.*41	B	03.11.2009	Eye
Finnraccoon	*A. phocae A.*96	C	19.01.2011	Paw
Mink	*A. phocae A.*108	D	04.10.2012	Skin
Fox	*A. phocae A.*128	B	21.04.2015	Uterus

2.3. Genotypical identification and characterization of the bacteria

The presence of phocaelysin encoding gene *phl* was determined with a previously described loop-mediated isothermal amplification (LAMP) assay. This was performed using a real-time fluorometer (Genie II®, OptiGene, Horsham, UK) (Abdulmawjood et al., 2016; Alssahen et al., 2018). All nine strains were additionally investigated by amplification and sequencing the 16S rRNA gene (Hassan et al., 2009; Sammra et al., 2014; Alssahen et al., 2018) and gene *phl* (Alssahen et al., 2018), the elongation factor tu encoding gene *tuf* (Wickhorst et al., 2016) and, as shown previously (Ülbegi-Mohyla et al., 2010; Sammra et al., 2013), the β subunit of bacterial RNA polymerase encoding gene *rpoB*.

Further epidemiological analyses were performed using whole genome sequencing data obtained for the nine *A. phocae* strains and the four control isolates. Genomic DNA was extracted using the Maxwell® RSC Blood DNA Kit (Promega Deutschland, Mannheim). Sequencing libraries were prepared using the Nextera DNA Flex Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Paired-end sequencing was performed on the Illumina NextSeq 500 benchtop sequencer using the NextSeq Reagent v2.5 300-cycle kit (Illumina) in 2 × 151 cycles. Raw reads were trimmed using fastp v0.19.5 (Chen et al., 2018) and *de novo* assembled using unicycler v0.4.4 (Wick et al., 2017). To determine the genomic relatedness, trimmed reads were imported into the BioNumerics 7.6 (Applied Maths, Gent, Belgium) for whole-genome SNP (wgSNP) analysis. As reference for wgSNP analysis, *A. phocae* DSM 10002^T (accession number: LT629804) and a concatenated *de novo* assembly obtained for strain *A. phocae A.*107 was used.

3. Results and discussion

The nine *A. phocae* strains investigated in the present study displayed, after cultivation on 5% sheep blood agar plates, a relatively wide zone of complete hemolysis. In addition, all nine strains showed a synergistic CAMP-like hemolysis with *Streptococcus agalactiae* and *Rhodococcus hoagii* as indicator strains and a reverse CAMP reaction in the area of incomplete staphylococcal β-haemolysis. The cultural properties and the biochemical properties determined with a

Table 2
Phenotypical properties of the nine *A. phocae* strains of the present study, the four previously investigated *A. phocae* strains and type strain *A. phocae* DSM 10002^T.

Biochemical properties	*A. phocae* (n = 9)	*A. phocae** (n = 4)	*A. phocae* DSM 10002^T*
Haemolysis on SBA	+(+) (9)	+(+) (2); +(2)	+(+)
CAMP-like haemolytic reactions**			
S. aureus β-haemolysin	- (9)	- (4)	–
S. agalactiae	+ (9)	+ (4)	+
Rhodococcus hoagii	+ (9)	+ (4)	+
Reverse CAMP reaction	+ (9)	+ (4)	+
Nitrate reduction	-[1] (9)	-[1] (4)	-[1]
Pyrazinamidase	-[1] (9)	-[1] (4)	-[1]
Pyrrolidonyl arylamidase	+[1] (8); -[1] (1)	+[1] (2); -[1] (2)	-[1]
Alkaline phosphatase	+[1] (9)	+[1] (4)	+[1]
β-Glucuronidase	-[1,2] (9)	-[1,2] (4)	-[1,2]
β-Galactosidase	-[1,2] (8), +[1,2](1)	+[1,2](4)	+[1,2]
α-Glucosidase	+[1,2] (9)	+[1,2](4)	+[1,2]
N-Acetyl-β-Glucosaminidase	+[1,2] (9)	+[1,2](4)	+[1,2]
Esculin	-[1] (9)	-[1] (4)	-[1]
Urease	-[1] (9)	-[1] (4)	-[1]
Gelatine	-[1] (9)	-[1] (4)	-[1]
Fermentation of:			
Glucose	+[1] (9)	+[1] (4)	+[1]
Ribose	+[1] (1); -[1] (8)	+[1] (1); -[1] (3)	+[1]
Xylose	-[1] (9)	-[1] (4)	-[1]
Mannitol	-[1] (9)	-[1] (4)	-[1]
Maltose	+[1] (9)	+[1] (3); (+)[1] (1)	+[1]
Lactose	+[1] (9)	+[1] (3); -[1] (1)	+[1]
Saccharose	-[1] (9)	-[1] (4)	+[1]
Glycogen	+[1] (5); (+)[1] (4)	+[1] (3); -[1] (1)	+[1]
Catalase	+ (9)	+[1] (2); -[1] (2)	+

* Results according to Ülbegi-Mohyla et al. (2009); Ülbegi-Mohyla (2010); Hijazin et al. (2013) and Alssahen et al., 2018; () = number of strains showing positive or negative reactions; SBA = sheep blood agar;** = synergistic or reverse CAMP-like reaction with indicator strains; the reactions are shown as follows: +(+) = enhanced positive reaction; + = positive reaction; (+) = weak reaction; - = negative reaction; [1] = Api-Coryne test system (bioMérieux, Nürtingen, Germany), [2] = 4-methylumbelliferyl conjugated substrates (Sigma, Steinheim, Germany).

commercial identification system, showed a close similarity of the nine strains among each other and corresponded generally to properties of previously investigated *A. phocae* strains and type strain *A. phocae* DSM 10002^T (Alssahen et al., 2018) (Table 2).

The MALDI-TOF MS analysis, comparable to previous results (Alssahen et al., 2018), allowed the identification of all nine *A. phocae* strains and the *A. phocae* control strains to the species level indicating that MALDI-TOF MS could also be used to reliably identify *A. phocae* strains isolated from fur animals. As shown by numerous authors MALDI-TOF MS appears to be a powerful tool for species classification of a broad spectrum of Gram-positive and Gram-negative bacteria (Seng et al., 2009; Murray, 2010; Bizzini et al., 2011), also including *A. phocae* (Hijazin et al., 2012; Alssahen et al., 2018). The results observed by MALDI-TOF MS of the strains of the present study yielded a correct identification but only a limited clustering of the nine *A. phocae* strains obtained from a single farm, the four previously investigated *A. phocae* strains and type strain *A. phocae* DSM 10002^T indicating the limitation of this technique for differentiation below the species level (Supplemental Fig. 1). The MALDI TOF mass-spectra of the *A. phocae* isolates are available by exchange via the MALDI-TOF user platform (http://www.maldi-up.ua-bw.de; Rau et al., 2016).

FT-IR spectroscopy, a promising technique for rapid and reliable identification of bacterial microorganisms, had already been used as tool for classification of *Listeria* (Janbu et al., 2008) and *Yersinia* species (Kuhm et al., 2009) and for identification of *Trueperella pyogenes* (Nagib et al., 2014). FT-IR also allowed a differentiation below the species level (Fetsch et al., 2014; Eisenberg et al., 2015, 2017). A cluster analysis of the IR spectra of the *A. phocae* strains of the present study clearly separated the nine *A. phocae* strains from farm A, the four *A. phocae* control strains from farms B, C and D and the reference strain *A. phocae* DSM 10002^T indicating the usefulness of FT-IR for differentiation on the subspecies level (Supplemental Fig. 2).

In a genotypic approach the previously developed LAMP assay, targeting phocaelysin encoding gene *phl* (Alssahen et al., 2018), also provided a rapid and reliable identification of all nine *A. phocae* strains isolated from minks of a single farm, the *A. phocae* control strains and type strain *A. phocae* DSM 10002^T. No cross reaction could be observed with various other species of genus *Arcanobacterium*. This could be demonstrated using a real-time fluorometer. Melting curve analysis, which is termed by Genie II® as anneal curve analysis, revealed a significant similarity among the investigated *A. phocae* strains. The melting temperature of the *A. phocae* specific amplicon was 84.4 °C (± 0.1 °C), (Supplemental Fig. 3). However, the presented LAMP assay did not allow a differentiation of the *A. phocae* on the subspecies level.

The nine *A. phocae* strains were additionally identified by sequencing the 16S rRNA gene with approximate sizes of 1350 bp with a sequence similarity of 100 % of the nine strains among each other and a sequence similarity between 99.6 % and 100 % to four previously characterized *A. phocae* strains and of 99.1 % sequence similarity to 16S

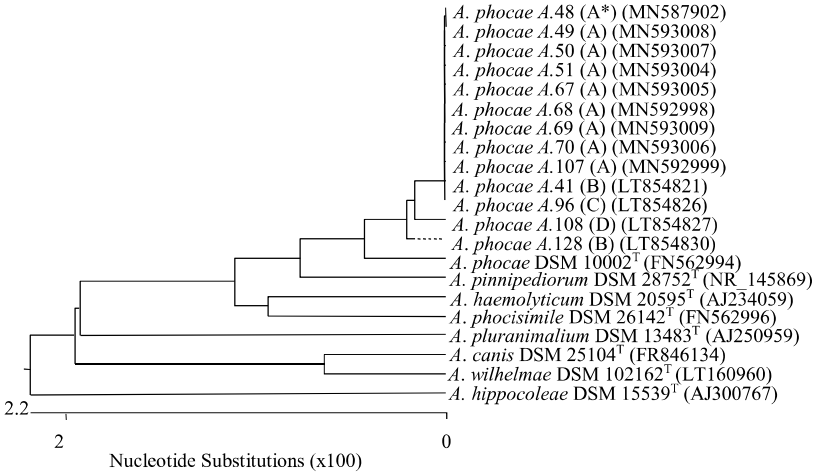


Fig. 1. Dendrogram analysis of 16S rRNA gene sequences of the nine *A. phocae* strains investigated in the present study in comparison with four previously characterized *A. phocae* strains, type strain *A. phocae* DSM 10002^T and type strains of other species of genus *Arcanobacterium* obtained from NCBI GenBank using the Clustal W method of DNASTAR/Lasergene MegAlign program (version 8.0.2). (*) = Farm.

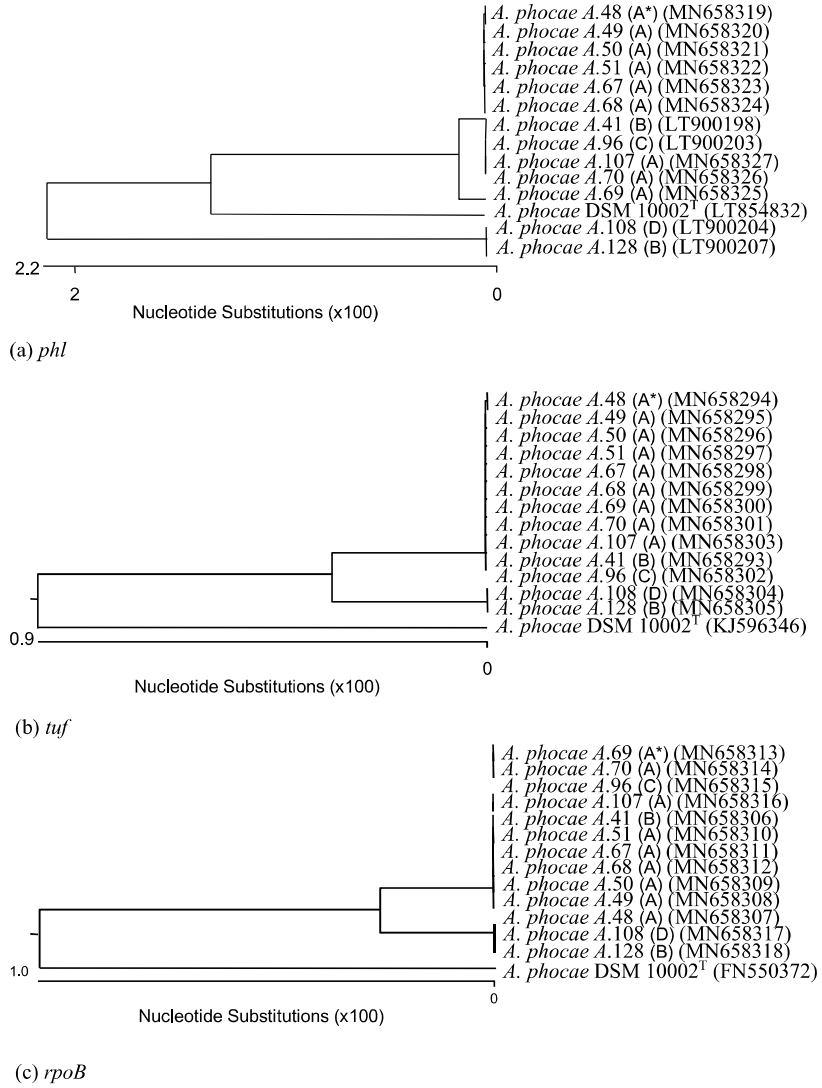


Fig. 2. Dendrogram analyses of the genes *phl* (a), *tuf* (b) and *rpoB* (c) of the nine *A. phocae* strains investigated in the present study in comparison with four previously characterized *A. phocae* strains and type strain *A. phocae* DSM 10002^{T} obtained from NCBI GenBank using the Clustal W method of DNASTAR/Lasergene MegAlign program (version 8.0.2). (*) = Farm.

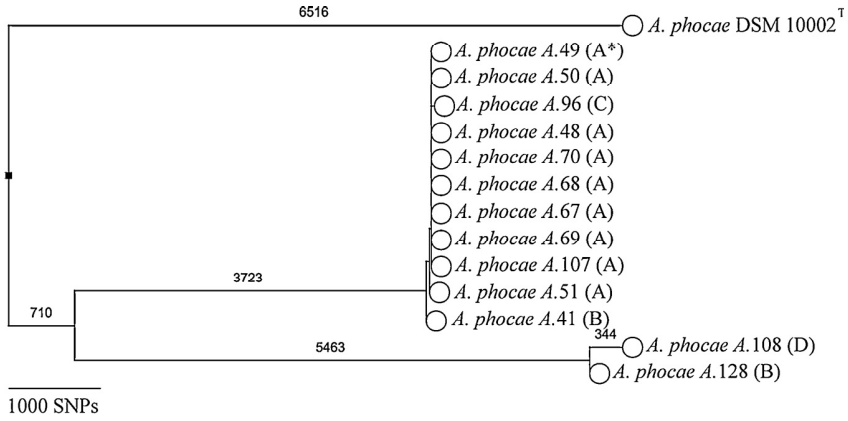
rRNA gene of type strain *A. phocae* DSM 10002^{T} (FN562994), (Fig. 1).

In addition, sequencing gene *phl* of the nine *A. phocae* strains showed a sequence similarity between 99.7 % and 100 % among each other and between 95.7 % and 100 % sequence similarity to the four previously characterized *A. phocae* strains and between 97.1 % and 97.2 % sequence similarity to the *phl* sequence of type strain *A. phocae* DSM 10002^{T} (Fig. 2a). In addition, the nine strains were characterized by sequencing gene *tuf* with a sequence similarity of 100 % among each other and between 99.4 % and 100 % sequence similarity to the four previously characterized *A. phocae* strains and a sequence similarity of 98.1 % to *tuf* gene of type strain *A. phocae* DSM 10002^{T} (Fig. 2b). Sequencing gene *rpoB* revealed a sequence similarity of 100 % of the nine *A. phocae* strains among each other and between 99.5 % and 100 % sequence similarity to four previously characterized *A. phocae* strains and a sequence similarity of 98.0 % to *rpoB* gene of type strain *A. phocae* DSM 10002^{T} (Fig. 2c). The GenBank accession numbers of the gene sequences of the *A. phocae* strains investigated in the present study are shown in Table 1 (Supplemental material).

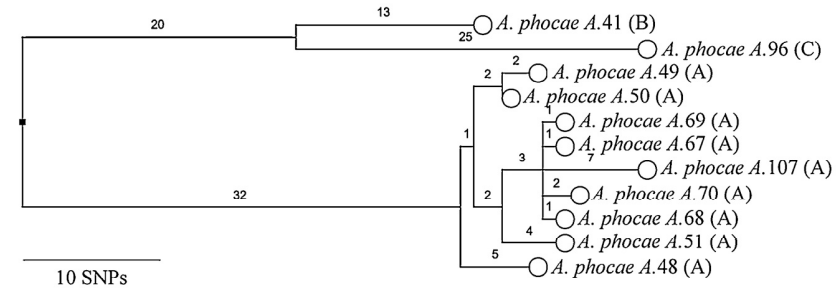
Comparable to the MALDI-TOF MS analysis, the 16S rRNA gene sequencing of the strains of the present study and sequencing of the genes *phl, tuf* and *rpoB*, respectively, all nine *A. phocae* strains of farm A and the control *A. phocae* A.41 of farm B and *A. phocae* A.96 of farm C formed a single branch, both catalase negative *A. phocae* strains *A. phocae* A.108 of farm D and *A. phocae* A.128 of farm B formed a second branch and type strain *A. phocae* DSM 10002^{T} formed a third branch, also indicating the limited use of this genotypic approach for subspecies typing.

The nine *A. phocae* strains were finally investigated for epidemiological relationships using wgSNP analysis (Table 1 in Supplemental material). The results revealed that the nine *A. phocae* strains of farm A of the present study were not closely related to the previously investigated *A. phocae* strains *A. phocae* A.108 of farm D, *A. phocae* A.128 of farm B and the type strain *A. phocae* DSM 10002^{T} (> 9000 SNPs, Fig. 3a), but distantly related to the strains *A. phocae* A.41 of farm B and *A. phocae* A.96 of farm C (≤ 90 SNPs, Fig. 3b). Among each other, all nine *A. phocae* strains of farm A were found to be highly similar (≤ 18 SNPs, Fig. 3b).

The relation shown by MALDI-TOF MS, FT-IR, by sequencing the various species specific target and by wgSNP analysis revealed a close relation of the nine *A. phocae* strains isolated from a single farm, and a difference to previously investigated *A. phocae* strains of other origin. The few SNP differences observed among the nine *A. phocae* strains of a single farm might have developed most likely from a common ancestor by evolutionary processes.



(a)



(b)

Fig. 3. (a) Neighbor-Joining tree derived from pairwise SNP distance of nine *A. phocae* strains investigated in the present study, four *A. phocae* strains isolated from other origin and *A. phocae* DSM 10002^T (reference: *A. phocae* DSM 10002^T (accession number: LT629804). (b) Neighbor-Joining tree derived from pairwise SNP distance of nine *A. phocae* strains investigated in the present study and two distantly related *A. phocae* strains from other origin (reference: concatenated assembly of *A. phocae* A.107).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2020.108618.

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Appendix 2.1 - Supplemental figures of Appendix 2

Supplemental table 1

GenBank accession/ENA run accession numbers of the gene and genome sequences of the nine *A. phocae* strains investigated in the present study, the four previously characterized *A. phocae* strains and the *A. phocae* type strain DSM 10002^T.

Strain code	16S rDNA	*phl*	*tuf*	*rpoB*	Genome sequences
A. phocae A.48	MN587902	MN658319	MN658294	MN658307	ERR3513993
A. phocae A.49	MN593008	MN658320	MN658295	MN658308	ERR3513994
A. phocae A.50	MN593007	MN658321	MN658296	MN658309	ERR3513995
A. phocae A.51	MN593004	MN658322	MN658297	MN658310	ERR3513996
A. phocae A.67	MN593005	MN658323	MN658298	MN658311	ERR3513997
A. phocae A.68	MN592998	MN658324	MN658299	MN658312	ERR3513998
A. phocae A.69	MN593009	MN658325	MN658300	MN658313	ERR3513999
A. phocae A.70	MN593006	MN658326	MN658301	MN658314	ERR3514000
A. phocae A.107	MN592999	MN658327	MN658303	MN658316	ERR3514001
A. phocae A.41	LT854821	LT900198	MN658293	MN658306	ERR3514002
A. phocae A.96	LT854826	LT900203	MN658302	MN658315	ERR3514003
A. phocae A.108	LT854827	LT900204	MN658304	MN658317	ERR3514004
A. phocae A.128	LT854830	LT900207	MN658305	MN658318	ERR3514005
A. phocae DSM 10002^T	FN562994	LT854832	KJ596346	FN550372	LT629804

Supplemental Figure 1

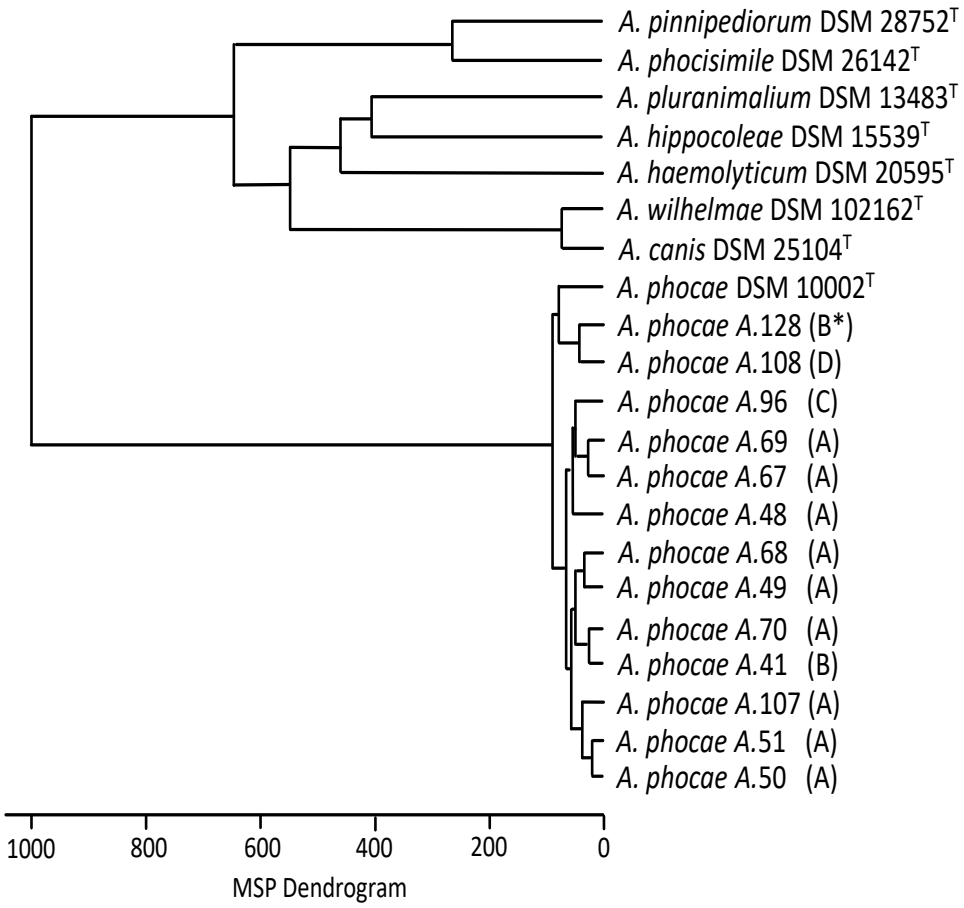

Fig. 1. Dendrogram of MALDI-TOF MS main spectra of the nine *A. phocae* strains investigated in the present study in comparison with four previously characterized *A. phocae* strains, *A. phocae* DSM 10002^T and other species of genus *Arcanobacterium*. The MALDI-TOF MS analysis was performed using MALDI Biotyper Version (4.0). Further information for the type strain MALDI reference spectra were listed on MALDI-UP, http://www.maldi-up.ua-bw.de (Rau et al., 2016). (*) = Farm.

Supplemental Figure 2

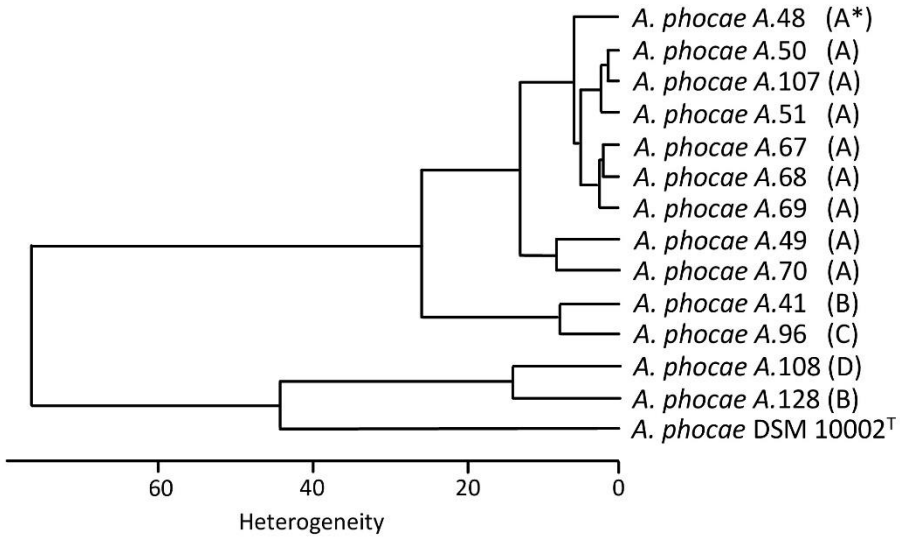

Fig. 2. Cluster analysis of infrared spectra of the *A. phocae* strains was performed by using the second derivatives of the spectra (n=2 for each isolate) in the spectral range of 500 to 1400 cm^{-1}. Ward's algorithm was applied. (*) = Farm.

Supplemental Figure 3

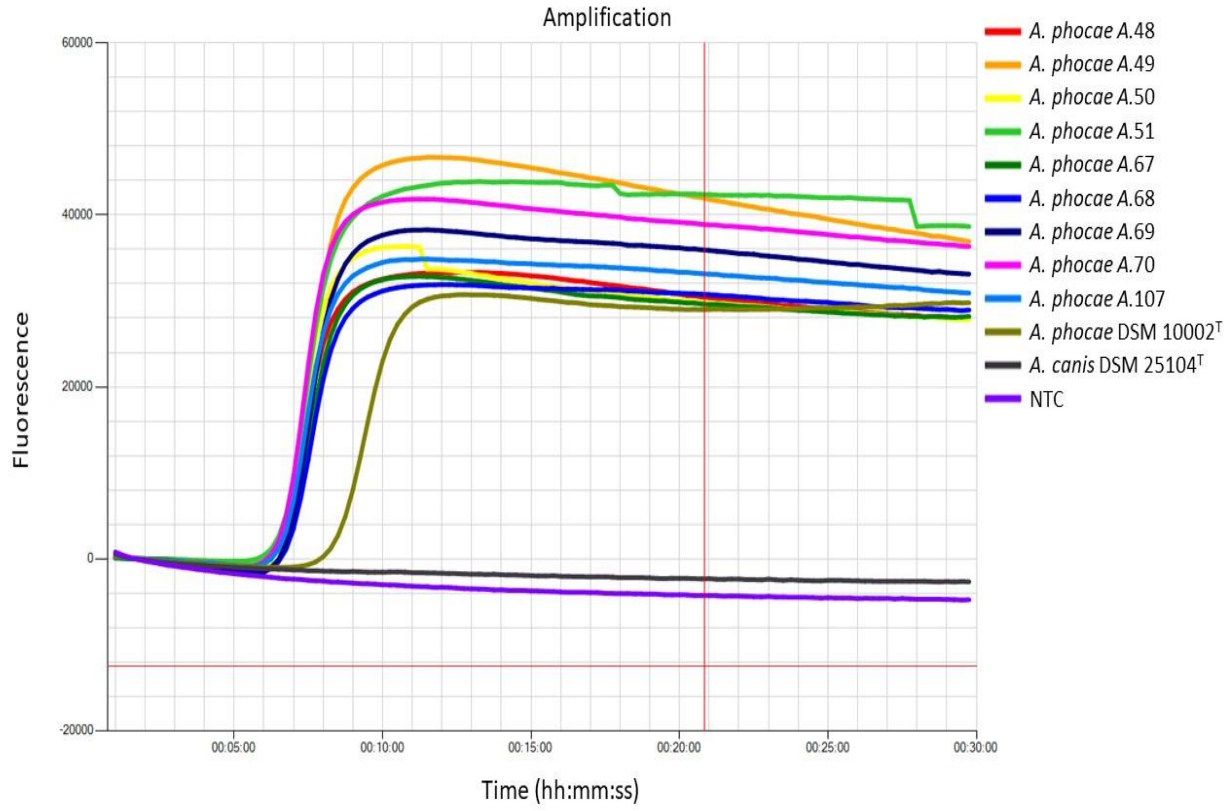

Fig. 3. Typical real-time amplification signal of *A. phocae phl* LAMP products of the nine *A. phocae* strains investigated in the present study and *A. phocae* DSM 10002^T; Negative reaction of *A. canis* DSM 25104^T and a negative control.

Chapter 4 - Discussion

Taxonomic classification

According to Salam et al. (2020) bacteria of genus *Arcanobacterium* (*A.*), belonging to the order *Actinomycetales* and to the family *Arcanobacteriaceae*, are facultative anaerobic, Gram-positive, non-acid fast, non-motile, rod-shaped bacteria. The name of this genus was descend from the word "arcanus", the Latin word for "secretive" or "mysterious". The first description of genus *Arcanobacterium* was by Collins et al. (1982), characterizing the species *A. haemolyticum*. *A. haemolyticum* was originally isolated from infected wounds of American soldiers and was previously classified *Corynebacterium haemolyticum* (MacLean et al., 1946). In 1997, Ramos et al. included the following species to genus *Arcanobacterium*; *A. phocae*, which was isolated from multiple tissues of sea mammals, *A. pyogenes*, which was recovered from infected wounds and abscesses of animals and humans and *A. bernardiae*, which was isolated from blood and abscesses of humans. Later, *A. pluranimalium*, which was isolated from a porpoise and a deer, was included to this genus (Lawson et al., 2001), followed by *A. hippocoleae* isolated from vaginal discharge of a mare (Hoyles et al., 2002) and *A. bialowiezense* and *A. bonasi*, which were isolated from prepuce of European bison bulls (Lehnen et al., 2006). In 2009, *A. abortisuis*, a species which was isolated from a placenta of a pig after abortion, had been included to genus *Arcanobacterium* (Azuma et al., 2009). However, genus *Arcanobacterium* was reclassified by chemotaxonomic and phylogenetic analyses by Yassin et al. (2011). The species *A. haemolyticum*, *A. phocae*, *A. pluranimalium* and *A. hippocoleae* still belong to genus *Arcanobacterium*, while *A. pyogenes*, *A. bialowiezense*, *A. bonasi* and *A. abortisuis* were transferred to the newly described genus *Trueperella*, as *T. pyogenes*, *T. bialowiezensis*, *T. bonasi* and *T. abortisuis* (Yassin et al., 2011). In the following years, novel species were included to genus *Arcanobacterium*, such as *A. canis*, which was recovered from an otitis externa of a seven year old, female English Bulldog (Hijazin et al., 2012d), *A. phocisimile*, which was isolated from vaginal swabs from common seal (Hijazin et al., 2013), *A. pinnipediorum*, which was isolated from an anal swab of a living male harbour seal of the German North Sea (Sammra et al., 2015), *A. wilhelmae*, which was isolated from the vaginal discharge of a rhinoceros (*Rhinoceros unicornis*) from

Stuttgart Zoo Wilhelma, Germany (Sammra et al., 2017), *A. bovis* which was isolated from a milk sample collected from the left forequarter of a 7 year old, pure Simmental beef cow at post-mortem in the north of Scotland (Sammra et al., 2020), *A. urinimassiliense* which was recovered from the urogenital tract of a nearly two-month old female infant suffering from rotavirus gastroenteritis (Diop et al., 2017) and *A. ihumii*, which was isolated from a vaginal swab of a woman living in Dielmo, Afrika (Fall et al., 2019). According to the German Collection of Microorganisms and Cell Culture (DSMZ) *A. urinimassiliense* and *A. ihumii* are not validly published species (https://lpsn.dsmz.de/genus/arcanobacterium).

The first description of some of the novel species of genus *Arcanobacterium*, such as *A. canis*, *A. phocisimile*, *A. pinnipediorum*, *A. wilhelmae* and *A. bovis* came from a working group at Justus-Liebig-University Gießen, Germany. The working group at Justus-Liebig-University, in cooperation with the German Federal Institute for Risk Assessment, also performed a complete genome analysis of *Arcanobacterium* strain 2701 isolated from a harbor seal. The genome of *Arcanobacterium* strain 2701 was compared to previously published complete genomes of species of genus *Arcanobacterium* and *Trueperella* through phylogenetic analysis of amino acid sequence comparison of 107 single-copy core genes with program bcgTree v1.1.0 and other tools. The result of this analysis showed that *Arcanobacterium* strain 2701 was most closely related to type strain *A. phocae* DSM 10002^{T} and also seems to represent a novel species of genus *Arcanobacterium* (Borowiak et al., 2020).

In 2007 multiple cases of pyoderma affecting fur animals spread across a wide region of Scandinavia, causing serious economic losses to fur industry. Nordgren et al. reported in 2014, during a retrospective case-control analysis, about the first isolation of *A. phocae* in affected fur animals. The primary fur animal species mentioned in this study were the captive mink (*Neovison vison*), blue foxes (*Vulpes lagopus*) and raccoon dogs (*Nyctereutes procyonoides*). *A. phocae* was isolated from the diseased animals together with other *Staphylococcus* and *Streptococcus* species. According to Nordgren et al. (2016a), the typical signs and histopathological findings of this fur animal epidemic necrotic pyoderma (FENP) were detected when naïve minks were infected with the tissue extract of mink with FENP using subcutaneous or intradermal but not a perorally infection route.

In this context the present study was designed to investigate *A. phocae* isolated from diseased foxes, minks and finnraccoons by phenotypic and genotypic properties. It was additionally of

interest to analyze the clonal relationship of *A. phocae* strains isolated from a single farm. The latter could be determined by molecular analysis, also including a whole genome analysis.

Identification of *Arcanobacterium phocae* isolated from fur animals by phenotypic properties, by MALDI-TOF MS analysis and by detection of phocaelysin encoding gene *phl* as probable novel target. Vet. Microbiol. 2018, 216: 45-51

According to a proposal of Yassin et al. (2011) genus *Arcanobacterium* (*A.*) includes four species, namely *A. haemolyticum*, *A. phocae*, *A. pluranimalium* and *A. hippocoleae.* Later, new species were added to this genus, such as *A. canis* and *A. phocisimile* (Hijazin et al., 2012d, 2013), *A. pinnipediorum* (Sammra et al., 2015), *A. wilhelmae* and *A. bovis* (Sammra et al., 2017, 2020), *A. urinimassiliense* (Diop et al., 2017) and *A. ihumii* (Fall et al., 2019).
However, *A. phocae* was first isolated and characterized in 1997 in mixed culture from various tissues and fluids in common seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) of the coastal waters around Scotland, UK (Ramos et al., 1997). Furthermore, *A. phocae* has been suggested to be the etiological agent of an emerging skin disease of fur animals called fur animal epidemic necrotic pyoderma (FENP). FENP, as multifactorial disease, is an emerging disease among Finnish fur animals in which *A. phocae* as well as other infectious factors, such as a novel *Streptococcus* sp. are involved (Nordgren et al., 2014).
In the present study twelve *Arcanobacterium phocae* strains isolated from fur animals in Finland, including foxes (*Vulpes lagopus*), minks (*Neovison vision*) and finnraccoons (*Nyctereutes procyonoides*), were investigated phenotypically and genotypically together with type strain *A. phocae* DSM 10002^{T}, *A. phocae* DSM 10003 and other reference strains of genus *Arcanobacterium* and *Trueperella* (Appendix 1, supplemental table 1).
The twelve strains were originally isolated as follows: Four strains were isolated from foxes (*A. phocae* 41 from the eye, *A. phocae* 91 from the skin, *A. phocae* 128 from the uterus, *A. phocae* 132 from the eye), six strains were isolated from minks (*A. phocae* 66 from the eye, *A. phocae* 83 from the paw, *A. phocae* 108 from the skin, *A. phocae* 122 from the lung, *A. phocae* 127 from the lung, *A. phocae* 870/4 from the skin) and two strains were isolated from finnraccons (*A. phocae* 89 from paw, *A. phocae* 96 from paw).

All twelve strains and the reference strains could be characterized phenotypically, by MALDI-TOF MS analysis and genotypically by sequencing the 16S rRNA gene and by sequencing of phocaelysin (PHL) encoding gene *phl* of *A. phocae* as a probable novel target. In addition, a newly designed loop-mediated isothermal amplification (LAMP) assay was used to determine this PHL encoding gene *phl*.

Among the twelve investigated strains ten strains displayed a relatively wide zone of complete hemolysis and two strains (*A. phocae* 108, *A. phocae* 128) a slightly reduced hemolysis on 5% sheep blood agar plates. In addition, all twelve strains showed a synergistic CAMP-like hemolysis with *S. agalactiae* and *R. equi* as indicator strains and a reverse CAMP reaction in the area of incomplete staphylococcal β-hemolysis. Additionally, using API Coryne test and various other biochemical tests, ten strains displayed positive catalase reactions, while two strains (*A. phocae* 108, *A. phocae* 128) reacted catalase negative. The twelve strains were uniformly positive for alkaline phosphatase, β-galactosidase, α-glucosidase, n-acetyl-β-glucosaminidase, glucose, maltose, xylose and lactose. All twelve strains additionally hydrolyzed starch and one strain showed a positive pyrazinamidase reaction, while the other eleven strains were pyrazinamidase negative. In addition, nine strains were ribose positive, seven strains were saccharose positive, ten strains were pyrrolidonyl arylamidase positive and nine strains were glycogen positive. *A. phocae* 108 and *A. phocae* 128 were pyrrolidonyl arylamidase negative. The twelve strains were uniformly negative for β-glucuronidase, esculin, urease, gelatine and serolysis on Löffler agar. These results were comparable to previous results from Ülbegi (2010) and Hijazin et al., (2013) (Appendix 1, table 1).

Referring to the MALDI-TOF MS analysis, all twelve strains could successfully be identified to the species level with score values between 2.2 and 2.4 and correspondingly cluster together with the reference strains *A. phocae* DSM 10002^{T} and *A. phocae* DSM 10003 (Appendix 1, supplemental figure 3). These results support the hitherto discussed MALDI-TOF MS analysis as powerful tool for species classification of a broad spectrum of Gram-positive and Gram-negative bacteria (Seng et al., 2009; Murray, 2010; Bizzini et al., 2011).

All twelve *A. phocae* strains of the present study and the reference strains *A. phocae* DSM 10002^{T} and *A. phocae* DSM 10003 were genotypically characterized by sequencing of the 16S rRNA gene and by amplification and sequencing of phocaelysin encoding gene *phl* as probable novel target. The sequences of the 16S rRNA gene of the twelve *A. phocae* strains of

the present study and the reference strains *A. phocae* DSM 10002^T and *A. phocae* DSM 10003 yielded a sequence similarity between 99.6% and 100% among each other and between 99.0% and 99.5% with the above mentioned reference strains, respectively (Appendix 1, supplemental figure 4). It was of interest that the 16S rRNA gene sequences of *A. phocae* 108 and *A. phocae* 128, both strains which were weakly hemolytic on sheep blood agar and negative in catalase and pyrrolidonyl arylamidase test, differed more pronounced to the 16S rRNA gene sequences of type strain *A. phocae* DSM 10002^T, *A. phocae* DSM 10003 and the remaining *A. phocae* strains investigated in the present study (Appendix 1, supplemental figure 4). In addition, all twelve *A. phocae* strains of the present study were characterized by amplification and sequencing of phocaelysin encoding gene *phl*. Newly designed oligonucleotide primer for amplification of gene *phl* could be achieved based on previously sequenced phocaelysin encoding gene *phl* of *A. phocae* DSM 10002^T (accession number: LT629804, region: 1304651 up to 1305874). Gene *phl* sequences of the twelve strains yielded a sequence similarity between 96.6% and 100% among each other and between 95.5% and 97.2% with the reference strains *A. phocae* DSM 10002^T and *A. phocae* DSM 10003. In addition, the phylogenetic analysis of the amino acid sequences of PHL of the 12 *A. phocae* strains of the present study, PHL of the reference strains *A. phocae* 10002^T and *A. phocae* 10003, arcanolysin (ALN) of *A. haemolyticum*, pyolysin (PLO) of *T. pyogenes* and of other pore forming toxins including streptolysin O (SLO) of *Streptococcus pyogenes*, intermedilysin (ILY) of *Streptococcus intermedius*, pneumolysin (PLY) of *Streptococcus pneumoniae* and listeriolysin O (HLY) of *Listeria monocytogenes* revealed a close relation of PHL to ALN and PLO, respectively and less pronounced to the other pore-forming toxins (Appendix 1, figure 1) indicating the close relation of these putative virulence factors of closely related species and genera.

A detailed analysis of the primary structure of amino acids of PHL of *A. phocae* DSM 10002^T compared to ALN of *A. haemolyticum* DSM 20595^T, described by Jost et al., (2011), yielded a variant undecapeptide for PHL. PHL and ALN both lack the conserved cysteine residue and displayed a typical tryptophan spacing (WxxWW) which differs to the consensus sequence (WxWW). PHL and ALN also showed the conserved threonine leucine pair in domain 4 known to be responsible for the interaction with membrane cholesterol (Appendix 1, figure 2A).

However, it was very interest that all twelve *A. phocae* strains of the present study and the reference strains *A. phocae* DSM 10002^{T} and *A. phocae* DSM 10003 uniformly displayed the undecapeptide sequence EATGLAWDPWW which differed to the undecapeptide sequence of ALN of *A. haemolyticum*, PLO of *T. pyogenes*, undecapeptides of other CDCs and the consensus sequence (Appendix 1, figure 2B).

Gene *phl* of *A. phocae* seems to be constantly present in all strains of this species and could be used for genotypic identification of *A. phocae*. This is comparable to gene *plo* of *T. pyogenes*, which seemed to be a constant characteristic of all investigated *T. pyogenes* strains (Billington et al., 1997; Ertaş et al., 2005; Ülbegi-Mohyla et al., 2010; Hijazin et al., 2011) and gene *pla* of *A. pluranimalium*, which was also used for molecular identification of this species (Moser et al., 2013; Balbutskaya et al., 2014; Risse et al., 2014; Wickhorst et al., 2016; Ningrum et al., 2017).

Loop mediated isothermal amplification (LAMP) assays were already used to identify various bacteria, such as *Leptospira* spp. (Koizumi et al., 2012), *Erysipelothrix rhusiopathiae* (Yamazaki et al., 2014), *Streptococcus equi* subsp. *zooepidemicus* (Kinoshita et al., 2014), *A. pluranimalium* and *T. pyogenes* (Abdulmawjood et al., 2015, 2016). In the present study all twelve *A. phocae* strains and the reference strains *A. phocae* DSM 10002^{T} and *A. phocae* DSM 10003 were identified using a LAMP assay, based on phocaelysin encoding gene *phl*. No cross reactions were observed with other species of genus *Arcanobacterium* and closely related genus *Trueperella* (Appendix 1, figure 3, table 2).

The phenotypic properties, the MALDI-TOF MS analysis and the genotypic properties investigating the 16S rRNA gene and the novel target gene *phl*, also including the LAMP assay based on gene *phl*, could successfully be used for identification of *A. phocae*. This might help to improve the identification of *A. phocae* in diagnostic laboratories in future. This might also help to elucidate the role this species plays in infections of seal, fur animals, such as mink, blue fox and finnraccoon, in other animals and possibly in humans.

Epidemiological analysis of *Arcanobacterium phocae* isolated from cases of mink dermatitis of a single farm. Vet. Microbiol. 2020, 243: 491-496

In 2014, *A. phocae* was recovered from tissues of fur animals which suffered from epidemic necrotic pyoderma (FENP), including minks, finnraccoons and foxes. This bacterium was found in some cases together with *Streptococcus* species (Nordgren et al., 2014). Furthermore, *A. phocae* was also isolated, together with *Staphylococcus delphini* and *Streptococcus canis*, in the pathogenesis of cases of pododermatitis of the Canadian mink (*Neovison vison*) (Chalmers et al., 2015). A link between feeding of seal meat and the emergence of pododermatitis in Canadian minks was proposed (Chalmers et al., 2015). However, according to Aaltonen et al., 2016 and Nordgren et al., 2016b environmental and immunological factors seem to contribute to the disease and the bacteria spread from farm to farm.

In the present study nine *A. phocae* strains were investigated for epidemiological relationships. These strains were isolated from cases of dermatitis from nine minks of a single farm in Finland (Appendix 2, table 1, farm A). In addition, for comparative purposes, four previously characterized *A. phocae* strains isolated from foxes, finnraccoon and mink of other farms in Finland (Appendix 2, table 1, farm B, C and D), the type strain *A. phocae* DSM 10002^{T} and other reference strains of genus *Arcanobacterium* were included.

All nine strains of this study were identified by cultural properties, by phenotypical properties, by MALDI-TOF MS and FT-IR analyses, genotypically by determination of the presence of gene *phl* using the above mentioned LAMP assay and by amplification and sequencing 16S rRNA gene and the target genes *phl*, *tuf* and *rpoB*. In addition, epidemiological analyses were performed using whole genome sequencing and whole genome single nucleotide polymorphism (wgSNP) analysis.

All nine *A. phocae* strains of the present study, showed, after cultivation on 5% sheep blood agar plates, a relatively wide zone of complete hemolysis. In addition, all nine strains displayed a synergistic CAMP-like hemolysis with *S. agalactiae* and *R. hoagii* as indicator strains and a reverse CAMP reaction in the area of incomplete staphylococcal β-hemolysis. The results of the commercial identification system API Coryne showed, together with cultural properties, a close similarity of the nine strains among each other and corresponded generally to properties of other previously investigated *A. phocae* strains and type strain *A.*

phocae DSM 10002^T (Appendix 2, table 2). The results of the MALDI-TOF MS analysis of the *A. phocae* strains of the present study allowed an identification of all nine strains to the species level. As shown by Hijazin et al. in 2012b MALDI-TOF MS appears to be a powerful tool for species identification of *A. phocae*. The results observed by MALDI-TOF MS analysis of the strains seems to be useful for identification of *A. phocae* to the species level but could not be used for differentiation below the species level (Appendix 2, supplemental figure 1).

However, comparable to the findings of Fetsch et al., 2014 and Eisenberg et al., 2015, 2017 investigating *S. aureus*, *Corynebacterium ulcerans* and *S. agalactiae*, respectively, FT-IR spectroscopy could also be used for identification of the nine *A. phocae* strains of the present study and for differentiation of the bacteria below the species level. The cluster analysis of the IR spectra of this study clearly separated the nine *A. phocae* strains of farm A, the four *A. phocae* control strains from farms B, C and D and the reference strain *A. phocae* DSM 10002^T, respectively (Appendix 2, supplemental figure 2).

In addition, the above mentioned LAMP assay based on gene *phl* could also be used for rapid and reliable identification of all *A. phocae* strains investigated in the present study, also including four *A. phocae* control strains and type strain *A. phocae* DSM 10002^T. The given results observed with the gene *phl* LAMP assay yielded no cross reactions with various other species of genus *Arcanobacterium.* However, the gene *phl* LAMP assay did not allow a differentiation to the subspecies level (Appendix 2, supplemental figure 3).

All nine *A. phocae* strains of the present study were additionally identified by sequencing 16S rRNA gene. The sequence similarity of the nine strains was 100% among each other and between 99.6 % and 100 % to 16S rRNA gene of type strain *A. phocae* DSM 10002^T (Appendix 2, figure 1).

The nine strains were additionally identified by sequencing the genes *phl*, *tuf* and *rpoB*. The gene *phl* sequence similarity of the nine strains was between 99.7% and 100% among each other and between 95.7 % and 100 % to gene *phl* of the four previously characterized *A. phocae* strains and between 97.1% and 97.2% to gene *phl* of type strain *A. phocae* DSM 10002^T (Appendix 2, figure 2a). The gene *tuf* sequence similarity of the nine strains was 100% among each other and between 99.4 % and 100 % to gene *tuf* of the four previously characterized *A. phocae* strains and 98.1 % to gene *tuf* of type strain *A. phocae* DSM 10002^T (Appendix 2, figure 2b). The gene *rpoB* sequence similarity was 100% among each other and

between 99.5 % and 100 % to gene *rpoB* of four previously characterized *A. phocae* strains and 98.0 % to gene *rpoB* of type strain *A. phocae* DSM 10002^T (Appendix 2, figure 2c). Comparable to the 16S rRNA gene sequencing of the strains of the present study and sequencing of the genes *phl*, *tuf* and *rpoB*, respectively, all nine *A. phocae* strains of farm A and the control strains *A. phocae* 41 of farm B and *A. phocae* 96 of farm C formed a single branch, both catalase negative *A. phocae* control strains *A. phocae* 108 of farm D and *A. phocae* 128 of farm B formed a second branch and type strain *A. phocae* DSM 10002^T formed a third branch, indicating only a limited use of this gene sequencing for subspecies typing. Sequencing of gene *tuf* was successfully used for identification of various bacteria of genus *Arcanobacterium*, such as *A. haemolyticum* (Sammra et al., 2014 b, c) and *A. hippocoleae* (Wickhorst et al., 2017b). In addition, sequencing of gene *rpoB* was also used for identification of bacteria of this genus, such as *A. pluranimalium* and *A. hippocoleae*, respectively (Wickhorst et al., 2016; 2017b).

However, all nine *A. phocae* strains of the present study were finally investigated for epidemiological relationships using whole genome single nucleotide polymorphism analysis (wgSNP). The results of wgSNP analysis showed that all nine *A. phocae* strain of a single farm (farm A) have a high similarity with ≤ 18 SNPs indicating a close relationship of the nine strains among each other (Appendix 2, figure 3b), while all nine *A. phocae* strains were not closely related to the previously investigated catalase and pyrrolidonyl arylamidase negative *A. phocae* strains (*A. phocae* 108 of farm D, *A. phocae* 128 of farm B) and to type strain *A. phocae* DSM 10002^T with > 9000 SNPs (Appendix 2, figure 3a). However, the nine *A. phocae* strains were distantly related to the strains *A. phocae* 41 of farm B and *A. phocae* 96 of farm C with ≤ 90 SNPs (Appendix 2, figure 3b).

Comparable wgSNP analyses could successfully be used to provide abundant information about epidemiological relationships and the genetic evolution in the population of various bacteria (Schürch et al., 2018), for *Mycobacterium tuberculosis* from various geographical areas (Gutacker et al., 2006) and for Methicillin-resistant *S. aureus* isolated in defined communities (McDonald et al., 2006).

The wgSNP results, partly the FT-IR analyses and sequencing of the various genes revealed that the nine *A. phocae* strains recovered from a single farm showed a close relation among each other and differed from previously investigated *A. phocae* strains isolated from other farms and animals in Finland and from the *A. phocae* type strain. This indicated a close

epidemiological relationship of the *A. phocae* strains isolated from a single farm and that the nine *A. phocae* strains of the present study might have developed from a common ancestor. The few SNP differences observed among the nine *A. phocae* strains of the single farm might have developed by evolutionary processes.

Chapter 5 - Summary

In the present study *A. phocae* strains isolated from fur animals in Finland, including foxes, minks and finnraccoons and nine additional *A. phocae* strains isolated from cases of mink dermatitis of a single farm in Finland were identified phenotypically and genotypically, the nine strains from a single farm also for epidemiological relationships by whole genome sequencing and single nucleotide polymorphism analysis.

The strains were characterized by determination of cultural properties, their hemolysis, by detection of synergistic and antagonistic CAMP-like hemolytic reactions, by determination of various biochemical properties and by investigating their peptidic profiles by MALDI-TOF MS and FT-IR analyses. A molecular identification of the *A. phocae* strains was performed by sequencing the 16S rRNA gene and by amplification and sequencing of phocaelysin encoding gene *phl* as novel target. Gene *phl* could also be used as target gene in a newly developed LAMP-assay. However, the putative virulence factor phocaelysin (PHL) of *A. phocae* seems to be present in all *A. phocae* strains and might play a role for pathogenicity of this bacterial species.

The nine *A. phocae* strains from a single farm were additionally characterized genotypically by sequencing the elongation factor tu encoding gene *tuf* and RNA polymerase encoding gene *rpoB* and by whole genome sequencing and single nucleotide polymorphism analysis. The latter and part of the other investigations revealed that the nine *A. phocae* strains from a single farm might have developed from a common ancestor.

All the data of the present study will help to identify *A. phocae* in future and might help to understand the pathogenic importance of this bacterial species in fur animals and in other animal species and possibly in humans.

Chapter 6 - Zusammenfassung

In der vorliegenden Studie wurden *A. phocae*-Stämme, isoliert von Pelztieren in Finnland, dies beinhaltete Füchse, Nerze und Marderhunde, und neun weitere *A. phocae*-Stämme, die aus Fällen von Nerzdermatitis einer einzelnen Farm in Finnland isoliert wurden, phänotypisch und genotypisch identifiziert, die neun Stämme aus einer einzelnen Farm zusätzlich für epidemiologische Untersuchungen auch durch Sequenzierung des gesamten Genoms und Analyse einzelner Nukleotidpolymorphismen. Die Charakterisierung der Stämme erfolgte durch Beurteilung der kulturellen Eigenschaften der Isolate, ihrer Hämolyse, durch Nachweis synergistischer und antagonistischer CAMP-ähnlicher hämolytischer Reaktionen, durch Nachweis verschiedener biochemischer Eigenschaften und durch Untersuchung ihrer Peptidprofile durch MALDI-TOF MS- und FT-IR-Analysen. Eine molekulare Identifizierung der *A. phocae*-Stämme konnte durch Sequenzierung des 16S rRNA Gens und durch Amplifikation und Sequenzierung des Phocaelysin-kodierenden Gens *phl* als neues Zielgen durchgeführt werden. Das Gen *phl* eignete sich auch als Zielgen in einem neu entwickelten LAMP-Assay. Der mutmaßliche Virulenzfaktor Phocaelysin (PHL) von *A. phocae* scheint bei allen *A. phocae*-Stämmen vorhanden zu sein und könnte eine Rolle für die Pathogenität dieser Bakterienart spielen.

Die neun *A. phocae*-Stämme aus einer einzelnen Farm wurden weitergehend genotypisch charakterisiert. Dies durch Sequenzierung des den Elongationsfaktor tu kodierenden Gens *tuf* und des die RNA-Polymerase kodierenden Gens *rpoB* und durch Sequenzierung des gesamten Genoms und anschließender Analyse einzelner Nukleotidpolymorphismen. Letzteres und ein Teil der zuvor durchgeführten Untersuchungen ergaben, dass die neun *A. phocae*-Stämme einer einzelnen Farm möglicherweise von einem gemeinsamen Vorfahren abstammen.

Die Daten der vorliegenden Studie könnten dazu beitragen *A. phocae* zukünftig zu identifizieren und die pathogene Bedeutung dieser Bakterienart bei Pelztieren und anderen Tierarten und möglicherweise auch beim Menschen zu verstehen.

Chapter 7 - References

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